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Abstract: *Litsea verticillata* Hance (Lauraceae), a Chinese medicine used for treatment of swelling caused by injury and snake biting, was the first plant identified by our National Institutes of Health (NIH)-funded International Cooperative Biodiversity Group (ICBG) project to exert anti-HIV activity. From this plant, we discovered a class of 8 novel litseane compounds as prototypic sesquiterpenes, all of which demonstrated anti-HIV activity. In subsequent studies, 26 additional compounds of different structural types were identified. During our continuing investigation of this plant species, we identified two new litseanes, litseaverticillols L and M, and a new sesquiterpene butenolide, litseasesquibutenolide. Litseaverticillols L and M were found to inhibit HIV-1 replication with an IC_{50} value of 49.6 μM. To further determine the antiviral properties of this plant, several relatively abundant isolates in the plant including a litseane compound, two eudesmane sesquiterpenes and three lignans were evaluated against additional 21 viral targets. The lignans **8** and **9** were shown to be active against Epstein-Barr Virus (EBV) with EC_{50} values of 22.0 μM (SI = 3.8) and 16.2 μM (SI > 6.2), respectively. Since many antiviral compounds have been discovered in *L. verticillata*, we further prepared 38 plant extracts made from the different plant parts in 9 additional *Litsea* species. These extracts were evaluated for their anti-HIV and cytotoxic activities, and four of the extracts, which ranged across three different species, displayed 97-100 % inhibitory effects against HIV replication without showing cytotoxicity to a panel of human cell lines at a concentration of 20 μg/mL.

Keywords: *Litsea*; Lauraceae; Litseane; Sesquiterpene butenolide; Antiviral activity; Anti-HIV activity; Structure determination

Introduction

Human Immunodeficiency Virus (HIV) was first established as the viral agent that causes acquired immunodeficiency syndrome (AIDS) in humans some 30 years ago (Barré-Sinoussi *et al*., 1983; Broder and Gallo, 1984). There are currently more than 20 drugs available on the market for the treatment of this disease. However, all of the available drugs require that HIV patients continue taking them for long periods of time, or even a life time because none of the drugs are curative. Further, the drug potency may be reduced at a later stage due to the chronic adverse effects and the emergence of drug-resistant strains (Greene *et al*., 2008). The current lack of a curative drug demands our continuous efforts to discover more effective anti-HIV therapeutic agents.

Plant compounds, known for their enormous numbers and their amazing structural diversity, are considered an excellent source for exploration for new and diverse antiviral agents. *Litsea verticillata* (Lauraceae) was found to be one of the first anti-HIV plant leads in our efforts to discover antiviral agents from the tropical plants in the Southern Asia area (Soejarto *et al*., 2006). The plant, grown up as a shrub or small tree 2-5 m tall, is found in southern Asia including Vietnam and Southern China, where the altitude is less than 1300. The plant has been used as a traditional Chinese medicine to relieve swelling caused by injury and snake biting (Jiangsu, 1986). Our previous studies on this plant have resulted in the isolation of 34 compounds including two lignans, five butenolides and 27 sesquiterpenes. Among these isolates, 21 were determined as new molecules, and 20 were found to have anti-HIV activity (Hoang *et al*., 2002; Zhang *et al*.,, 2001, 2003a, 2003b, 2005). The sesquiterpenes belong to 13 different skeletal types, including the novel structural class we designated as litseane. Confirmation of the new unique litseane structural

skeleton has been confirmed by synthesis of selected members of our isolates by three research groups. From 2003 to 2006, the Vassilikogiannakis group accomplished the total synthesis of litseaverticillols A-H by means of a biomimetic sequence of transformations involving the singlet oxygen ($^1O_2$)-initiated cascade reaction as the key step (Vassilikogiannakis and Stratakis, 2003; Vassilikogiannakis *et al*., 2004, 2005; Margaros *et al*., 2006; Montagnon *et al*., 2008). In 2006, the Kuwahara group first accomplished the enantioselective total synthesis of the (1*R*, 5*S*)-stereoisomer of litseaverticillols A and B from homogeranic acid in six steps by employing the Evans asymmetric aldol reaction and a microwave-promoted cyclization of a stannylated thiol ester intermediate as the C-C bond-forming steps (Morita and Kuwahara, 2006; Morita *et al*., 2006). In 2007, the enantioselective total synthesis of the litseaverticillols C and K was achieved by the Mohapatra group using D-glucose as starting material and by employing the ring closing metathesis (RCM) and Wittig reactions as the key steps (Mohapatra *et al*., 2007).

In an effort to further elucidate the antiviral constituents of *L. verticillata*, we identified three additional new sesquiterpenes: litseaverticillols L/M (**1**/**2**) and litseasesquibutenolide (**3**) (structures shown in Fig. 1). These compounds were evaluated for their anti-HIV activity. We further assessed the antiviral activity of a number of previously obtained natural compounds from this plant against 21 viral targets available at NIAID (National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA).

Litsea is a plant genus in the Lauraceae family. There are about 200 species found in the genus all over the world. Apart from *L. verticillata*, a number of the other *Litsea* species such as *L. cubeba* and *L. garrettii* have a long history of use as traditional medicines. *L. cubeba* has been used as a Chinese medicine for treatment of indigestion, diarrhea, toothache, vomiting and fall caused injury (State, 1999) and *L. garrettii* has been used as a Dai minority medicine for treatment of skin itching,

swelling and body ache (State, 2005). Due to the antiviral effects of the compounds found in the plant *L. verticillata*, we further investigated the anti-HIV activity of 38 extracts belonging to 9 additional *Litsea* plant species. Four (4) of the extracts in three species including *L. balansae*, *L. lancifolia* and *L. monopetala* were found to be able to inhibit HIV replication by 97-100% at a concentration of 20 μg/mL without showing cytotoxicity to a panel of human cell lines.

The current paper describes the isolation, identification/structure elucidation and biological evaluation of the isolated compounds from *L. verticillata* as well as the biological activity evaluation of 41 *Litsea* plant extracts.

Materials and Methods

General Experimental Procedures

Optical rotations were measured with a Perkin-Elmer model 241 polarimeter. IR spectra were recorded on a Jasco FT/IR-410 spectrometer, equipped with a Specac Silver Gate ATR system by applying a film on a Germanium plate. 1D and 2D NMR spectra were recorded on a Bruker DRX-500 MHz spectrometer. Chemical shifts (δ) were expressed in ppm with reference to the solvent signals ($CDCl_3$; ^1H: 7.24 ppm; ^{13}C: 77.00 pm), and coupling constants (J) are reported in Hz. All NMR experiments were obtained by using standard pulse sequences supplied by the vendor. Column chromatography was carried out on silica gel (200–400 mesh, Natland International Corporation). Reversed-phase flash chromatography was accomplished with RP-18 silica gel (40–63 μm, EM Science), and reversed-phase preparative HPLC was carried out on a Waters 600E Delivery System pump, equipped with a Waters 996 photodiode detector, and a

Watrex GROM-Saphir 110 C18 column (120 Å, 12 μm, 300 × 40 mm^2) or a Phenomenex LUNA-C-18 column (120 Å, 12 μm, 250 × 50 mm^2). Thin-layer chromatography was performed on Whatman glass-backed plates coated with 0.25 mm layers of Silica gel 60. HRTOFMS spectra were recorded on a Micromass QTOF-2 spectrometer, and CIMS and HRCIMS spectra were recorded on a Finnigan FTMS 2001 spectrometer.

Plant Material

L. balansae Lecomte: The stem sample (SV0014) was collected on Nov 23, 1998 Bong, and was documented by voucher specimen Soejarto et al. 10396. The leaf, twig and flower sample (SV0418) was collected on July 27, 1999, and was documented by voucher specimens Soejarto et al. 10918. The stem bark sample (SV0419) was collected on July 27, 1999, and was documented by voucher specimens Soejarto et al. 10918. The bark sample (SV0420) was collected on July 27, 1999, and was documented by voucher specimens Soejarto et al. 10918. The leaf sample (SV5186) of *L. balansae* Lecomte was collected in 1995, and was documented by voucher specimens MAFB-1425.

L. baviensis Lecomte: The leaf, twig and flower bud sample (SV0357) of *L. baviensis* Lecomte was collected on April 5, 1999 Bong, and was documented by voucher specimens Soejarto et al. 10686. The bark sample (SV0358) was collected on April 5, 1999 and was documented by voucher specimens Soejarto et al. 10686. The stem bark sample (SV0359) was collected on April 5, 1999, and was documented by voucher specimens Soejarto et al. 10686. The root sample (SV0360) was collected on April 5, 1999, and was documented by voucher specimens Soejarto et al. 10686. The leaf and twig sample (SV0614) was collected on September 17, 1999, and was documented by voucher specimens NMC_533. The fruit sample (SV0616) was

collected on September 17, 1999, and was documented by voucher specimens NMC_533.

L. chartacea (Wall. ex Nees) Hook. f.: The leave and twig sample (SV0382) of was collected on June 4, 1999 at Bong, CPNP, and was documented by voucher specimens Soejarto et al. 10694. The bark sample (SV0383) was collected on June 4, 1999 Bong, CPNP, and was documented by voucher specimens Soejarto et al. 10694.

L. cubeba (Lour.) Pers: The stem sample (SV0244) of was collected on March 21, 1999, and was documented by voucher specimens Soejarto et al. 10653. The leaf and twig sample (SV0245) was collected on March 21, 1999,, and was documented by voucher specimens Soejarto et al. 10653. The stem bark sample (SV4163) was collected on July 18, 2000, and was documented by voucher specimens Phan Ke Loc 10362. The root sample (SV4164) was collected on July 18, 2000, and was documented by voucher specimens Phan Ke Loc 10362.. The bark sample (SV4166) was collected on July 18, 2000, and was documented by voucher specimens Phan Ke Loc 10362.

L. garrettii Gamble: The leaf sample (SV5040) of was collected on 1995, and was documented by voucher specimens Kanh & On 1144-A. The leaf and twig sample (SV2224) was collected on August 25, 2000, and was documented by voucher specimens CNMC_1050.

L. griffithii: The bark branch sample (SV2225) of was collected on August 25, 2000, and was documented by voucher specimens NMC_1050. The fruit sample (SV2226) was collected on August 25, 2000, and was documented by voucher specimens NMC_1050. The stem bark sample (SV2227) was collected on August 25, 2000, and was documented by voucher specimens NMC_1050.

L. lanciflolia (Roxb. ex Nees) Benth. et Hook.: The leaf, twig and flower bud sample (SV0219) of was collected on March 20, 1999, and was documented by voucher specimens

Soejarto et al. 10631. The stem bark sample (SV0220) was collected on March 20, 1999, and was documented by voucher specimens Soejarto et al. 10631. The root sample (SV0221) was collected on March 20, 1999, and was documented by voucher specimens Soejarto et al. 10631.

L. monopetala (Roxb.) Pers.: The leaf, twig and flower bud sample (SV0172) of was collected on March 19, 1999, and was documented by voucher specimens Soejarto et al. 10596. The stem bark sample (SV0173) of was collected on March 19, 1999, and was documented by voucher specimens Soejarto et al. 10596. The leaf, twig and flower sample (SV0907) was collected on May 16, 2000 and was documented by voucher specimens Soejarto et al. 11505. The bark sample (SV0908) was collected on May 16, 2000, and was documented by voucher specimens Soejarto et al. 11505. The stem bark sample (SV0909) was collected on May 16, 2000, and was documented by voucher specimens Soejarto et al. 11505. The root sample (SV0910) was collected on May 16, 2000, and was documented by voucher specimens Soejarto et al. 11505. The leaf sample (SV5188) was collected in 1995, and was documented by voucher specimens MAFA_1053. The stem bark sample (SV5189) was collected on 1995, and was documented by voucher specimens MAFA_1053.

L. robusta Blume: The stem bark sample (SV0191) of was collected on March 19, 1999, and was documented by voucher specimens Soejarto et al. 10611. The root sample (SV0192) was collected on March 19, 1999, and was documented by voucher specimens Soejarto et al. 10611. The leaf and twig sample (SV0193) was collected on March 19, 1999, and was documented by voucher specimens Soejarto et al. 10611. The bark sample (SV0416) was collected on July 27, 1999, and was documented by voucher specimens Soejarto et al. 10917.

L. verticillata Hance: The leaf, twig and flower bud sample (SV0001) was collected on November 22, 1998, and was documented by voucher specimens Soejarto et al. 10379. The bark

sample (SV0002) was collected on November 22, 1998, and was documented by voucher specimens Soejarto et al. 10379. The leaf sample (SV5064) was collected on 1995, and was documented by voucher specimens Kanh & On 1254-B.

A large quantity of the plant sample (SVA0001, 4.5 kg, voucher specimens *Soejarto et al*. 11003) was subsequently re-collected at the same site of SV0001 at CPNP on November 17, 1999, for complete isolation work. Duplicate voucher specimens of both collections were deposited at the herbaria of CPNP, Institute of Ecology and Biological Resources (HN) of the Vietnam Academy of Science and Technology in Hanoi, Hanoi University of Pharmacy in Hanoi, and John G. Searle Herbarium of the Field Museum (F) (Chicago, IL, USA).

Preparation of the Crude Plant Extracts

All initially collected plant materials (about 50 g each) were extracted with $CHCl_3$ and prepared as $CHCl_3$ extracts. The dried and milled leaves and twigs (4.5 kg) of the recollected plant materials of *L. verticillata* were extracted with MeOH, and subsequently defatted with *n*-hexane and partitioned with $CHCl_3$ to afford a $CHCl_3$ extract (93.0 g).

Isolation of Compounds

The resulting extract (93.0 g) was processed as previously described (Zhang *et al*., 2005) to afford the anti-HIV fractions F17 and F18. F17 (1.31 g) was subjected to preparative HPLC separation on a Phenomenex LUNA-C18 column (solvent system: MeOH/H_2O 1:1) to afford the active fraction F31 (12 mg), which was subjected to further preparative HPLC separation on the

GROM-Saphir 110 C18 column, and elution with MeCN/H_2O 3:7 to afford litseaverticillol I/J (**1**/**2**, 1.51 mg). F18 (0.92 g) was subjected to preparative HPLC separation on a GROM-Saphir 110 C18 column (solvent system: MeOH/H_2O 7:3) to afford the active fraction F48 (59 mg), which was subjected to further preparative HPLC separation on the GROM-Saphir 110 C18 column, and elution with MeCN/H_2O 4:6 to afford litseasesquibutenolide (**3**, 0.56 mg).

*Litseaverticillols L/M (**1**/**2**)*

Colorless gum; $[\alpha]_D^{23}$ 0° (*c* 0.10, $CHCl_3$); UV (MeOH) λ_{max} (AU) 222.4 (1.15), 316.3 (0.02) nm; IR (film) ν_{max} 3414 (*br*), 2973, 2926, 2854, 1702, 1652, 1452, 1378, 1322, 1185, 1072, 1036 cm^{-1}; ^1H NMR and ^{13}C NMR spectroscopic data, see Table 1; CIMS *m/z* (rel. int.) 300 $[M+NH_4]^+$, 283 $[M+H]^+$, 251 $[M-OMe+H]^+$ (100), 231 $[M-H_2O-OMe+H]^+$; HRCIMS *m/z* 283.1924 $[M+H]^+$ (Calcd for $C_{16}H_{27}O_4$: 283.1909); HRTOFMS *m/z* 251.1649 $[M-OMe+H]^+$ (Calcd for $C_{15}H_{23}O_3$: 251.1647).

*Litseasesquibutenolide (**3**)*

Colorless gum; $[\alpha]_D^{23}$ -15° (*c* 0.04, $CHCl_3$); UV (MeOH) λ_{max} (AU) 200.0 (0.75), 280.4 (0.05) nm; IR (film) ν_{max} 2966, 2817, 1769, 1708, 1652, 1459, 1384, 1266, 1153, 1104, 923 cm^{-1}; ^1H NMR and ^{13}C NMR spectroscopic data, see Table 1; CIMS *m/z* (rel. int.) 298 $[M+NH_4]^+$ (100), 281 $[M+H]^+$, 249 $[M-OMe+H]^+$ (100); HRCIMS *m/z* 281.1737 $[M+H]^+$ (Calcd for $C_{16}H_{25}O_4$: 281.1747)

Anti-HIV and Cytotoxicity Assays

Anti-HIV and cytotoxicity assays were performed in parallel utilizing the green fluorescent protein (GFP)-based HOG·R5 reporter cell line that was constructed and developed specifically for quantitating HIV-1 infectivity. The system was validated and adapted as a moderately high-throughput procedure for screening natural products for anti-HIV activity in our laboratory (Hoang *et al*., 2002; Zhang *et al*., 2005). Briefly, a reporter cell line for quantitating HIV-1 replication was developed using HOS (human osteosarcoma) cells rendered susceptible to HIV-1 infection by the transfection of genes for CD4 and CCR5, the co-receptor utilized by macrophage-tropic (R5) HIV-1 isolates. This microtiter assay is based on the transactivation of a stably integrated HIV-1 LTR-green fluorescent protein (GFP) transcription unit. Upon HIV-1 entry into these HOS target cells, Tat expression increases the HIV LTR-directed transcription of the GFP gene as demonstrated by the increased fluorescence of detergent lysates of infected cells relative to that of uninfected controls. Procedures adopted for the assay were as described previously. The positive control compound used was 3TC (Lamivudine), which had an IC_{50} value of approximately 1.2 μM in the HOG·R5 system utilizing the assay conditions described above. This nucleoside reverse transcriptase inhibitor and the virus stock of HIV-1IIIB/H9 were obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH.

Antiviral Activity Evaluated at NIAID

Under a collaborative agreement with NIAID (National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA), selected compounds were evaluated at the Southern

Research Institute (2000 9th Avenue South, Post Office Box 55305, Birmingham, AL35255-5305, USA), for their antiviral potential against 21 viral targets: flu-A (Solomon Island/03/2006 H1N1, the Wisconsin/67/2005 H3N2, and Vietnam/1203/2004H H5N1 strains) and flu B (Malaysia/2506/2004 strain) viruses tested in Madin-Darby canine kidney (MDCK) cells, rhinovirus type-2 (HGP strain) virus tested in Hela cervical cancer cells, adenovirus (65089/Chicago strain) tested in A-549 lung cancer cells, parainfluenza (PIV: 14702 strain) and respiratory syncytial (RSVA: A2 strain) viruses tested in MA-104 monkey kidney cells, Epstein-Barr virus (EBV) tested in Akata lymphoma cells, severe acute respiratory syndrome (SARS: Urbani strain), Rift Valley Fever (MP-12 strain), Tacribe (TRVL11573 strain) and West Nile (WNV: New York strain) viruses tested in vero-76 monkey kidney cells, hepatitis C virus (HCV) tested in Huh7 ET liver cells, hepatitis B virus (HBV) tested in Hep G2 liver cells, human papilloma virus (HPV), measles (Chicago strain) tested in CV-1 monkey kidney cells, Varicella zoster viurs (VZV), and herpes simplex-1 and -2 viruses (HSV-1 and HSV-2) and human cytomegalovirus (HCMV) tested in human foreskin fibroblast (HFF) cells.

Results and Discussion

Through bioassay-guided fractionation of the $CHCl_3$ extract of the leaves and twigs of *L. verticillata*, F17 and F18 were identified as two anti-HIV fractions, exhibiting 81 and 83 % inhibition against HIV-1 replication in HOG·R5 cells (Hoang *et al.*, 2002) at 20 μg/mL, respectively. Further separation of fraction F17 led to the isolation of litseaverticillols L/M (**1**/**2**), and work-up of fraction F18 resulted in the isolation of litseasesquibutenolide (**3**).

Compounds **1** and **2** were isolated as an inseparable mixture in a 1:1 ratio. Our attempts to

separate the two molecules using two different preparative HPLC columns (Phenomenex, LUNA phenyl-hexyl, 15 μm, 250 × 50 mm; Grom Saphir 110 C18, 12 μm, 300 × 40 mm) and several different solvent systems were unsuccessful. This mirrors the situations that we and the other research groups have previously encountered using a variety of different techniques to separate pairs of compounds possessing a side chain formed from two or more isoprene units and containing an oxy group at the far end carbon(s) (Zhang *et al*., 2003a, 2006; Montagnon *et al*., 2008).

Most of the ^{1}H NMR signals of **1**/**2** completely overlapped each other (Table 1), indicating a mixture of two compounds bearing a close structural relationship. However, the ^{13}C signals for 10 of the 16 carbons were clearly distinctive (maximum chemical shift divergence = 0.41 ppm). The 16 carbons were characterized by the ^{13}C NMR and DEPT spectra as four non-oxymethyls (δ 20.7/20.6, 19.0, 17.2/16.9 and 10.2), an oxymethyl (δ 49.1), two methylenes (δ 37.0/36.8 and 29.4/29.3), a non-oxymethine (δ 56.4/56.3), two oxymethines (δ 76.4 and 76.3/76.0), two olefinic methines (δ 155.0/154.9 and 119.3/118.9), an oxy-quaternary carbon (δ 77.0), two olefinic quaternary carbon (δ 142.8 and 142.0/141.8), and a quaternary carbonyl group (δ 206.3/206.2) (Table 1). The similarity of the NMR spectral data of **1/2** to those of the previously identified litseanes suggested that **1**/**2** possess the same structural skeleton of the litseane sesquiterpenes (Zhang *et al*., 2001, 2003a). The spectral data of **1**/**2** are most similar to the litseane litseaverticillol D (**4**). As in **4**, compounds **1**/**2** were also found to possess a 1-oxy-1-methyl-ethyl group [$(Me)_2C(O)$-] and a sub-structural unit (**unit A**: C1 to C8) of 3-(5-hydroxy-3-methyl-2-oxo-cyclopent-3-enyl)-2-methyl-allyl through analysis of the ^{1}H-^{1}H COSY, HMQC, and HMBC spectral data (Fig. 2). However, the NMR data indicated that **1**/**2** differ from **4** by the presence of a 1-hydroxy-ethyl group at C-9 and C-10 [$-CH_2CH(OH)$-] [δ1.54

(m), 1.44 (m), and 3.45/3.40 (br d, J = 10.0/10.1 Hz); δ 29.4/29.3 (t) and 76.3/76.0 (d)] and a methoxy group [δ 3.20 (s) and δ 49.1 (q)]. There is no second double bond, as in the case of **4**, being observed in the NMR spectra of **1**/**2**. The ^{1}H-^{1}H COSY couplings between the methylene proton signals of the 1-hydroxy-ethyl group and the methylene proton signals [δ 2.35 (m) and 2.14 (m)] of **unit A** connected the two methylene carbons in **1**/**2**, while the presence of the HMBC correlations of the proton signal at δ 3.45 (br d, J = 10.0 Hz) to the ^{13}C signals of the three carbons of the 1-oxy-1-methyl-ethyl group [δ 77.0 (s), 20.7 or 20.6 (q), and 19.0 (q)] bonded the oxy-methine carbon of the 1-hydroxy-ethyl group to the oxy-quaternary carbon (C-11) in the 1-oxy-1-methyl-ethyl group. The methoxy group is also bonded to C-11 due to the presence of the HMBC correlation between the methoxy proton signal and the ^{13}C signal of C-11. The planar structures of **1**/**2** were then elucidated to be 4-hydroxy-5-(5-hydroxy-6-methoxy-2, 6-dimethyl-hept-1-enyl)-2-methyl-cyclopent-2-enone, which has a molecular formula of $C_{16}H_{26}O_4$ as determined by the HRCIMS ($[M+H]^+$ (*m/z* 283.1924, calcd. 283.1909) and HRTOFMS ($[M-OMe+H]^+$ (*m/z* 251.1649, calcd. 251.1647).

The geometric and chiral (C-1 and C-5) configurations of **1**/**2** were determined to be the same as those in **4** due to their similar chemical shifts and coupling patterns at these carbon positions, and on the basis of the observation of an ROE correlation between H-5 [δ 3.15 (dd, J = 9.0, 2.5 Hz)/3.14 (dd, J = 9.1, 2.4 Hz)] and H-14 [δ 1.75/1.74 (3H, s)], as well as the ROE correlation between H-1 [δ 4.59 (br s)] and H-6 [δ 5.07 (dq, J = 8.9, 1.2 Hz)/5.05 (dq, J = 8.8, 1.2 Hz)] (Fig. 3). Based on the above data, **1** and **2** were determined to be epimeric at C-10, which mirrors the structural relationship of litseaverticillols F and G (Zhang *et al*., 2003a). The presence of this very flexible side chain at C-5 contributed significantly to the compounds being unable to be separated. The structures for the two compounds (**1**/**2**) were thus determined as 1α,

10α-dihydroxy-11-methoxy-(*E*)-litse-2, 6-dien-4-one and 1α, 10β-dihydroxy-11-methoxy-(*E*)-litse-2, 6-dien-4-one, and given the trivial names of litseaverticillols L and M, respectively.

Compound **3** with a molecular formula of $C_{16}H_{24}O_4$ according to the HRCIMS ($[M+H]^+$ *m/z* 281.1737, calcd. 281.1747), was shown to be composed of an α, β-conjugated ester group, a second C-C double bond, two tertiary methyls, two secondary methyls, an oxy-methyl, three methylenes, a methine, a dioxy-substituted carbon, and a keto carbonyl carbon as evidenced by the ^1H and ^{13}C NMR spectral data (Table 1). Analysis of the ^1H-^1H COSY, HMQC and HMBC (Fig. 4) spectral data in the same manner employed for the structure determination of **1**/**2** led to the elucidation of a sub-structural unit of 3, 7-dimethyl-6-oxo-oct-2-enyl group (**unit B**) for **3**.

The presence of HMBC correlations of the proton signals at δ 2.72 (dd, J = 15.2, 7.4 Hz, H-5a) and 2.46 (dd, J = 15.8, 7.2 Hz, H-5b) to the ^{13}C signal at δ 111.5 (s) suggested the attachment of **unit B** to a dioxy-substituted carbon (C-4), which was then connected by an α, β-conjugated ester group evidenced by the presence of HMBC correlations between the proton signal at δ 5.88 (q, J = 1.6 Hz) and the ^{13}C signal of C-4, and between H-5a and the ^{13}C signal at δ 164.8 (s, C-3). The methoxy group and the second methyl group in **3** were assigned to C-4 and C-3 respectively, as supported by the presence of HMBC correlations of the methoxy proton signals at δ 3.15 (s) to the ^{13}C signal of C-4, and the methyl proton signals at δ 1.94 (d, J = 1.6 Hz) to the^{13}C signals of C-2 [δ 120.3 (d)], -3 and -4. Five double-bond equivalents were calculated from the molecular formula ($C_{16}H_{24}O_4$) of **3**, four of which were accounted for by the presence of two carbon-carbon double bonds and two carbonyl double bonds. The remaining unassigned unsaturated bond equivalent must be involved in the formation of a butenolide ring structure in **3**. The two proton doublet signals at δ 5.88 (J = 1.6 Hz) and 1.94 (J = 1.6 Hz) and the

^{13}C NMR signals at δ 168.7, 164.8, 120.3, 111.1, and 12.7 assignable to the butenolide moiety are very similar to those of the known compound, actinolide A (Kim *et al*., 2002). The double bond on the side chain was assigned as *E* configuration, which resulted in significant upfield of the ^{13}C NMR chemical shift of C-14 (δ 16.6 of **3** *vs* δ 25.6) and downfield of the ^{13}C NMR chemical shift of C-8 (δ 33.4 of **3** *vs* δ 39.7) in comparison with the sesquiterpene butenolides with a *Z* configured double bond (Vassilikogiannakis *et al*., 2005). Compound **3** was thus elucidated as 5-(2*E*-3, 7-dimethyl-6-oxo-oct-2-enyl)-5-methoxy-4-methyl-5*H*-furan-2-one, and was given the trivial name of litseasesquibutenolide.

During the initial bioactivity evaluation, the total $CHCl_3$ extract of *L. verticillata* inhibited HIV-1 replication by 76% at a concentration of 20 μg/mL with no apparent toxicity at the same concentration. Follow-up bioassay-guided fractionation of the recollected plant materials yielded two active fractions (F-17 and F-18), from which compounds **1**-**3** were isolated. Litseaverticillols L/M (**1**/**2**) exhibited anti-HIV activity with an IC_{50} value of 49.6 μM and no toxicity to the host HOG·R5 cells at a concentration of 70 μM, which is at a similar level of potency as litseaverticillol D (**4**) and other litseanes (Zhang *et al*., 2003a). Compound **3** lacked inhibitory activity against HIV-1 replication at a concentration of 70 μM .

Including litseaverticillols L/M (**1**/**2**) and litseasesquibutenolide (**3**), we have now identified a total of 39 natural compounds from *L. verticillata*. While lignans are the most abundant compounds produced in this plant, litseanes are considered as minor components with litseaverticillol A as the most abundant with a yield of 0.0016%. In cooperation with NIH NIAID (National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA) under a collaborative agreement, six compounds obtained from this plant, including litseaverticillol A (**5**), two eudesmane sesquiterpenes [verticillatol (**6**) and eudesm-4(15)-ene-1β, 6α-diol (**7**)] and three

353 lignans [epicexcelcin (**8**), demethoxyepicexcelcin (**9**), and sesamin (**10**)] were evaluated for their
354 antiviral potential against other viruses using a battery of 21 viral targets (Table 2). Of the
355 compounds tested, the lignans **8** and **9** were shown to be active against Epstein-Barr Virus (EBV)
356 (DNA hybridization assay using Akata cells) with EC_{50} values of 22.0 μM (selective index = 3.8)
357 and 16.2 μM (selective index > 6.2), respectively. Compound **8** also displayed slight activity
358 against human cytomegalovirus (HCMV) [cytopathogenic effect (CPE) inhibition in HFF cells]
359 with an EC_{50} value of 58.3 μM (selective index > 5.1). In addition, compound 5 showed
360 inhibitory activity against severe acute respiratory syndrome (SARS) virus with an EC_{50} value of
361 68.4 μM (selective index = 2.8).

362 We have identified many different types of bioactive compounds from *L. verticillata*. It is thus
363 worthy for us to further investigate the antiviral properties of the other plant species in the genus
364 *Litsea*. Consequently, we investigated 38 extracts in 9 additional *Litsea* plant species: *L. balansae*, *L.*
365 *baviensis*, *L. chartacea*, *L. cubeba*, *L. garrettii*, *L. griffithii*, *L. lanciflolia*, *L. monopetala*, and *L.*
366 *robusta*. Different parts (leaves, twigs, barks, stem barks and roots) of these plants were evaluated
367 for their anti-HIV activity as well as their cytotoxicity against a panel of human cell lines
368 comprising four cancer cell lines (KB, LNCaP, Col2 and Lu1), a primary umbilical vein cell line
369 (HUVEC) and a telomerase-immortalized normal cell line (hTERT) at a concentration of 20 μg/mL
370 (Table 3). As a result, an extract (SV0420) in *L. balansae*, two extracts (SV0220 and SV0221) in *L.*
371 *lanciflolia* and an extract (SV0173) in *L. monopetala* demonstrated 97-100 % inhibitory activity
372 against HIV replication without showing cytotoxicity to the tested human cell lines; an extract
373 (SV0014) in *L. balansae* demonstrated 100 % inhibitory activity against HIV replication without
374 showing cytotoxicity to the hTERT and HUVEC cell lines; an extract (SV0418) in *L. balansae*, two
375 extracts (SV0244 and SV0245) in *L. cubeba* and an extract (SV5189) in *L. monopetala*

demonstrated approximately 50 % inhibitory activity against HIV replication without showing cytotoxicity to the tested human cell lines.

In summary, with the isolation of litseaverticillols L/M (**1**/**2**), a total of 13 litseane sesquiterpenes have now been identified. Among these, litseaverticillols A-H and L/M were found to be naturally occurring molecules, while litseaverticillols I-K were compounds prepared through total synthesis. The sesquiterpene butenolides such as litseasesquibutenolide (**3**) may be considered as biosynthetic precursors of the litseane sesquiterpenes, as suggested from the biomimetic synthesis of litseaverticillols A-H using sesquiterpene butenolides as precursors (Vassilikogiannakis and Stratakis, 2003; Vassilikogiannakis *et al*., 2004, 2005; Margaros *et al*., 2006; Montagnon *et al*., 2008). Although all of our litseanes showed inhibitory activity against HIV replication, their selectivity indices of 2-3 are in the sub-optimal range for drug development. Our laboratory is committed to synthesizing a library of litseane analogues in an attempt to enhance the anti-HIV potency of the compounds while reducing their toxicity. Analogues with improved selective indices may be considered potential anti-HIV drug candidates for further development. The lignan epicexcelcin (**8**) is the most abundant compound found in *L. verticillata*. Its antiviral activity against several viral targets including EBV and HCMV renders it a potential lead compound for the synthesis of additional analogues for bioactivity evaluation and potential drug development. After evaluating 41 extracts made from various plant parts from 10 *Litsea* plant species, we have determined *L. balansae*, *L. lancifolia* and *L. monopetala* as three additional anti-HIV plant leads, which are worthy for further phytochemical exploration to discover other potential novel anti-HIV compounds.

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Legends and Notes

Figure 1. Chemical structures of compounds **1**-**10**.

Figure 2. Selected HMBC correlations for compound **1**/**2** ($CDCl_3$).

Figure 3. Selected ROESY correlations for compound **1**/**2** ($CDCl_3$).

Figure 4. Selected HMBC correlations for compound **3** ($CDCl_3$).

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Spectroscopic Data of Compounds 1-3 in $CDCl_3$

Notes:

[a] Coupling constants *J* in Hz are shown in parentheses, and δ values are given in ppm.

[b] Multiplicities in parentheses represent: s (quaternary carbon), d (CH), t (CH_2), and q (CH_3).

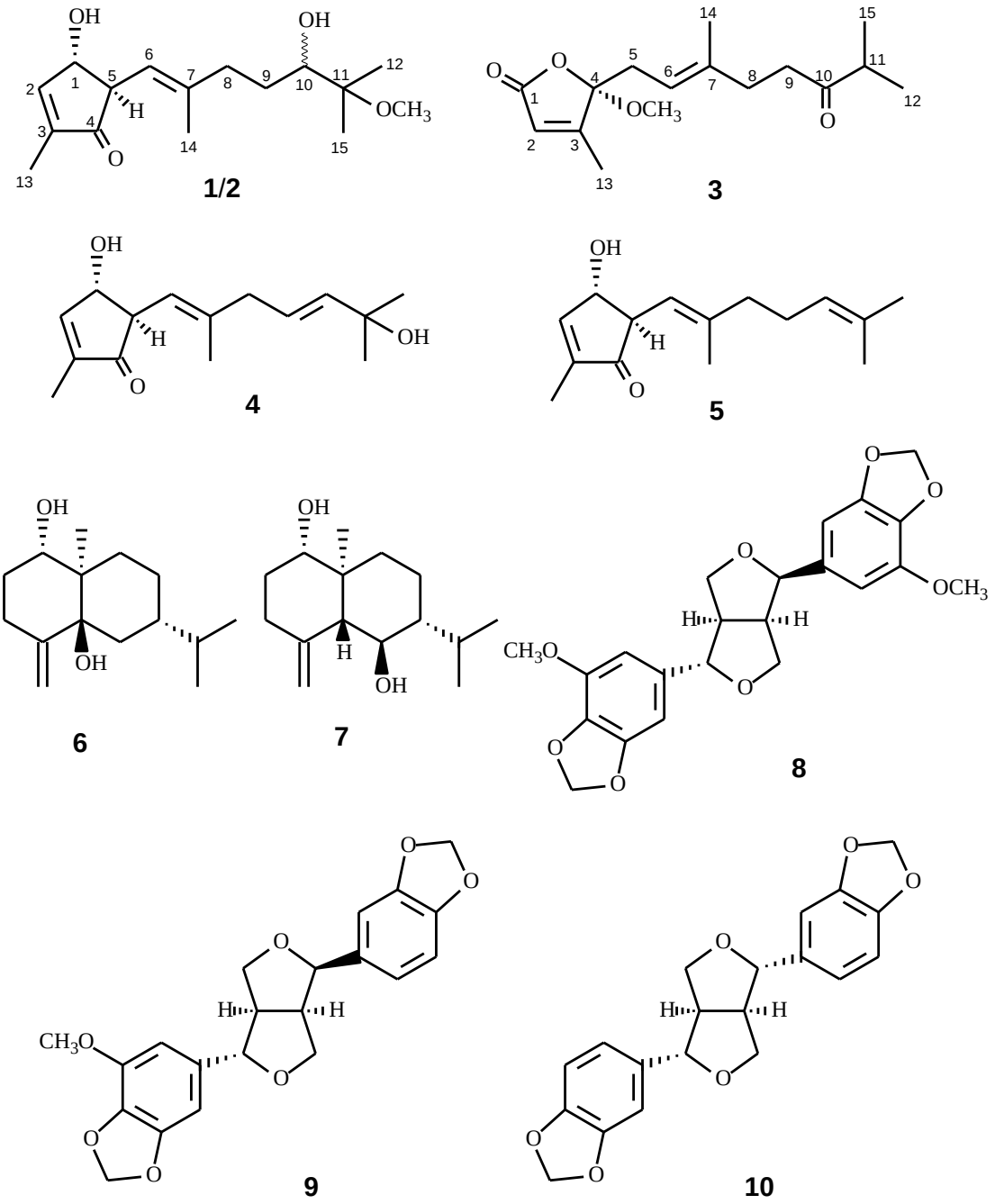
[c] Multiplicities in parentheses represent: s (singlet), d (doublet), t (triplet), br s (broad singlet), br d, (broad doublet), dd (doublet of doublet), dt (doublet of triplet), and tq (triplet of quartet), qu (quintet), se (septet).

Table 2. Antiviral Activities of Compounds 5-10

Table 3. Anti-HIV and Cytotoxic Activities of *Litsea* Plant Extracts

Notes: Samples were tested for their anti-HIV activities at 20 μg/mL; BR: barks; FB: flower buds; FL: flowers; LF: leaves; RT: roots; SR: stem barks; TW: twigs; HOG.R5: green fluorescent protein-based reporter cell line for anti-HIV activity; KB: human oral epidermoid carcinoma cells; LNCaP: human

prostate carcinoma cells; Col2: human colon carcinoma cells; hTERT: human telomerase-immortalized normal cells; HUVEC: human umbilical vein endothelial; Lu1: human lung carcinoma cells.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OH
OCH3
H
O
1/2
O
O
OCH3
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3
OH
H
OH
O
4
OH
H
O
5
OH
OH
6
OH
H
OH
7
O
O
O
OCH3
H
H
CH3O
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O
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8
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O
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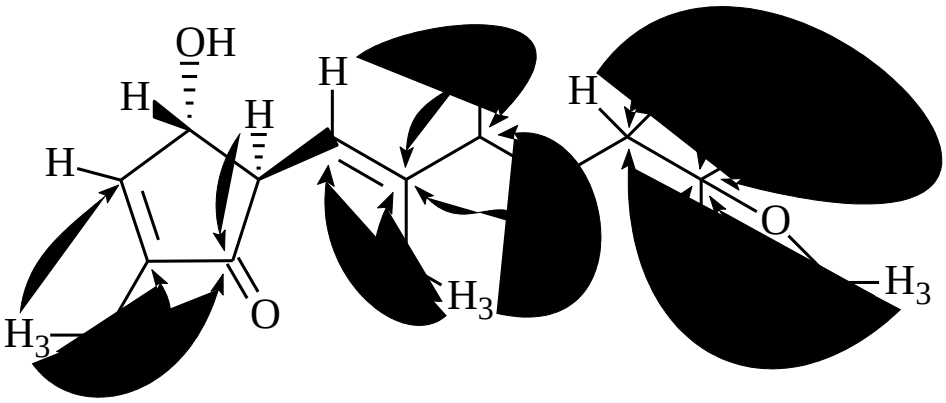
10

Figure 1.



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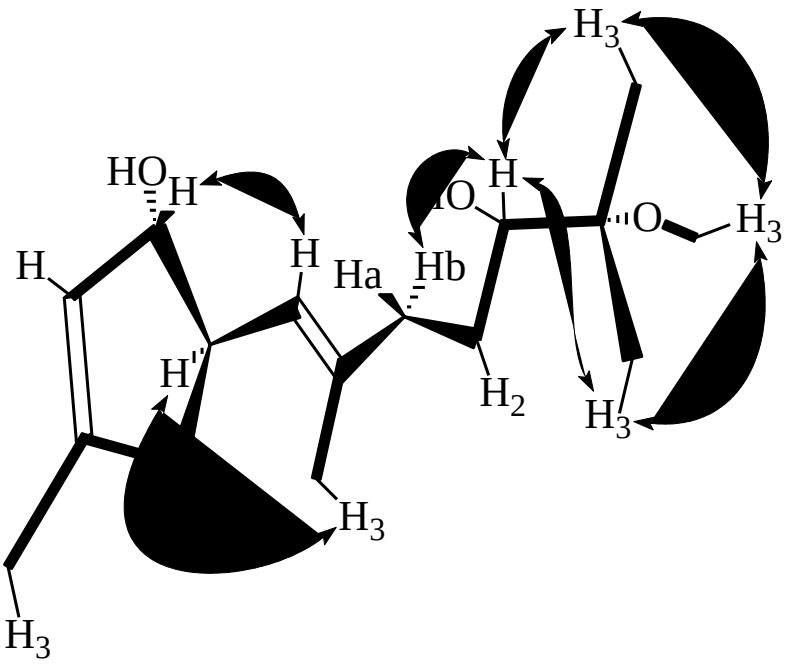
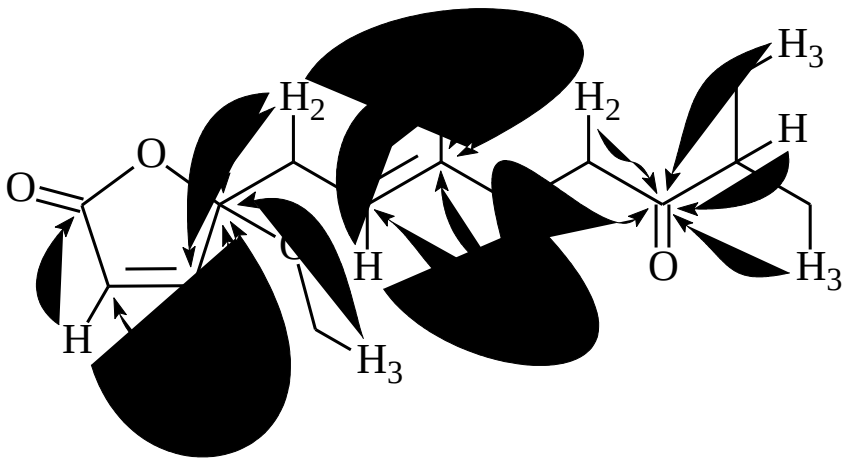
506 Figure 2.



507

508 Figure 3.



509

510 Figure 4.

511

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Spectroscopic Data of Compounds 1-3 in $CDCl_3$[a]

Position	**1/2**		**3**	
	δ_C[b]	δ_H[c]	δ_C	δ_H
1	76.4 (d)	4.59 (br s)	168.7 (s)	
2	155.0 or 154.9 (d)	7.12 (qu, 1.4)	120.3 (d)	5.88 (q, 1.6)
3	142.8 (s)		164.8 (s)	
4	206.3 or 206.2 (s)		111.1 (s)	
5	56.4 or 56.3 (d)	3.15 or 3.14 (dd, 9.0, 2.5 or 9.1, 2.4)	34.1 (t)	
5a				2.72 (dd, 15.2, 7.4)
5b				2.46 (dd, 15.8, 7.2)
6	119.3 or 118.9 (d)	5.07 or 5.05 (dq, 8.9, 1.2 or 8.8, 1.2)	115.4 (d)	4.92 (tq, 7.3, 1.3)
7	142.0 or 141.8 (s)		139.5 (s)	
8	37.0 or 36.8 (t)		33.4 (t)	2.21 (t, 7.4)
8a		2.35 (m)		
8b		2.14 (m)		
9	29.4 or 29.3 (t)		38.7 (t)	
9a		1.54 (m)		2.51 (ABdt, 17.0, 7.7)
9b		1.44, m		2.45 (ABdt, 17.0, 6.9)
10	76.3 or 76.0 (d)	3.45 or 3.40 (br d, 10.0 or 10.1)	214.1 (s)	
11	77.0 (s)		40.8 (d)	2.57 (se, 6.9)
12	20.7 or 20.6 (q)	1.11 or 1.10 (s)	18.23 (q)	1.06 (d, 6.9)
13	10.2 (q)	1.79 (t, 1.3)	12.7 (q)	1.94 (d, 1.6)
14	17.2 or 16.9 (q)	1.75 or 1.74 (s)	16.6 (q)	1.62 (d, 1.0)
15	19.0 (q)	1.08 or 1.07 (s)	18.28 (q)	1.05 (d, 6.9)
OCH_3	49.1 (q)	3.20 (s)	50.5 (q)	3.15 (s)

Table 2. Antiviral Activities of Compounds 5-10

Virus	Antiviral Activity (EC_{50}: μM; Selective Index)					
	5	**6**	**7**	**8**	**9**	**10**
Adeno	–	–	–	>50	–	–
HBV	–	–	–	>300	>300	–
EBV	–	–	–	22, 3.8	16.2, >6.2	–
Flu A (H1N1)	>50	>100	>400	>140	>260	>130
Flu A (H3N2)	>50	>85	>190	>90	>260	>280
Flu A (H5N1)	>130	>230	>400	>240	>260	>280
Flu B (H5N1)	>15	–	–	>240	–	–
HCMV	–	–	–	58.3, >5.1	–	–
HCV	–	–	–	>20	>20	–
HPV	–	–	–	>60.4	–	–
HSV-1	–	–	–	>300	–	–
HSV-2	–	–	–	>280	–	–
Measles	–	–	–	>50	–	–
PIV	–	–	–	>100	–	–
Rhinovirus Type 2	–	–	–	>100	–	–
Rift Valley Fever	–	–	–	>240	–	–
RSVA	–	–	–	>100	–	–
SARS	68.4, 2.8		>210	>240	>130	
Tacaribe	–	–	–	>150	–	–
VZV	–	–	–	>300	–	–
WNV	–	–	–	>240	–	–

532 **Table 3. Anti-HIV and Cytotoxic Activities of *Litsea* Plant Extracts**

Plant Species	Code	Plant Part	Anti-HIV Activity	Cytotoxicity (IC_{50}: μg/mL)					
				KB	LNCaP	Col2	hTERT	Lu1	HUVEC
L. balansae	SV0014	ST	100	4.0	4.7	>20	>20	>20	>20
	SV0418	LF+TW+FL	55	>20	>20	>20	>20	>20	>20
	SV0419	SB	100	5.0	12.3	>20	7.0	10.9	14.9
	SV0420	BR	100	>20	>20	>20	>20	>20	>20
	SV5186	LF	29	>20	>20	>20	>20	>20	>20
L. baviensis	SV0357	LF+TW+FB	0	>20	>20	>20	>20	>20	>20
	SV0358	BR	5	>20	>20	>20	>20	>20	>20
	SV0359	SB	4	>20	>20	>20	>20	>20	>20
	SV0360	RT	20	>20	>20	>20	>20	>20	>20
	SV0614	LF+TW	14	>20	>20	>20	>20	>20	>20
	SV0616	FR	25	>20	>20	>20	>20	>20	>20
L. chartacea	SV0382	LF+TW	0	>20	>20	>20	>20	>20	>20
	SV0383	BR	17	>20	>20	>20	>20	>20	>20
L. cubeba	SV0244	ST	47	>20	>20	>20	>20	>20	>20
	SV0245	LF+TW	49	>20	>20	>20	>20	>20	>20
	SV4163	SB	40	>20	>20	>20	>20	>20	>20
	SV4164	RT	26	>20	>20	>20	>20	>20	>20
	SV4166	BR	34	>20	>20	>20	>20	>20	>20
L. garrettii	SV5040	SB	37	>20	>20	>20	>20	>20	>20
L. griffithii	SV2224	LF+TW	0	>20	>20	>20	>20	>20	>20
	SV2225	BR	7	>20	>20	>20	>20	>20	>20
	SV2226	FR	18	>20	>20	>20	>20	>20	>20
	SV2227	SB		9.8	9.4	10.1	12.0	>20	11.7
L. lancifolia	SV0219	LF+TW+FB	0	>20	>20	>20	>20	>20	>20
	SV0220	SB	100	>20	>20	>20	>20	>20	>20
	SV0221	RT	97	>20	>20	>20	>20	>20	>20
L. monopetala	SV0172	LF+TW+FB	5	>20	>20	>20	>20	>20	>20
	SV0173	SB	100	>20	>20	>20	>20	>20	>20
	SV0907	LF+TW+FL	5	>20	>20	>20	>20	>20	>20
	SV0908	BR	0	>20	9.0	>20	18.0	>20	10.1
	SV0909	SB	100	>20	>20	>20	19.0	>20	9.5
	SV0910	RT	100	>20	9.0	>20	14.0	>20	9.0
	SV5188	LF	35	–	>20	–	–	–	–
	SV5189	SB	47	>20	>20	–	–	–	–
L. robusta	SV0191	SB	16	>20	>20	>20	>20	>20	>20
	SV0192	RT	19	>20	>20	>20	>20	>20	>20
	SV0193	LF+TW	32	>20	>20	>20	>20	>20	>20
	SV0416	BR	35	>20	>20	>20	>20	>20	>20
L. verticillata	SV0001	LF+TW+FB	77	>20	>20	>20	>20	>20	>20
	SV0002	BR	50	>20	>20	>20	>20	>20	>20
	SV5064	LF	38	>20	>20	>20	>20	>20	>20

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