Introduction

Plants from the Litsea species (Lauraceae) are rich in lignans with a broad range of biological activities \cite{1-4}. The dried fruits and roots of *L. cubeba*, called “Bi-cheng-qie” and “Dou-chi-jiang” in Chinese, were recorded in *Chinese Pharmacopeia* and *Chinese Materia Medica* as two important traditional Chinese herbs, which can promote qi circulation to relieve pain, dispel wind, and eliminate dampness \cite{5,6}. Previous chemical investigation of the fruits and roots of *L. cubeba* revealed that aporphine-type alkaloids and lignans are the major active components of *L. cubeba* with respect to their anti-thrombotic, anti-inflammatory, and anti-nociceptive properties \cite{3,4,7-11}.

Our previous investigation of the twigs of *L. cubeba* found 55 aromatic compounds including 30 lignans from the H$_2$O- and EtOAc-soluble fraction of ethanolic extract. Notably, some lignans showed potent hepatoprotective, HDAC1 inhibitory, and anti-inflammatory activities \cite{10-14}. As part of an ongoing search for biological natural products with structure diversity from *L. cubeba* \cite{15-20}, we report herein the isolation, structure elucidation, and biological assays of five new sesquineolignans (1–5) (Fig. 1) from the remaining EtOAc-soluble fractions.

Results and Discussion

Compound 1 was isolated as white amorphous powder. The IR spectrum displayed absorptions of hydroxy (3422 cm$^{-1}$), ester (1680 cm$^{-1}$), and aromatic (1612 cm$^{-1}$) functionalities. Its molecular formula C$_{30}$H$_{32}$O$_{9}$ with fifteen degrees of unsaturation, was assigned by HR-ESI-MS ion peak at m/z 559.1936 (Caled. for C$_{30}$H$_{32}$O$_{9}$). The NMR data of 1 showed resonances attributed to a 1, 2, 4-substituted benzene ring ($\delta$$_H$ 7.07 (1H, d, $J = 1.5$ Hz, H-2), 6.82 (1H, d, $J = 8.0$ Hz, H-5), and 6.91 (1H, dd, $J = 8.0$, 1.5 Hz, H-6); $\delta$$_C$ 133.7 (C-1), 110.6 (C-2), 148.4 (C-3), 147.5 (C-4), 115.7 (C-5), and 119.9 (C-6)) and a 1, 2, 3, 5-substituted benzene ring ($\delta$$_H$ 6.77 (1H, brs, H-2') and 6.78 (1H, brs, H-6'); $\delta$$_C$ 136.7 (C-1'), 114.2 (C-2'), 145.0 (C-3'), 147.3 (C-4'), 128.6 (C-5'), and 117.3 (C-6')). Additionally, $^1$H and $^{13}$C NMR data revealed the presence of two methines [($\delta$$_H$ 5.51 (1H, d, $J = 7.5$ Hz, H-
7) and 3.83 (1H, m, H-8); δC 88.9 (C-7) and 51.6 (C-8)], four methylenes [δH 4.55 (1H, dd, J = 11.5, 5.5 Hz, H-9a), 4.41 (1H, dd, J = 11.5, 8.0 Hz, H-9b), 2.64 (2H, t, J = 7.5 Hz, H-2'), 1.79 (2H, m, H-8') and 3.56 (2H, td, J = 6.5, 4.5 Hz, H-2''); δC 66.0 (C-9), 32.6 (C-7'), 35.9 (C-8'), and 61.7 (C-9')] and two aromatic methoxy groups [δH 3.79, 3.84 (each 3H, s, 3-and 3'-OMe); δC 56.4 and 56.3 (3-and 3'-OMe)]. The above NMR data were consistent with the aglycone portion of a lignan glycoside isolated from the title plant by our group [18], suggesting a 4, 9, 9'-trihydroxy-3, 3'-dimethoxy-4', 7-epoxy-8, 5'-neolignan moiety in 1. This deduction was confirmed by 2D NMR COSY and HMBC correlations as depicted in Fig. 2. Furthermore, an E-feruloyl moiety was readily assigned by the remaining characteristic NMR signals (Table 1) [13]. Therefore, 1 was a sesquinolignan containing a 4, 9, 9'-trihydroxy-3, 3'-dimethoxy-4', 7-epoxy-8, 5'-neolignan and an E-feruloyl moiety. Finally, the E-feruloyl moiety was linked to C-9 to form an ester bond, thereby completing the planar structure of 1, according to the key HMBC correlation from C-9 to H-7'. The coupling constant of H-7 (J = 7.5 Hz) required a trans relationship of H-7/H-8 as previously reported for 4', 7-epoxy-8, 5'-neolignans [20, 21]. NOESY correlations of H-7 with H2-9 and H-8 with H-2(6) were in full support of the trans-arrangement between H-7 and H-8. The absolute configuration of 1 was established as 7R, 8S by the negative Cotton effects at 212 nm and 297 nm in its ECD spectrum (Fig. S10, Supporting Information), showing an ECD curve trend similar to that of (7R, 8S)-3, 3'-O-methylcedrusin 9-p-coumarate [22] and litsecol B, the latter of which was a known sesquinolignol obtained from the roots and stems of L. cubeba [2]. Thus, the structure of 1 was assigned as (−)-(7R, 8S)-9-O-(E)-feruloyl-4, 9, 9'-trihydroxy-3, 3'-dimethoxy-4', 7-epoxy-8, 5'-neolignan.

Compound 2 was obtained as white powder with the molecular formula C31H30O10 established by HR-ESI-MS ion peak at m/z 585.1753 [M + Na]+ (Calcd. for C31H30O10 Na 585.1731). The 1H and 13C NMR spectra of 2 were very similar to those of litsecol B [2]. However, the data for 4 were elucidated as (−)-(7R, 8S, 7'E)-9-O-(E)-feruloyl-4, 9-dihydroxy-3, 5, 3'-trimethoxy-4', 7-epoxy-8, 5'-neolignan-7-ene-al.

It was obvious that compound 5 was the 5-demethoxy derivative of 4 because the molecular formula of 5 had a CH2O less than that of 4 and the NMR resonances for the symmetrically tetrasubstituted aromatic ring with two methoxy groups of 4 were replaced by resonances attributed to a 1, 2, 4-trisubstituted benzene moiety and one methoxy group of 5 (Table 1). This deduction was confirmed by the 2D COSY, HSQC, HMBC, and NOESY NMR experiments of 5. Therefore, the structure of 5 was established as (−)-(7R, 8S, 7'E)-9-O-(E)-feruloyl-4, 9-dihydroxy-3, 5, 3'-trimethoxy-4', 7-epoxy-8, 5'-neolignan-7-ene-9'-al.

Compounds 1–5 were evaluated for their inhibitory activity against NO production in LPS-induced RAW264.7 macrophage cells. Compounds 1–5 showed moderate inhibi-
macrophages were also evaluated for their inhibitory effects against LPS-induced NO production in RAW264.7 macrophages, with IC₅₀ values of 16.2, 20.2, 22.1, 15.1, and 16.6 μmol L⁻¹, respectively. Dexamethasone was used as the positive control with an IC₅₀ value of 7.5 μmol L⁻¹. At concentrations up to 100 μmol L⁻¹, compounds 1–5 were not cytotoxic to LPS-induced RAW264.7 cells. Compounds 1–5 were also evaluated for their inhibitory activities against TNF-α secretion in mouse peritoneal macrophages [21], PTP1B (protein tyrosine phosphatase 1B) [18], acetaminophen-induced HepG2 cell injury [19], and cytotoxic properties toward HCT-8 colon, A2780 ovary, BGC-823 stomach, Bel-7402 hepatoma, and A549 lung cell lines [20], but inactive at 10 μmol L⁻¹.

### Experimental

#### General experimental procedures

Optical rotation values were measured with a JASCO P-2000 automatic digital polarimeter (JASCO, Tokyo, Japan).
UV spectra were recorded on a JASCO V-650 spectrophotometer (JASCO, Tokyo, Japan). CD spectra were recorded on a JASCO J-815 spectropolarimeter (JASCO, Tokyo, Japan). IR (4000–400 cm⁻¹) spectra (KBr pellets) were recorded on a Nicolet 5700 FT-IR spectrophotometer (Thermo Electron Corporation, Madison, WI, USA). NMR spectra were obtained on a Bruker spectrometer (600 MHz for 1H, 150 MHz for 13C) or a Varian INOVA spectrometer (500 MHz for 1H, 125 MHz for 13C) equipped with an inverse detection probe (Bruker Corp., Karlsruhe, Germany). Chemical shifts were given in δ (ppm) using the peak signals of the solvent acetone-d6 as references from Cambridge Isotope Laboratories, and coupling constants are reported in Hz. ESI-MS and HR-ESI-MS data were measured using an AccuToFCS JMS-T100CS spectrometer (Agilent Technologies, Ltd., Santa Clara, CA, USA). Silica gel GF254 prepared for TLC and silica gel (200 to 300 mesh) for column chromatography were obtained from Qingdao Marine Chemical Factory (Qingdao, China). Sephadex LH-20 was a product of Pharmacia Biotech AB (Uppsala, Sweden). HPLC analyses were performed on a Waters HPLC instrument (Waters, USA) equipped with a Waters 600 pump, a Waters 2487 dual absorbance detector, and a Grace (250 mm × 10 mm) semipreparative column packed with C18 (5 μm). HPLC analyses were performed on a Waters HPLC instrument (Waters, USA) equipped with a Waters 600 pump, a Waters 2487 dual absorbance detector, and a Grace (250 mm × 10 mm) semipreparative column packed with C18 (5 μm). All the reagents were of HPLC or analytical grade and prepared over C18 silica gel, eluting with a step gradient from 20% to 95% MeOH in H2O to give 10 fractions (F1−F10). Fraction F5 (1.5 g) was separated on Sephadex LH-20 eluting with CHCl3–MeOH–PE (5 : 5 : 1, V/V) to give four subfractions, and the second subfraction was purified by reversed-phase semipreparative (RP18, 5 μm, 230 nm, MeOH–H2O, 68 : 32, 1.5 mL·min⁻¹) to afford 1 (5.2 mg, tR 29.8 min). Using the same HPLC system, the third subfraction afforded 4 (7.3 mg, tR 36.9 min) and 5 (12 mg, tR 34.1 min). Fraction F8.5 (0.8 g) was chromatographed over Sephadex LH-20 eluting with CHCl3–MeOH–PE (5 : 5 : 1, V/V) to give three subfractions, and the second subfraction was purified by reversed-phase semipreparative HPLC (RP18, 5 μm, 230 nm, MeOH–H2O, 67 : 33, 2.5 mL·min⁻¹) to give 2 (6.5 mg, tR 15.9 min) and 3 (8.2 mg, tR 21.3 min).

(−)-(7R, 8S)-9-O-(E)-feruloyl-4, 9, 9′-trihydroxy-3, 3′-dime-thoxy-4′, 7-epoxy-8, 5′-neolignan (1)

White amorphous powder; [α]D20° = −12.0 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 207 (1.43), 234 (0.52), 287 (0.30), 327 (0.32) nm; ECD (MeOH) δmax (Δε) 212 (−4.75), 297 (1.86) nm; IR (KBr) νmax 3418, 2934, 2849, 1680, 1632, 1516, 1464, 1430, 1385, 1273, 1207, 1143, 1034, 846, 821, 723 cm⁻¹; 1H NMR (acetone-d6, 500 MHz) and 13C NMR (acetone-d6, 125 MHz) data, see Table 1; HR-ESI-MS m/z 559.1936 [M + Na]+ (Calcd. for C30H33O12Na, 559.1939).  

(−)-(7R, 8S)-9-O-(E)-feruloyl-4, 9′-trihydroxy-3, 3′-dimethoxy-4′, 7-epoxy-8, 5′-neolignan (2)

White amorphous powder; [α]D20° = −28.0 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 205 (1.10), 230 (0.53), 286 (0.39), 328 (0.51) nm; ECD (MeOH) δmax (Δε) 220 (−1.04), 240 (0.75), 297 (−1.55) nm; IR (KBr) νmax 3422, 2920, 2851, 1680, 1516, 1464, 1430, 1385, 1273, 1207, 1143, 1034, 846, 821, 776, 737 cm⁻¹; 1H NMR (acetone-d6, 500 MHz) and 13C NMR (acetone-d6, 125 MHz) data, see Table 1; HR-ESI-MS m/z 557.1794 [M + Na]+ (Calcd. for C30H33O12Na, 557.1782).

(−)-(7R, 8S)-9-O-(E)-feruloyl-4, 9′-dihydroxy-3, 3′-trihydroxy-4′, 7-epoxy-8, 5′-neolignan (3)

White amorphous powder; [α]D20° = −25.0 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 205 (1.10), 230 (0.53), 286 (0.39), 328 (0.51) nm; IR (KBr) νmax 3418, 2934, 2849, 1702, 1667, 1632, 1599, 1516, 1462, 1431, 1384, 1331, 1272, 1215, 1154, 5 μm; 230 nm, MeOH–H2O, 67 : 33, 2.5 mL·min⁻¹) to give 2 (6.5 mg, tR 15.9 min) and 3 (8.2 mg, tR 21.3 min).

(−)-(7R, 8S)-9-O-(E)-feruloyl-4, 9′-trihydroxy-3, 3′-dime-thoxy-4′, 7-epoxy-8, 5′-neolignan (4)
1032, 974, 852, 821, 776, 737 cm⁻¹; ¹H NMR (acetone-d₆, 500 MHz) and ¹³C NMR (acetone-d₆, 125 MHz) data, see Table 1; HR-ESI-MS m/z 571.1927 [M + Na⁺] (Calcd. for C₄₁H₃₂O₅Na, 571.1939).

White amorphous powder; [α]₂⁵D = 18.0 (c 0.1, MeOH); ECD (MeOH) λmax (Δε) 216 (−0.13), 235 (0.24), 314 (−0.32) nm; ¹H NMR (acetone-d₆, 600 MHz) and ¹³C NMR (acetone-d₆, 150 MHz) data, see Table 1; HR-ESI-MS m/z 585.1753 [M + Na⁺] (Calcd. for C₃₃H₃₇O₇Na, 585.1731).

(--)-(7R, 8S, 7′E)-9-O-(E)-feruloyl-4, 9-dihydroxy-3, 3′-dimethoxy-4', 7-epoxy-8, 5′-neolignan-7′-ene-9′-al (4)

White amorphous powder; [α]₂⁵D = 20.0 (c 0.1, MeOH); ¹H NMR (acetone-d₆, 600 MHz) and ¹³C NMR (acetone-d₆, 150 MHz) data, see Table 1; HR-ESI-MS m/z 533.1780 [M + H⁺] (Calcd. for C₂₆H₂₅O₇H, 533.1806).

Nitric Oxide (NO) production in RAW264.7 macrophages

RAW 264.7 macrophages were cultured in RPMI 1640 medium (Hyclone, Logan, UT) containing 10% FBS. The compounds were dissolved in DMSO and further diluted in medium to produce different concentrations. The cell mixture and culture medium were dispensed into 96-well plates (2 × 10⁴ cells/well) and maintained at 37 °C under an atmosphere of 5% CO₂. After preincubation for 24 h, serial dilutions of the test compounds were added into the cells, up to the maximum concentration of 25 μmol·L⁻¹, followed by addition of LPS to a concentration of 1 μg·mL⁻¹ before incubation for 18 h. The amount of NO was assessed by determining the nitrite concentration in the cultured RAW264.7 macrophage supernatants with Griess reagent. Aliquots of supernatants with Griess reagent. Aliquots of supernatants were incubated in sequence, with 50 μL 1% phosphoric acid solution. The sample absorbance was measured at 570 nm by a 2104 Envision Multilabel Plate Reader. Dexamethasone was used as a positive control.

Cytotoxicity

The cytotoxicity of compounds 1−5 were tested in vitro using human HCT-8 colon, A2780 ovary, BGC-823 stomach, and Bel-7402 hepatoma, and A549 lung cell lines. MTT assay was used as previously described[3].

References


[3] Zhang SY, Guo Q, Gao XL, et al. A phytochemical and pharma-


[6] Editorial Committee of Chinese Materia Medica, State Administra-
tion Bureau of Traditional Chinese Medicine. Chinese Ma-


[8] Wu YC, Liou JY, Duh CY, et al. Litebamine, a new phenan-


[13] Li XT, Xia H, Wang LY, et al. Lignans from the twigs of Lit-


[19] Li R, Zhang JF, Wu YZ, et al. Structures and biological evalu-


