Lignans from the Twigs of *Litsea cubeba* and Their Bioactivities

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**Abstract:** *Litsea cubeba*, an important medicinal plant, is widely used as a traditional Chinese medicine and spice. Using cytotoxicity-guided fractionation, nine new lignans 1–9 and ten known analogues 10–19 were obtained from the EtOH extract of the twigs of *L. cubeba*. Their structures were assigned by extensive 1D- and 2D-NMR experiments, and the absolute configurations were resolved by specific rotation and a combination of experimental and theoretically calculated electronic circular dichroism (ECD) spectra. In the cytotoxicity assay, 7′,9-epoxylignans with feruloyl or cinnamoyl groups (compounds 7–9, 13 and 14) were selectively cytotoxic against NCI-H1650 cell line, while the dibenzylbutyro lactone lignans 17–19 exerted cytotoxicities against HCT-116 and A2780 cell lines. The results highlighted the structure-activity relationship importance of a feruloyl or a cinnamoyl moiety at C-9′ or C-7 ketone in 7′,9-epoxylignans. Furthermore, compound 11 was moderate active toward protein tyrosine phosphatase 1B (PTP1B) with an IC_{50} value of 13.5 µM, and compounds 4–6, 11 and 12 displayed inhibitory activity against LPS-induced NO production in RAW264.7 macrophages, with IC_{50} values of 46.8, 50.1, 58.6, 47.5, and 66.5 µM, respectively.

**Keywords:** *Litsea cubeba*; cytotoxicity; isolation and elucidation; lignans

1. Introduction

Plants from the *Litsea* species (Lauraceae) are widely distributed in tropical or subtropical areas. *Litsea cubeba*, mainly grown in the east and south of China, is broadly used as a traditional Chinese medicine and spice. “Bi-cheng-qie” and “dou-chi-jiang”, the dried fruits and roots of *L. cubeba*, respectively, have been documented in the Chinese Pharmacopoeia and *Chinese Materia Medica* as two important traditional Chinese medicines for the treatment of various ailments, including coronary disease, cerebral apoplexy, asthma, and rheumatic arthritis [1–3]. Moreover, *Litsea cubeba* fruits are also important spices and great sources of essential oils which are often used as flavor enhancers in foods, cigarettes, and cosmetics [4]. Previous phytochemical investigation of the fruits and roots of *L. cubeba* have reported the discovery of aporphine-type alkaloids, lignans, and phenolic constituents [5–11]. Among them, aporphine-type alkaloids and lignans were considered as the major active principles of
this plant due to their antithrombotic, anti-inflammatory, and antinociceptive properties [8,9,12–15]. Since there are few reports on the phytochemicals of twigs of L. cubeba, a recent study on L. cubeba twigs by our group led to the characterization of 36 aromatic glycosides from the the water-soluble fraction of an ethanolic extract. Interestingly, some lignan glycosides showed potent hepatoprotective and HDAC1 inhibitory activity [16,17]. In the present study, we have investigated the constituents of the EtOAc-soluble fraction of the ethanolic extract of L. cubeba twigs. Bioassay-guided isolation of a fraction with cytotoxicity against HCT-116, NCI-H1650, and A2780 cell lines (IC50 = 28.3, 11.5, and 16.8 μg/mL, respectively) led to the discovery of nine new lignans (Figure 1). The structures of 1–9 were elucidated by spectroscopic methods, and their absolute configurations were determined by optical rotations and a combination of experimental and theoretically calculated electronic circular dichroism (ECD) spectra. Detailed herein are the isolation, structural elucidation, and bioactivity assay of compounds 1–19.

2. Results and Discussions

2.1. Structure Elucidation

The EtOAc extract of the twigs of L. cubeba was subjected to column chromatography on silica gel to give 13 fractions (F1–F13). Cytotoxicity assays found that F9 displayed potent activities against HCT-116, NCI-H1650, and A270 cell lines. Fractionation of F9 by Sephadex LH-20, RP-18, preparative TLC, and preparative HPLC led to the discovery of nine new lignans 1–9 and ten analogues 10–19 (Figure 1). Compound 1 was obtained as a white amorphous powder. The presence of amide (1643 cm−1), aromatic ring (1611, 1516, and 1459 cm−1), and hydroxy (3372 cm−1) functionalities were evident in its IR spectrum. Its molecular formula of C39H33NO6 with fifteen degrees of unsaturation was established by HREIMS based on the [M + H]+ ion at m/z 552.2234 (calcd. 552.2228) and 13C-NMR spectrum. In the 1H-NMR spectrum recorded in acetone-d6, the signals for an aromatic singlet integrated for two protons at δ 6.39 (2H, s, H-2′ and H-6′), a methoxy singlet integrated for six protons at δ 3.67 (6H, s, OMe×6), suggested a 1-substituted-3,5-dimethoxy-4-hydroxybenzene ring in 1. Signals of a singlet proton at δ 6.74 and two methoxy protons at δ 3.86 and 3.58 revealed a pentasubstituted aromatic ring attached two methoxy groups. These 1H-NMR signals, together with another two singlet protons...
The complete trans protons at 2HMBC correlations with the amide carbon at a 3-methoxytyramine moiety (Table 1; Table 2). In the HMBC spectrum of 1 isolated from this plant [20], with the only difference being the replacement of one of a tyramine group by a 3-methoxytyramine moiety (Table 1; Table 2). In the HMBC spectrum of 1 displayed 30 carbon signals, of which twelve could be assigned to be a tyramine moiety (δC 131.2, 130.5 × 2, 116.0 × 2, 156.6, 35.6, 42.2) and four methoxy groups (δC 56.6 × 2, 56.5, 60.4), and the remaining eighteen carbons were consistent with the 2,7'-cyclolignan-7-en skeleton. The complete 1H- and 13C-NMR assignments of 1 were made by a combination of 1D- and 2D-NMR experiments. In the HMBC spectrum of 1, the two or three bonds long range correlations from H-6 to C-2, C-4, and C-7, from H-7 to C-2, C-6, C-9, and C-8', from H-7' to C-3, C-8, C-2' (C-6'), and C-9', from H-8' to C-2, C-7, C-9, and C-1', from H-2'9' to C-8 and C-7', and from the methoxy protons at δH 3.58 to C-3' (C-5') (Figure 2) confirmed the 2,7'-cyclolignan-7-en type lignan containing a 3,5-dimethoxy-4-hydroxy-benzene moiety. The NOESY correlation observed between H-6 and the methoxy protons at δH 3.86 together with the HMBC correlation observed for these methoxy protons and C-5 gave the evidence for the location of one methoxy group at C-5. Key HMBC cross-peaks, such as between methoxy protons at δH 3.58 and C-3, as well as between OH proton at δH 7.76 and C-4, served to locate this methoxy and OH group at C-3 and C-4, respectively. Furthermore, the tyramine was linked to C-9 to form an amine bond, according to the HMBC correlations from both H2-8' and NH proton to C-9. Therefore, these data completed the planar structure of 1 as N-[2-(4-hydroxyphenyl)-ethyl]-4,4'-trihydroxy-3,5,3',5'-tetramethoxy-2,7'-cyclolignan-7-en-9-amide. H-7″ showed the dihedral angle for the vicinal protons of H-7' and H-8' was nearly 90°, requiring a trans relationship of H-7' and H-8'. This assignment was also supported by the NOESY correlations of H-7' with H-9', and H-8' with H-2' (H-6'). Finally, the negative optical rotation of 1 demonstrated the 7'R,8'S absolute configuration of 1 [18,19]. Hence, compound 1 was defined as (−)(7'R,8'S)-N-[2-(4-hydroxyphenyl)-ethyl]-4,4',9'-trihydroxy-3,5,3',5'-tetramethoxy-2,7'-cyclolignan-7-en-9-amide.

Compound 2 was isolated as a white amorphous powder. The IR spectrum exhibited absorptions of hydroxy (3362 cm⁻¹), amide (1649 cm⁻¹), and aromatic (1612 and 1516 cm⁻¹) moieties. Its molecular formula was deduced as C₃₉H₄₂N₂O₁₁ from the negative HRESIMS at m/z 713.2719 [M − H]⁻ (calcld. 713.2716) and the 13C-NMR spectrum. This indicated twenty degrees of unsaturation. The NMR spectra of 2 were very similar to those of compound 10, a known lignan diamide that was also isolated from this plant [20], with the only difference being the replacement of one of a tyramine group by a 3-methoxytyramine moiety (Table 1; Table 2). In the HMBC spectrum of 2, H₂-7″ showed HMBC correlations with the amide carbon at δC 171.4, which indicated that the 3-methoxytyramine moiety was connected to C-9' via an amide bond (Figure 2). In the 1D NOE difference spectrum of 2, H-8' was enhanced upon irradiation of H-2' (H-6'). This enhancement, together with H-7' presented in a singlet, revealed a trans-orientational relationship of H-7' and H-8'. Finally, on the basis of the negative optical rotation of 2 and biosynthetic considerations, the structure of compound 2 was defined as (−)(7'R,8'S)-N₁-[2-(4-hydroxyphenyl)-ethyl]-N₂-[2-(4-hydroxy-3-methoxyphenyl)-ethyl]-4,4'-dihydroxy-3,5,3',5'-tetramethoxy-2,7'-cyclolignan-7-en-9,9'-diamide.
Table 1. $^1$H-NMR Data ($\delta_H$ (mult, $J$, Hz)) of Compounds 1-9 in Acetone-d$_6$.

| No. | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 2   |     |     |     |     |     |     |     |     |     |
| 5   |     |     |     |     |     |     |     |     |     |
| 6   | 6.74 s | 6.69 s | 6.60 s |     |     |     |     |     |     |
| 7   | 7.19 s | 7.18 s | 7.21 s |     |     |     |     |     |     |
| 8   |     |     |     |     |     |     |     |     |     |
| 9   |     |     |     |     |     |     |     |     |     |
| 2'  |     |     |     |     |     |     |     |     |     |
| 5'  |     |     |     |     |     |     |     |     |     |
| 6'  | 6.39 s | 6.38 s | 6.38 s |     |     |     |     |     |     |
| 7'  | 4.62 s | 5.03 s | 5.03 s |     |     |     |     |     |     |
| 8'  | 3.14 dd (7.5, 7.5) | 3.66 s | 3.67 s | 1.99 m | 1.99 m | 1.99 m | 3.01 m | 2.61 m |     |
| 9'  | 3.59 m 3.28 m |     |     |     |     |     |     |     |     |
| 2'' |     |     |     |     |     |     |     |     |     |
| 3'' |     |     |     |     |     |     |     |     |     |
| 5'' | 6.71 d (8.5) | 6.72 d (8.5) | 6.71 d (7.8) |     |     |     |     |     |     |
| 6'' | 6.98 d (8.5) | 6.98 d (8.5) | 6.98 d (8.5) | 6.61 dd (7.1, 1.8) |     |     |     |     |     |
| 7'' | 2.69 t (7.5) | 2.70 t (7.0) | 2.72 t (7.2) |     |     |     |     |     |     |
| 8'' | 3.39 dt (7.4, 4.5) | 3.41 t (6.0) | 3.47 m, 3.39 m |     |     |     |     |     |     |
| 2''' |     |     |     |     |     |     |     |     |     |
| 3''' |     |     |     |     |     |     |     |     |     |
| 5''' | 6.69 d (8.0) | 6.70 d (8.4) |     |     |     |     |     |     |     |
| 6''' | 6.55 dd (8.0, 1.5) | 6.93 d (8.4) |     |     |     |     |     |     |     |
| 7''' | 2.58 t (7.0) | 2.56 t (7.2) |     |     |     |     |     |     |     |
| 9''' | 3.28 (7.0) |     |     |     |     |     |     |     |     |
| OMe-3 | 3.58 s | 3.69 s | 3.69 s | 3.75 s | 3.73 s | 3.75 s | 3.84 s | 3.79 s | 3.80 s |
| OMe-5 | 3.86 s | 3.85 s | 3.85 s |     |     |     |     |     |     |
| OMe-7 |     |     |     |     |     |     |     |     |     |
| OMe-3' | 3.67 s | 3.67 s | 3.67 s |     |     |     |     |     |     |
| OMe-5' | 3.67 s | 3.67 s |     |     |     |     |     |     |     |
| OMe-3'' | 3.78 s | 3.88 s |     |     |     |     |     |     |     |
| OMe-5'' |     |     |     |     |     |     |     |     |     |
| OMe-3''' |     |     |     |     |     |     |     |     |     |
| OH-4 | 7.76 s | 7.78 s | 7.79 s | 7.29 s | 6.91 s | 7.27 s |     | 7.09 s |     |
| OH-4' | 6.90 s | 6.91 s | 6.91 s | 7.26 s | 6.89 s | 7.24 s |     | 6.98 s |     |
| OH-4'' |     |     |     |     |     |     |     |     |     |
| OH-4''' | 7.26 s | 8.07 s |     |     |     |     |     |     |     |
| NH | 7.45 t (4.5) | 7.81 t (4.5), 7.59 t (4.5) | 7.72 t (4.5), 7.59 t (4.5) |     |     |     |     |     |     |

$^a$ $^1$H-NMR data (δ) were measured at 600 MHz or 500 MHz. The assignments were based on $^1$H-$^1$H COSY, HSQC, and HMBC experiments.
Table 2. $^{13}$C-NMR Data ($\delta$C) for Compounds 1–9 in Acetone-$d_6$.

| No. | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1   | 132.0 | 123.8 | 123.8 | 132.9 | 132.4 | 132.9 | 129.6 | 131.8 | 134.5 |
| 2   | 124.6 | 126.5 | 126.5 | 113.2 | 107.2 | 113.2 | 107.2 | 106.9 | 106.9 |
| 3   | 147.0 | 146.4 | 146.4 | 148.1 | 148.5 | 148.1 | 148.4 | 148.9 | 148.5 |
| 4   | 141.8 | 142.4 | 142.4 | 145.5 | 134.9 | 145.5 | 142.2 | 135.2 | 136.0 |
| 5   | 148.2 | 148.1 | 148.1 | 115.5 | 148.5 | 115.5 | 148.4 | 148.9 | 148.5 |
| 6   | 108.0 | 108.3 | 108.2 | 122.3 | 107.2 | 122.3 | 107.2 | 106.9 | 106.9 |
| 7   | 131.5 | 132.5 | 133.6 | 35.4  | 35.9  | 35.4  | 198.2 | 34.2  | 82.6  |
| 8   | 124.4 | 128.3 | 128.4 | 40.7  | 40.6  | 40.8  | 47.6  | 43.6  | 48.1  |
| 9   | 169.1 | 169.8 | 169.6 | 65.2  | 65.2  | 65.2  | 71.1  | 73.3  | 70.3  |

| 1′  | 135.9 | 135.1 | 135.1 | 133.4 | 131.8 | 133.4 | 132.9 | 134.6 | 131.7 |
| 2′  | 106.4 | 106.4 | 106.4 | 113.2 | 107.3 | 113.2 | 104.7 | 104.3 | 104.4 |
| 3′  | 148.3 | 148.4 | 148.4 | 148.1 | 148.5 | 148.1 | 148.6 | 148.7 | 148.8 |
| 4′  | 135.3 | 135.5 | 135.5 | 145.6 | 135.0 | 145.5 | 136.3 | 136.0 | 136.5 |
| 5′  | 148.3 | 148.4 | 148.4 | 115.4 | 148.5 | 115.4 | 148.6 | 148.7 | 148.8 |
| 6′  | 106.4 | 106.4 | 106.4 | 122.3 | 107.3 | 122.3 | 107.2 | 104.3 | 104.4 |
| 7′  | 39.0  | 39.5  | 39.6  | 34.9  | 35.4  | 35.0  | 84.9  | 84.5  | 85.1  |
| 8′  | 46.1  | 49.1  | 49.1  | 44.1  | 44.1  | 44.2  | 51.5  | 50.3  | 49.4  |
| 9′  | 64.6  | 171.4 | 171.4 | 62.1  | 62.1  | 62.1  | 62.8  | 63.4  | 63.6  |
| 1″  | 131.2 | 131.1 | 131.7 | 126.1 | 127.4 | 127.5 | 127.2 | 126.0 | 127.3 |
| 2″  | 130.5 | 130.6 | 113.1 | 106.8 | 111.3 | 11.3  | 11.0  | 106.7 | 111.3 |
| 3″  | 116.0 | 116.1 | 148.2 | 148.9 | 148.7 | 148.8 | 148.6 | 148.7 | 148.8 |
| 4″  | 156.6 | 156.7 | 145.9 | 139.4 | 150.1 | 150.1 | 149.9 | 139.5 | 150.1 |
| 5″  | 116.0 | 115.7 | 148.9 | 116.1 | 116.0 | 115.9 | 148.6 | 116.1 | 116.1 |
| 6″  | 130.5 | 130.6 | 122.0 | 106.8 | 123.9 | 124.0 | 123.7 | 106.7 | 123.8 |
| 7″  | 35.6  | 35.5  | 36.0  | 145.9 | 145.6 | 145.6 | 145.6 | 146.2 | 145.8 |
| 8″  | 42.2  | 42.4  | 42.3  | 116.2 | 116.0 | 116.0 | 115.1 | 115.9 | 114.8 |
| 9″  | 167.5 | 167.6 | 167.5 | 167.5 | 166.7 | 167.3 | 167.3 | 167.3 | 167.3 |

| 1‴  | 131.8 | 131.2 | 113.0 | 130.6 | 148.2 | 116.0 | 145.8 | 156.6 | 115.6 | 116.0 |
| 2‴  | 36.1  | 35.7  | 41.9  | 42.1  | 56.5  | 56.5  | 56.1  | 56.7  | 56.6  | 56.6 |
| 3‴  | 41.9  | 42.1  | 56.6  | 56.5  | 56.1  | 56.7  | 56.6  | 56.6  | 56.6  | 56.6 |
| 4‴  | 56.5  | 56.2  | 60.3  | 60.3  | 56.5  | 56.5  | 56.1  | 56.7  | 56.6  | 56.6 |
| 5‴  | 56.5  | 56.2  | 60.3  | 60.3  | 56.5  | 56.5  | 56.1  | 56.7  | 56.6  | 56.6 |
| 6‴  | 56.5  | 56.2  | 60.3  | 60.3  | 56.5  | 56.5  | 56.1  | 56.7  | 56.6  | 56.6 |
| 7‴  | 56.5  | 56.2  | 60.3  | 60.3  | 56.5  | 56.5  | 56.1  | 56.7  | 56.6  | 56.6 |
| 8‴  | 56.5  | 56.2  | 60.3  | 60.3  | 56.5  | 56.5  | 56.1  | 56.7  | 56.6  | 56.6 |

* $^{13}$C-NMR data (δC) were measured at 150 MHz or 125 MHz. The assignments were based on $^1$H-$^1$H COSY, HSQC, and HMBC experiments.

Compound 3 gave the same molecular formula, C$_{36}$H$_{42}$N$_2$O$_{11}$, as that of 2 by analysis of the HRESIMS. Compound 3 shared almost identical UV, IR, and $^1$H- and $^{13}$C-NMR features to those of 2, which suggested that they both contained the 4,4′-dihydroxy-3,5,3′,5′-tetramethoxy-2,7′-cyclolignan-7-en-9,9′-diamide core, a tyramine, and a 3-methoxytyramine moiety.

Further analysis of 2D-NMR data permitted the tyramine and 3-methoxytyramine moieties to be located at C-9′ and C-9 in 3, the reverse of 2, via the amide bonds (Figure 2), respectively. Analysis of the 1D NOE difference spectrum of 3 and its optical rotation indicated that 3 had the same absolute configuration as 2. Therefore, the structure of 3 was confirmed as (−)-(7′R,8′S)-.
Compound 4 was obtained as a yellow solid and its molecular formula was deduced as C_{34}H_{56}O_{10} from HRESIMS. The IR spectrum exhibited absorption bands at 3391, 1608, and 1516 cm\(^{-1}\) due to the aromatic and hydroxy groups. The NMR data of 4 showed signals similar with secoisolariciresinol (Table 1; Table 2) [21,22]. However, both the H\(_2\)-9 and C-9 were shifted downfield when compared with secoisolariciresinol. Besides, the \(^1\)H- and \(^{13}\)C-NMR signals attributed to a trans-cinnamylxylo unit were present (Table 1; Table 2). These were consistent with the substitution of the trans-cinnamylxylo at C-9, which was verified by the key HMBC cross-peak from H-9 to C-9\(^\text{′}\). The positive optical rotation of 4 supported the same (8S,8′S) configuration as that of the known compound (+)-(8S,8′S)-9,9′-di-O-(E)-feruloyl-secoisolariciresinol (11), which has been also isolated from this plant [12]. The (8S,8′S) configuration was confirmed by the evidence that compound 4 showed optical rotation opposite to that of (−)-1-O-feruloylsecoisolariciresinol [21]. Thus, the structure of 4 was defined as (+)-(8S,8′S)-9-O-(E)-cinnamoyl-secoisolariciresinol.

The molecular formula of compound 5 was C_{32}H_{58}O_{11} from the HRESIMS data. Analysis of the 1D- and 2D-NMR data revealed that its planar structure was completely identical to the known lignan, (−)-(8R,8′R)-9-O-(E)-feruloyl-5,5′-dimethoxysecoisolariciresinol, but their specific rotation was inverse [23]. Taking into account that 4 was the 5-methoxy analogue of 5 and they displayed similar specific rotation, it is proposed that they both have the (8S,8′S) configuration. Thus, the structure of 5 was defined as (+)-(8S,8′S)-9-O-(E)-feruloyl-5,5′-dimethoxysecoisolariciresinol.

The planar structure of 6 was proved to be identical to (−)-(8R,8′R)-9-O-(E)-feruloyl-secoisolariciresinol (different nomenclature was used in literature [21]) after analysis of the HRMS, and 1D- and 2D-NMR data of 6. However, the optical rotation of 6 was opposite for (−)-(8R,8′R)-9-O-(E)-feruloyl-secoisolariciresinol [21]. Thus, the structure of 6 was determined as (+)-(8S,8′S)-9-O-(E)-feruloyl-secoisolariciresinol.

Compound 7, an amorphous powder, was determined to have the molecular formula of C_{32}H_{54}O_{12} by HRESIMS. The NMR spectra of 7 were similar to the co-occurring (+)-9′-O-trans-feruloyl-5,5′-dimethoxyariciresinol (13) [24], with the only difference being the replacement of the CH\(_2\) group by a ketone. These data demonstrated the presence of a ketone moiety at C-7 in 7. This inference was confirmed by the HMBC cross-peak of H-2(6)/C-7, H\(_2\)-9/C-7, and H-8′/C-7. The coupling constant of H-7′ (\(J = 7.5\) Hz) indicated a trans relationship of H-7′/H-8′. The presence of correlations of H-7′/H\(_2\)-9′ and H-2(6)/H-8′ and the absence of H-8/H\(_2\)-9′ were observed in the NOESY spectrum of 7, which confirmed that H-7′ was oriented opposite to H-8 and H-8′. The absolute configuration of 7 was established by quantum chemical ECD calculation (Supplementary Materials). The calculated ECD curve for 8R,7′,S,8′R-isomer matched well with the experimental ECD spectrum of 7 (Figure 3), which suggested compound 7 had the (8R,7′S,8′R) absolute configurations. Based on these observations, the structures of 7 was assigned as (+)-(8R,7′S,8′R)-9′-O-(E)-feruloyl-5,5′-dimethoxyariciresinol-7-one.

The molecular formula of compound 8 was C_{33}H_{58}O_{12} as indicated by the HRESIMS. The NMR spectra of 8 and (+)-9′-O-trans-feruloyl-5,5′-dimethoxyariciresinol were closely comparable [24], except for the replacement of (E)-feruloyl group by the (E)-cinnamoyl group. The structure of 8 was confirmed by the 2D-NMR HSQC, COSY, HMBC, and NOESY data. Also, the NOESY correlations of H-7′/H\(_2\)-9′ and H\(_2\)-7′/H\(_2\)-9′ revealed that compounds 7 and 8 have the same relative configuration. Therefore, on the positive optical rotation of 8 and biosynthetic considerations, the structure of 8 was deduced as (+)-(8R,7′S,8′R)-9′-O-(E)-cinnamoyl-5,5′-dimethoxyariciresinol.

Compound 9 was shown to have the molecular formula of C_{33}H_{58}O_{12}, as established by the HRESIMS. The \(^1\)H- and \(^{13}\)C-NMR spectra of 9 closely resembled those of 7, the only discernable difference being the presence of a new methoxy moiety and lack of a ketone moiety in 9, suggesting that compound 9 contains a methoxy moiety rather than a ketone moiety at C-7. This was confirmed from the COSY correlation of H-7/H-8 and HMBC correlation of OMe/C-7. In the NOESY spectrum of
9, the NOE correlations of H-7/H2-9′ and H-7′/H2-9′ also verified that H-7′ was oriented opposite to H-8 and H-8′. Thus, the structure of 9 was defined as 9′-O-(E)-feruloyl-5,7,5′-trimethoxy-lariciresinol.

The known compounds were identified as 1,2-dihydro-6,8-dimethoxy-7-hydroxy-1-(3,5-dimethoxy-4-hydroxyphenyl)-N1,N2-bis-[2-(4-hydroxyphenyl)ethyl]-2,3-naphthalene dicarboxamide (10) [20], (+)-9,9′-O-di-(E)-feruloyl-5,5′-dimethoxy secoisolariciresinol (11) [25], (+)-9,9′-O-di-(E)-feruloyl-secoisolariciresinol (12) [12], (+)-9′-O-(E)-feruloyl-5,5′-dimethoxylariciresinol (13) [24], (+)-9′-O-(E)-feruloyl-5′-methoxylariciresinol (14) [26], (+)-5,5′-dimethoxylariciresinol (15) [27], (+)-5′-methoxylariciresinol (16) [28], arctigenin (17), matairesinol (18) [29], and (7E,8R′)-didehydroarctigenin (19) [30], respectively, by spectroscopic analysis and comparison of the data obtained with literature values.

![Figure 3](image-url)

**Figure 3.** The experimental ECD spectrum of 7 (black), and the calculated ECD spectra of (8R,7′S,8′R)-7 (red) and (8S,7′R,8′S)-7 (blue).

### 2.2. Biological Activities of Compounds 1–19

#### 2.2.1. Cytotoxic Activity

The task of IC₅₀ assessment for all isolates against human colon cancer (HCT-116), human non-small-cell lung carcinoma (NCI-H1650), and human ovarian cancer (A2780) cell lines began immediately following the purification and characterization of each lignan.

Of the compounds, only 7′,9-epoxylignans with feruloyl or cinnamoyl group (compounds 7–9, 13 and 14) were selectively cytotoxic against NCI-H1650 cell line, with IC₅₀ values of less than 20 μM. These results suggested the presence of a feruloyl or a cinnamoyl moiety at C-9′ in 7′,9-epoxylignans is essential for cytotoxicity against NCI-H1650 cell line. It is noteworthy that compound 7 displayed 4-6 folds more active than 8, 9, 13, and 14, indicating that the presence of the C-7 ketone could enhance the bioactivity. In addition, the dibenzylbutyro lactone lignans (17–19) exerted cytotoxicities against HCT-116 and A2780 cell lines, with IC₅₀ values ranging from 0.28 to 18.47 μM (Table 3), but less potent than the positive control taxol (IC₅₀ = 0.005 and 0.02 μM, respectively). Interestingly, the addition of the double bond at C-7–C-8 on 19 resulted in 4–40 folds less active than 17 and 18. This implied that the C-7–C-8 double bond could reduce the cytotoxicity, especially against the A2780 cell line.

#### 2.2.2. Inhibitory Activity of Protein Tyrosine Phosphatase 1B

The isolates were also evaluated for inhibitory activities against protein tyrosine phosphatase 1B (PTP1B). Only compound 11 was moderate active toward PTP1B with an IC₅₀ value of 13.5 μM. The positive control oleancic acid gave an IC₅₀ value of 3.82 μM.
2.2.3. Anti-Inflammatory Activity

The inhibitory activity of compounds 1–19 against LPS-induced NO production in RAW264.7 macrophages was examined in this study. As a result, compounds 4–6, 11 and 12 displayed inhibitions against LPS-induced NO production in RAW264.7 macrophages, with IC<sub>50</sub> values of 46.8, 50.1, 58.6, 47.5, and 66.5 µM, respectively. Dexamethasone was used as positive control with an IC<sub>50</sub> value of 9.5 µM.

| Compound | IC<sub>50</sub> (µM) |
|----------|-----------------------|
|          | HCT-116 | NCI-H1650 | A2780 |
| 1        | >20     | >20       | >20   |
| 2        | >20     | >20       | >20   |
| 3        | >20     | >20       | >20   |
| 4        | >20     | >20       | >20   |
| 5        | >20     | >20       | >20   |
| 6        | >20     | >20       | >20   |
| 7        | >20     | 2.47      | >20   |
| 8        | >20     | 11.25     | >20   |
| 9        | >20     | 13.16     | >20   |
| 10       | >20     | >20       | >20   |
| 11       | >20     | >20       | >20   |
| 12       | >20     | >20       | >20   |
| 13       | >20     | 9.68      | >20   |
| 14       | >20     | 10.52     | >20   |
| 15       | >20     | >20       | >20   |
| 16       | >20     | >20       | >20   |
| 17       | 3.25    | >20       | 0.28  |
| 18       | 13.95   | >20       | 1.53  |
| 19       | 18.47   | >20       | 12.8  |
| Taxol<sup>a</sup> | 0.005 | 1.28 | 0.02 |

<sup>a</sup> Taxol was used as a positive control.

3. Materials and Methods

3.1. General Experimental Procedures

Optical rotations were measured on an Autopol III automatic polarimeter (Rudolph Research, Hackettstown, NJ, USA). UV spectra were measured on a Cary 300 spectrometer (Agilent, Melbourne, Australia). ECD spectra were recorded on a J-815 spectrometer (JASCO, Tokyo, Japan). IR spectra were acquired on an Impact 400 FT-IR Spectrophotometer (Nicolet, Madison, WI, USA). Standard pulse sequences were used for all NMR experiments, which were run on either a Bruker spectrometer (600 MHz for <sup>1</sup>H or 150 MHz for <sup>13</sup>C, Karlsruhe, Germany) or a Varian INOVA spectrometer (500 MHz for <sup>1</sup>H or 125 MHz for <sup>13</sup>C, Palo Alto, CA, USA) equipped with an inverse detection probe. Residual solvent shifts for acetone-<sup>d6</sup> were referenced to δ<sub>H</sub> 2.05, δ<sub>C</sub> 206.7 and 29.9, respectively. Accurate mass measurements were obtained on a Q-Trap LC/MS/MS (Turbo ionspray source) spectrometer (Sciex, Toronto, ON, Canada). Column chromatography (CC) was run using silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden). HPLC separation was done on Waters HPLC components (Milford, MA, USA) comprising of a Waters 600 pump, a Waters 600 controller, a Waters 2487 dual λ absorbance, with GRACE preparative (250 x 19 mm) Rp C<sub>18</sub> (5 µm) columns.
3.2. Plant Material

The twigs of Litsea cubeba were collected in Zhaotong, Yunnan Province, People’s Republic of China, in May 2013, and identified by Prof. Gan-Peng Li at Yunnan Minzu University. A herbarium specimen was deposited in at the Herbarium of the Department of Medicinal Plants, Institute of Materia Medica, Beijing 100050, People’s Republic of China (herbarium No. 2013-05-10).

3.3. Extraction and Isolation

The air-dried twigs of L. cubeba (12 kg) were ground and extracted using 30.0 L of 95% EtOH under ambient temperature for 3 × 48 h. The EtOH extract was concentrated in vacuo and the residue was suspended in H2O, then partitioned with EtOAc, to afford EtOAc and H2O soluble extracts.

The EtOAc fraction (300 g) was chromatographed over silica gel (1500 g), eluting with a gradient of acetone (0−100%) in petroleum ether, and 13 fractions (F1−F13) was obtained based on the TLC analysis. F9 (12.0 g), which showed potent cytotoxicity against HCT-116, NCI-H1650, and A270 cell lines, was subjected to the reversed-phase flash chromatography over C-18 silica gel, eluting with a step gradient from 20 to 95% MeOH in H2O, to give 15 fractions (F9.1−F9.15). F9.8 (1.5 g) was separated on Sephadex LH-20 eluting with petroleum CHCl3-MeOH (1:1) to give three subfractions. The second subfraction was further purified by preparative TLC with CHCl3-acetone (20:1) to give 17 (25 mg), 18 (18 mg), and 19 (23 mg). The fourth subfraction was purified by reversed-phase preparative HPLC (RP18, 5 µm, 254 nm, MeOH-H2O, 75:25) to yield 1 (9.2 mg). The second and third subfractions were further purified by preparative TLC developed with CHCl3-MeOH (15:1) to afford 15 (52 mg), 16 (35 mg), and 18 (29 mg). F6.9 (1.0 g) was fractionated on a Sephadex LH-20 column using CHCl3-MeOH (1:1) as the eluent to yield five corresponding subfractions. Compound 10 (55 mg) was crystallized from a Me2CO solution of the second subfraction. The third subfraction was further purified by preparative TLC with CHCl3-MeOH (20:1) to give 17 (17 mg) and 19 (8 mg). The fourth subfraction was purified by reversed-phase preparative HPLC (RP18, 5 µm, 254 nm, MeOH-H2O, 85:15) to give 2 (56 mg), 3 (21 mg), and 14 (23 mg). Using the same HPLC system, the fifth subfraction afforded 7 (27 mg), 8 (12 mg) and 9 (8 mg), and 13 (17 mg). F6.10 (1.2 g) was chromatographed over Sephadex LH-20 eluting with CHCl3-MeOH (1:1), and then further separated by reversed-phase preparative HPLC (RP18, 5 µm, 254 nm, MeOH-H2O, 90:10), to afford 4 (8 mg) and 5 (5 mg). F8.11 (0.8 g) was fractionated on a Sephadex LH-20 column with CHCl3-MeOH (1:1) as the eluent to give three subfractions. The second and third subfractions were further purified by reversed-phase preparative HPLC (RP18, 5 µm, 254 nm, MeOH-H2O, 90:10) to afford 6 (12 mg), 11 (23 mg), and 12 (15 mg).

3.4. (−)-(7′R,8′S)-N-[2-(4-Hydroxyphenyl)-ethyl]-4,4′,9′-trihydroxy-3,5,3′,5′-tetramethoxy-2,7′-cyclolignan-7-en-9-amide (1)

White, amorphous powder. [α]D 20 −35.0 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 204 (4.04), 200 (2.32), 245 (2.12), 324 (1.13) nm; IR (KBr) νmax 3372, 2935, 2849, 1643, 1611, 1516, 1459, 1427, 1329, 1286, 1218, 1115, 1030, 961, 912, 834, 646 cm−1; 1H-NMR (acetone-d6, 500 MHz) and 13C-NMR (acetone-d6, 125 MHz) data, see Table 1; Table 2; ESIMS m/z 574 [M + Na]+ and 550 [M − H]−; HRESIMS m/z 552.2234 [M + H]+ (calcd. for C39H33N2O9, 552.2228) and 574.2048 [M + Na]+ (calcd. for C39H33N2O9Na, 574.2048).

3.5. (−)-(7′R,8′S)-N1-[2-(4-Hydroxyphenyl)-ethyl]-N2-[2-(4-hydroxy-3-methoxyphenyl)-ethyl]-4,4′-dihydroxy-3,3′,5′,5′′-tetramethoxy-2,7′′-cyclolignan-7-en-9,9′-diamide (2)

White, amorphous powder. [α]D 20 −23.0 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 204 (4.11), 250 (0.86), 281 (0.30), 328 (0.42) nm; IR (KBr) νmax 3362, 2919, 2851, 1736, 1649, 1612, 1516, 1464, 1424, 1372, 1328, 1274, 1217, 1115, 1035, 890, 834, 802, 721, 640 cm−1; 1H-NMR (acetone-d6, 600 MHz) and 13C-NMR (acetone-d6, 150 MHz) data, see Table 1; Table 2; ESIMS m/z 713 [M − H]−; HRESIMS m/z 713.2719 [M − H]− (calcd. for C39H41N2O11, 713.2716).
3.6. (−)-(7R,8S)-N-[(2-(4-Hydroxy-3-methoxyphenyl)-ethyl]-N2-[(2-(4-hydroxyphenyl)-ethyl]-4,4-dihydroxy-3,5,3',5'-tetramethoxy-2,7'-cyclolignan-7-en-9,9'-diamide (3)

White, amorphous powder. [α]D20 25.0 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 204 (4.12), 248 (0.82), 285 (0.27), 333 (0.45) nm; IR (KBr) νmax 3391, 2920, 2851, 1647, 1611, 1541, 1517, 1465, 1425, 1367, 1278, 1203, 1116, 1035, 932, 888, 829, 801, 722, 650, 599 cm−1; 1H-NMR (acetone-d6, 600 MHz) and 13C-NMR (acetone-d6, 150 MHz) data, see Table 1; Table 2; ESIMS m/z ESIMS m/z 713 [M – H]+; HRESIMS m/z 713.2715 [M – H]+ (calcd. for C39H41N2O11, 713.2716).

3.7. (+)-(8S,8'S)-9-O-(E)-Cinnamoyl-secoisolariciresinol (4)

Yellow solid. [α]D20 +18.2 (c 0.05, MeOH); UV (MeOH) λmax (log ε) 204 (4.12), 230 (0.82), 287 (0.39), 329 (0.78) nm; IR (KBr) νmax 3391, 2920, 2850, 1683, 1645, 1608, 1516, 1463, 1428, 1375, 1341, 1272, 1237, 1155, 1119, 1033, 875, 820, 799, 721, 631 cm−1; 1H-NMR (acetone-d6, 500 MHz) and 13C-NMR (acetone-d6, 125 MHz) data, see Table 1; Table 2; ESIMS m/z 567 [M – H]+; HRESIMS m/z 569.2387 [M + H]+ (calcd. for C31H37NO10, 569.2381) and 591.2204 [M + Na]+ (calcd. for C31H38O11Na, 621.2306).

3.8. (+)-(8S,8'S)-9-O-(E)-Feruloyl-5,5'-dimethoxysecoisolariciresinol (5)

Yellow solid. [α]D20 +22.2 (c 0.05, MeOH); UV (MeOH) λmax (log ε) 206 (4.22), 234 (0.84), 284 (0.36), 326 (0.82) nm; IR (KBr) νmax 3394, 2921, 2850, 1696, 1604, 1517, 1461, 1428, 1370, 1328, 1273, 1218, 1161, 1117, 1033, 984, 915, 825, 721, 645, 604 cm−1; 1H-NMR (acetone-d6, 600 MHz) and 13C-NMR (acetone-d6, 150 MHz) data, see Table 1; Table 2; HRESIMS m/z 621.2299 [M + Na]+ (calcd. for C32H38O11Na, 621.2306).

3.9. (+)-(8S,8'S)-9-O-(E)-Feruloyl-secisorilaciresinol (6)

Yellow solid. [α]D20 +25.2 (c 0.1, MeOH); IR (KBr) νmax 3367, 2928, 2855, 1683, 1601, 1516, 1454, 1431, 1375, 1271, 1207, 1154, 1033, 935, 846, 801, 724 cm−1; 1H-NMR (acetone-d6, 600 MHz) and 13C-NMR (acetone-d6, 150 MHz) data, see Table 1; Table 2; HRESIMS m/z 537.2134 [M – H]+ (calcd. for C30H33O6, 537.2130).

3.10. (+)-(8R,7'S,8'R)-9'-O-(E)-Feruloyl-5,5'-dimethoxyariciresinol-7-one (7)

Amorphous powder. [α]D20 +19.5 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 211 (4.01), 234 (2.12), 318 (1.96) nm; ECD (MeOH) 331 (Δε = −0.37), 288 (Δε + 0.73), 222 (Δε + 2.01); IR (KBr) νmax 3409, 2940, 2843, 1701, 1665, 1604, 1516, 1461, 1425, 1371, 1323, 1271, 1215, 1169, 1116, 1032, 983, 912, 845, 827, 765, 712, 662 cm−1; 1H-NMR (acetone-d6, 500 MHz) and 13C-NMR (acetone-d6, 125 MHz) data, see Table 1; Table 2; ESIMS m/z 609 [M – H]+; HRESIMS m/z 609.1980 [M – H]+ (calcd. for C32H35O12, 609.1978).

3.11. (+)-(8R,7'S,8'R)-9'-O-(E)-Cinnamoyl-5,5'-dimethoxyariciresinol (8)

Amorphous powder. [α]D20 +23.0 (c 0.1, MeOH); IR (KBr) νmax 3425, 2937, 2845, 1703, 1612, 1516, 1461, 1427, 1331, 1282, 1218, 1154, 1117, 1041, 980, 913, 832, 719 cm−1; 1H-NMR (acetone-d6, 600 MHz) and 13C-NMR (acetone-d6, 150 MHz) data, see Table 1; Table 2; HRESIMS m/z 625.2297 [M – H]+ (calcd. for C33H37O12, 625.2291).

3.12. 9'-O-(E)-Feruloyl-5,5'-trimethoxyariciresinol (9)

Amorphous powder. [α]D20 +21.0 (c 0.1, MeOH); IR (KBr) νmax 3395, 2933, 2849, 1701, 1610, 1517, 1462, 1428, 1372, 1324, 1270, 1214, 1159, 1116, 1033, 983, 909, 831, 703 cm−1; 1H-NMR (acetone-d6, 500 MHz) and 13C-NMR (acetone-d6, 125 MHz) data, see Table 1; Table 2; HRESIMS m/z 625.2297 [M – H]+ (calcd. for C33H37O12, 625.2291).
3.13. Cytotoxicity Assay

The cytotoxic activity was determined against human colon cancer (HCT-116), human non-small-cell lung carcinoma (NCI-H1650), and human ovarian cancer (A2780) cell lines which were bought from the Cell Bank of Shanghai Institute of Cell Biology (Chinese Academy of Sciences) and originally obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in RPMI 1640 (GIBCO, New York, NY, USA) supplemented with 10% fetal calf serum (Life Technologies, Carlsbad, CA, USA), penicillin G (100 U/mL), and streptomycin (100 µg/mL) at 37 °C in a 5% CO₂ and seeded in 96-well plates (CLS3635, Corning®, Sigma, Santa Clara, CA, USA) at a cell density of 3000 per well over night, and then were treated with various diluted concentrations (each concentration was arranged triple) of compounds 1–19, which were prepared with DMSO (Sigma) to 100 µM stock solution and stored in −20 °C in advance. After 24 h of treatment, 10 µL of MTT (5 mg/mL in PBS) was then added directly to all wells and the plates were placed in the dark at 37 °C for 3 h incubation. Cell viability was measured by observing absorbance at 570 nm on a SpectraMax® microplate reader (Molecular Devices, Silicon Valley, CA, USA). IC₅₀ values were calculated using Microsoft Excel software (version 2010, Redmond, WA, USA). Taxol was used as a positive control.

3.14. PTP1B Inhibition Assay

The recombinant GST-hPTP1B (glutathione S-transferase-human protein tyrosine phosphatase 1B) bacteria pellets were purified by a GST bead column. The dephosphorylation of para-nitrophenyl phosphate (p-NPP) was catalyzed to para-nitrophenol by PTP1B. Enzyme activity involving an end-point assay, which intensified the yellow color, was measured at a wavelength of 405 nm. All compounds were dissolved in 100% dimethyl sulfoxide (DMSO), and reactions, including controls, were performed at a final concentration of 10% DMSO. Selected compounds were first evaluated for their ability to inhibit the PTPase reaction at a 10 µM concentration at 30 °C for 10 min, in a reaction system with 3 mM p-NPP in HEPES assay buffer (pH 7.0). The reaction was initiated by addition of the enzyme and quenched by addition of 1 M NaOH. The amount of the produced p-nitrophenol was determined at 405 nm using a microplate spectrophotometer (uQuant, Bio-Tek, Winooski, VT, USA). IC₅₀ values were evaluated using a sigmoidal dose-response (variable slope) curve-fitting program of GraphPad Prism 4.0 software (La Jolla, CA, USA). Oleanolic acid was used as a positive control.

3.15. Nitric Oxide (NO) Production in RAW264.7 Macrophages

The RAW 264.7 macrophages were cultured in The RPMI 1640 medium (Hyclone, Logan, UT, USA) 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 × 105 cells/well) and maintained at 37 °C under 5% CO₂. After preincubation for 24 h, serial dilutions of the test compounds were added into the cells, up to the maximum concentration 25 µM, then added with LPS to a concentration 1 µg/mL and continued to incubate for 18 h. The amount of NO was assessed by determined the nitrite concentration in the cultured RAW264.7 macrophage supernatants with Griess reagent. Aliquots of supernatants (100 µL) were incubated, in sequence, with 50 µL 1% sulphanilamide and 50 µL 1% naphthylethylenediamine in 2.5% phosphoric acid solution. The sample absorbance was measured at 570 nm by a 2104 Envision Multilabel Plate Reader (PerkinElmer, Inc., Waltham, MA, USA). Dexamethasone was used as a positive control.

4. Conclusions

In summary, bioassay-guided isolation of cytotoxic fractionsof the twigs of L. cubeba revealed the presence of nine new lignans 1–9 and ten analogues 10–19. Initially, all of the isolated compounds were evaluated against HCT-116, NCI-H1650, and A2780 tumor cell lines. Of the compounds, only 7′,9-epoxylignans with feruloyl or cinnamoyl group (7–9, 13 and 14) were selectively cytotoxic against NCI-H1650 cell line, with IC₅₀ values of less than 20 µM, whereas, the dibenzylbutyro lactone lignans

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17–19 exerted cytotoxicity against HCT-116 and A2780 cell lines, with IC_{50} values ranging from 0.28 to 18.47 µM. The results highlighted the structure-activity relationship importance of a feruloyl or a cinnamoyl moiety at C-9’ or/and C-7 ketone in 7’,9-epoxylignans. The isolates were also examined for inhibitory activities against PTP1B and LPS-induced NO production in RAW264.7 macrophages. As a result, compound 11 was moderate active toward PTP1B with an IC_{50} value of 13.5 µM and compounds 4–6, 11 and 12 displayed inhibitions against LPS-induced NO production in RAW264.7 macrophages, with IC_{50} values of 46.8, 50.1, 58.6, 47.5, and 66.5 µM, respectively. The present results provide additional phytochemical and bioactive information of this medicinal and spiced plant.

Supplementary Materials: The following are available online, IR, UV, HRMS, NMR and ECD spectra of compounds 1–9 as well as other supporting data.

Author Contributions: X.S. conceived and designed the experiments; X.L. and Y.Q. realized the evaluation of bioactivities; H.X., L.W. and G.X. performed the isolation, structural elucidation and wrote the paper; S.L. analyzed the results and revised the paper.

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Sample Availability: Samples of the compounds 1–19 are available from the authors.

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