

## Litsea Species as Potential Antiviral Plant Sources

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# ***Litsea* Species as Potential Antiviral Plants Source**

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1

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

28  
29 **Keywords:** *Litsea*; Lauraceae; Litseane; Sesquiterpene butenolide; Antiviral activity;  
30 Anti-HIV activity; Structure determination

31

## 32 **Introduction**

33

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

42 Plant compounds, known for their enormous numbers and their amazing structural diversity,  
43 are considered an excellent source for exploration for new and diverse antiviral agents. *Litsea*  
44 *verticillata* (Lauraceae) was found to be one of the first anti-HIV plant leads in our efforts to  
45 discover antiviral agents from the tropical plants in the Southern Asia area (Soejarto *et al.*, 2006).  
46 The plant, grown up as a shrub or small tree 2-5 m tall, is found in southern Asia including Vietnam  
47 and Southern China, where the altitude is less than 1300. The plant has been used as a traditional  
48 Chinese medicine to relieve swelling caused by injury and snake biting (Jiangsu, 1986). Our  
49 previous studies on this plant have resulted in the isolation of 34 compounds including two lignans,  
50 five butenolides and 27 sesquiterpenes. Among these isolates, 21 were determined as new  
51 molecules, and 20 were found to have anti-HIV activity (Hoang *et al.*, 2002; Zhang *et al.*, 2001,  
52 2003a, 2003b, 2005). The sesquiterpenes belong to 13 different skeletal types, including the novel  
53 structural class we designated as litseane. Confirmation of the new unique litseane structural

54 skeleton has been confirmed by synthesis of selected members of our isolates by three research  
55 groups. From 2003 to 2006, the Vassilikogiannakis group accomplished the total synthesis of  
56 litseaverticillols A-H by means of a biomimetic sequence of transformations involving the singlet  
57 oxygen ( $^1\text{O}_2$ )-initiated cascade reaction as the key step (Vassilikogiannakis and Stratakis, 2003;  
58 Vassilikogiannakis *et al.*, 2004, 2005; Margaros *et al.*, 2006; Montagnon *et al.*, 2008). In 2006, the  
59 Kuwahara group first accomplished the enantioselective total synthesis of the (1*R*, 5*S*)-stereoisomer  
60 of litseaverticillols A and B from homogermanic acid in six steps by employing the Evans  
61 asymmetric aldol reaction and a microwave-promoted cyclization of a stannylated thiol ester  
62 intermediate as the C-C bond-forming steps (Morita and Kuwahara, 2006; Morita *et al.*, 2006). In  
63 2007, the enantioselective total synthesis of the litseaverticillols C and K was achieved by the  
64 Mohapatra group using D-glucose as starting material and by employing the ring closing metathesis  
65 (RCM) and Wittig reactions as the key steps (Mohapatra *et al.*, 2007).

66 In an effort to further elucidate the antiviral constituents of *L. verticillata*, we identified three  
67 additional new sesquiterpenes: litseaverticillols L/M (**1/2**) and litseasesquibutenolide (**3**) (structures  
68 shown in Fig. 1). These compounds were evaluated for their anti-HIV activity. We further assessed  
69 the antiviral activity of a number of previously obtained natural compounds from this plant against  
70 21 viral targets available at NIAID (National Institute of Allergy and Infectious Diseases, National  
71 Institutes of Health, USA).

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

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

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

85

## 86 **Materials and Methods**

87

### 88 *General Experimental Procedures*

89

90 Optical rotations were measured with a Perkin-Elmer model 241 polarimeter. IR spectra were  
91 recorded on a Jasco FT/IR-410 spectrometer, equipped with a Specac Silver Gate ATR system by  
92 applying a film on a Germanium plate. 1D and 2D NMR spectra were recorded on a Bruker  
93 DRX-500 MHz spectrometer. Chemical shifts ( $\delta$ ) were expressed in ppm with reference to the  
94 solvent signals (CDCl<sub>3</sub>; <sup>1</sup>H: 7.24 ppm; <sup>13</sup>C: 77.00 pm), and coupling constants (*J*) are reported in  
95 Hz. All NMR experiments were obtained by using standard pulse sequences supplied by the  
96 vendor. Column chromatography was carried out on silica gel (200–400 mesh, Natland  
97 International Corporation). Reversed-phase flash chromatography was accomplished with RP-18  
98 silica gel (40–63 µm, EM Science), and reversed-phase preparative HPLC was carried out on a  
99 Waters 600E Delivery System pump, equipped with a Waters 996 photodiode detector, and a

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

105

#### 106 *Plant Material*

107 *L. balansae* Lecomte: The stem sample (SV0014) was collected on Nov 23, 1998 Bong, and was  
108 documented by voucher specimen Soejarto et al. 10396. The leaf, twig and flower sample  
109 (SV0418) was collected on July 27, 1999, and was documented by voucher specimens Soejarto et  
110 al. 10918. The stem bark sample (SV0419) was collected on July 27, 1999, and was documented  
111 by voucher specimens Soejarto et al. 10918. The bark sample (SV0420) was collected on July 27,  
112 1999, and was documented by voucher specimens Soejarto et al. 10918. The leaf sample  
113 (SV5186) of *L. balansae* Lecomte was collected in 1995, and was documented by voucher  
114 specimens MAFB-1425.

115 *L. baviensis* Lecomte: The leaf, twig and flower bud sample (SV0357) of *L. baviensis*  
116 Lecomte was collected on April 5, 1999 Bong, and was documented by voucher specimens  
117 Soejarto et al. 10686. The bark sample (SV0358) was collected on April 5, 1999 and was  
118 documented by voucher specimens Soejarto et al. 10686. The stem bark sample (SV0359) was  
119 collected on April 5, 1999, and was documented by voucher specimens Soejarto et al. 10686. The  
120 root sample (SV0360) was collected on April 5, 1999, and was documented by voucher  
121 specimens Soejarto et al. 10686. The leaf and twig sample (SV0614) was collected on September  
122 17, 1999, and was documented by voucher specimens NMC\_533. The fruit sample (SV0616) was



123 collected on September 17, 1999, and was documented by voucher specimens NMC\_533.

124 *L. chartacea* (Wall. ex Nees) Hook. f.: The leaf and twig sample (SV0382) of was collected  
125 on June 4, 1999 at Bong, CPNP, and was documented by voucher specimens Soejarto et al.  
126 10694. The bark sample (SV0383) was collected on June 4, 1999 Bong, CPNP, and was  
127 documented by voucher specimens Soejarto et al. 10694.

128 *L. cubeba* (Lour.) Pers: The stem sample (SV0244) of was collected on March 21, 1999, and  
129 was documented by voucher specimens Soejarto et al. 10653. The leaf and twig sample (SV0245)  
130 was collected on March 21, 1999,, and was documented by voucher specimens Soejarto et al.  
131 10653. The stem bark sample (SV4163) was collected on July 18, 2000, and was documented by  
132 voucher specimens Phan Ke Loc 10362. The root sample (SV4164) was collected on July 18,  
133 2000, and was documented by voucher specimens Phan Ke Loc 10362.. The bark sample  
134 (SV4166) was collected on July 18, 2000, and was documented by voucher specimens Phan Ke  
135 Loc 10362.

136 *L. garrettii* Gamble: The leaf sample (SV5040) of was collected on 1995, and was  
137 documented by voucher specimens Kanh & On 1144-A. The leaf and twig sample (SV2224) was  
138 collected on August 25, 2000, and was documented by voucher specimens CNMC\_1050.

139 *L. griffithii*: The bark branch sample (SV2225) of was collected on August 25, 2000, and was  
140 documented by voucher specimens NMC\_1050. The fruit sample (SV2226) was collected on  
141 August 25, 2000, and was documented by voucher specimens NMC\_1050. The stem bark sample  
142 (SV2227) was collected on August 25, 2000, and was documented by voucher specimens  
143 NMC\_1050.

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

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

149 *L. monopetala* (Roxb.) Pers.: The leaf, twig and flower bud sample (SV0172) of was  
150 collected on March 19, 1999, and was documented by voucher specimens Soejarto et al. 10596.  
151 The stem bark sample (SV0173) of was collected on March 19, 1999, and was documented by  
152 voucher specimens Soejarto et al. 10596. The leaf, twig and flower sample (SV0907) was  
153 collected on May 16, 2000 and was documented by voucher specimens Soejarto et al. 11505. The  
154 bark sample (SV0908) was collected on May 16, 2000, and was documented by voucher  
155 specimens Soejarto et al. 11505. The stem bark sample (SV0909) was collected on May 16, 2000,  
156 and was documented by voucher specimens Soejarto et al. 11505. The root sample (SV0910) was  
157 collected on May 16, 2000, and was documented by voucher specimens Soejarto et al. 11505.  
158 The leaf sample (SV5188) was collected in 1995, and was documented by voucher specimens  
159 MAFA\_1053. The stem bark sample (SV5189) was collected on 1995, and was documented by  
160 voucher specimens MAFA\_1053.

161 *L. robusta* Blume: The stem bark sample (SV0191) of was collected on March 19, 1999, and  
162 was documented by voucher specimens Soejarto et al. 10611. The root sample (SV0192) was  
163 collected on March 19, 1999, and was documented by voucher specimens Soejarto et al. 10611.  
164 The leaf and twig sample (SV0193) was collected on March 19, 1999, and was documented by  
165 voucher specimens Soejarto et al. 10611. The bark sample (SV0416) was collected on July 27,  
166 1999, and was documented by voucher specimens Soejarto et al. 10917.

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

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

172 A large quantity of the plant sample (SVA0001, 4.5 kg, voucher specimens *Soejarto et al.*  
173 11003) was subsequently re-collected at the same site of SV0001 at CPNP on November 17,  
174 1999, for complete isolation work. Duplicate voucher specimens of both collections were  
175 deposited at the herbaria of CPNP, Institute of Ecology and Biological Resources (HN) of the  
176 Vietnam Academy of Science and Technology in Hanoi, Hanoi University of Pharmacy in Hanoi,  
177 and John G. Searle Herbarium of the Field Museum (F) (Chicago, IL, USA).

178

#### 179 *Preparation of the Crude Plant Extracts*

180

181 All initially collected plant materials (about 50 g each) were extracted with CHCl<sub>3</sub> and prepared  
182 as CHCl<sub>3</sub> extracts. The dried and milled leaves and twigs (4.5 kg) of the recollected plant  
183 materials of *L. verticillata* were extracted with MeOH, and subsequently defatted with *n*-hexane  
184 and partitioned with CHCl<sub>3</sub> to afford a CHCl<sub>3</sub> extract (93.0 g).

185

#### 186 *Isolation of Compounds*

187

188 The resulting extract (93.0 g) was processed as previously described (Zhang *et al.*, 2005) to  
189 afford the anti-HIV fractions F17 and F18. F17 (1.31 g) was subjected to preparative HPLC  
190 separation on a Phenomenex LUNA-C18 column (solvent system: MeOH/H<sub>2</sub>O 1:1) to afford the  
191 active fraction F31 (12 mg), which was subjected to further preparative HPLC separation on the

192 GROM-Saphir 110 C18 column, and elution with MeCN/H<sub>2</sub>O 3:7 to afford litseaverticillol I/J  
193 (**1/2**, 1.51 mg). F18 (0.92 g) was subjected to preparative HPLC separation on a GROM-Saphir  
194 110 C18 column (solvent system: MeOH/H<sub>2</sub>O 7:3) to afford the active fraction F48 (59 mg),  
195 which was subjected to further preparative HPLC separation on the GROM-Saphir 110 C18  
196 column, and elution with MeCN/H<sub>2</sub>O 4:6 to afford litseasesquibutenolide (**3**, 0.56 mg).

197

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

199

200 Colorless gum;  $[\alpha]_{\text{D}}^{23}$  0° (*c* 0.10, CHCl<sub>3</sub>); UV (MeOH)  $\lambda_{\text{max}}$  (AU) 222.4 (1.15), 316.3 (0.02) nm;  
201 IR (film)  $\nu_{\text{max}}$  3414 (*br*), 2973, 2926, 2854, 1702, 1652, 1452, 1378, 1322, 1185, 1072, 1036 cm<sup>-1</sup>;  
202 <sup>1</sup>H NMR and <sup>13</sup>C NMR spectroscopic data, see Table 1; CIMS *m/z* (rel. int.) 300 [M+NH<sub>4</sub>]<sup>+</sup>, 283  
203 [M+H]<sup>+</sup>, 251 [M-OMe+H]<sup>+</sup> (100), 231 [M-H<sub>2</sub>O-OMe+H]<sup>+</sup>; HRCIMS *m/z* 283.1924 [M+H]<sup>+</sup>  
204 (Calcd for C<sub>16</sub>H<sub>27</sub>O<sub>4</sub>: 283.1909); HRTOFMS *m/z* 251.1649 [M-OMe+H]<sup>+</sup> (Calcd for C<sub>15</sub>H<sub>23</sub>O<sub>3</sub>:  
205 251.1647).

206

207 *Litseasesquibutenolide (3)*

208

209 Colorless gum;  $[\alpha]_{\text{D}}^{23}$  -15° (*c* 0.04, CHCl<sub>3</sub>); UV (MeOH)  $\lambda_{\text{max}}$  (AU) 200.0 (0.75), 280.4 (0.05)  
210 nm; IR (film)  $\nu_{\text{max}}$  2966, 2817, 1769, 1708, 1652, 1459, 1384, 1266, 1153, 1104, 923 cm<sup>-1</sup>; <sup>1</sup>H  
211 NMR and <sup>13</sup>C NMR spectroscopic data, see Table 1; CIMS *m/z* (rel. int.) 298 [M+NH<sub>4</sub>]<sup>+</sup> (100),  
212 281 [M+H]<sup>+</sup>, 249 [M-OMe+H]<sup>+</sup> (100); HRCIMS *m/z* 281.1737 [M+H]<sup>+</sup> (Calcd for C<sub>16</sub>H<sub>25</sub>O<sub>4</sub>:  
213 281.1747)

214

215 *Anti-HIV and Cytotoxicity Assays*

216

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

233

234 *Antiviral Activity Evaluated at NIAID*

235

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

238 Research Institute (2000 9<sup>th</sup> Avenue South, Post Office Box 55305, Birmingham, AL35255-5305,  
239 USA), for their antiviral potential against 21 viral targets: flu-A (Solomon Island/03/2006 H1N1,  
240 the Wisconsin/67/2005 H3N2, and Vietnam/1203/2004H H5N1 strains) and flu B  
241 (Malaysia/2506/2004 strain) viruses tested in Madin-Darby canine kidney (MDCK) cells,  
242 rhinovirus type-2 (HGP strain) virus tested in Hela cervical cancer cells, adenovirus  
243 (65089/Chicago strain) tested in A-549 lung cancer cells, parainfluenza (PIV: 14702 strain) and  
244 respiratory syncytial (RSVA: A2 strain) viruses tested in MA-104 monkey kidney cells,  
245 Epstein-Barr virus (EBV) tested in Akata lymphoma cells, severe acute respiratory syndrome  
246 (SARS: Urbani strain), Rift Valley Fever (MP-12 strain), Tacribe (TRVL11573 strain) and West  
247 Nile (WNV: New York strain) viruses tested in vero-76 monkey kidney cells, hepatitis C virus  
248 (HCV) tested in Huh7 ET liver cells, hepatitis B virus (HBV) tested in Hep G2 liver cells, human  
249 papilloma virus (HPV), measles (Chicago strain) tested in CV-1 monkey kidney cells, Varicella  
250 zoster viurs (VZV), and herpes simplex-1 and -2 viruses (HSV-1 and HSV-2) and human  
251 cytomegalovirus (HCMV) tested in human foreskin fibroblast (HFF) cells.

252

## 253 **Results and Discussion**

254

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

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

261 separate the two molecules using two different preparative HPLC columns (Phenomenex, LUNA  
262 phenyl-hexyl, 15  $\mu\text{m}$ , 250  $\times$  50 mm; Grom Saphir 110 C18, 12  $\mu\text{m}$ , 300  $\times$  40 mm) and several  
263 different solvent systems were unsuccessful. This mirrors the situations that we and the other  
264 research groups have previously encountered using a variety of different techniques to separate  
265 pairs of compounds possessing a side chain formed from two or more isoprene units and  
266 containing an oxy group at the far end carbon(s) (Zhang *et al.*, 2003a, 2006; Montagnon *et al.*,  
267 2008).

268 Most of the  $^1\text{H}$  NMR signals of **1/2** completely overlapped each other (Table 1), indicating a  
269 mixture of two compounds bearing a close structural relationship. However, the  $^{13}\text{C}$  signals for 10  
270 of the 16 carbons were clearly distinctive (maximum chemical shift divergence = 0.41 ppm). The  
271 16 carbons were characterized by the  $^{13}\text{C}$  NMR and DEPT spectra as four non-oxymethyls ( $\delta$   
272 20.7/20.6, 19.0, 17.2/16.9 and 10.2), an oxymethyl ( $\delta$  49.1), two methylenes ( $\delta$  37.0/36.8 and  
273 29.4/29.3), a non-oxymethine ( $\delta$  56.4/56.3), two oxymethines ( $\delta$  76.4 and 76.3/76.0), two olefinic  
274 methines ( $\delta$  155.0/154.9 and 119.3/118.9), an oxy-quaternary carbon ( $\delta$  77.0), two olefinic  
275 quaternary carbon ( $\delta$  142.8 and 142.0/141.8), and a quaternary carbonyl group ( $\delta$  206.3/206.2)  
276 (Table 1). The similarity of the NMR spectral data of **1/2** to those of the previously identified  
277 litseanes suggested that **1/2** possess the same structural skeleton of the litseane sesquiterpenes  
278 (Zhang *et al.*, 2001, 2003a). The spectral data of **1/2** are most similar to the litseane  
279 litseaverticillol D (**4**). As in **4**, compounds **1/2** were also found to possess a 1-oxy-1-methyl-ethyl  
280 group [(Me) $_2$ C(O)-] and a sub-structural unit (**unit A**: C1 to C8) of  
281 3-(5-hydroxy-3-methyl-2-oxo-cyclopent-3-enyl)-2-methyl-allyl through analysis of the  $^1\text{H}$ - $^1\text{H}$   
282 COSY, HMQC, and HMBC spectral data (Fig. 2). However, the NMR data indicated that **1/2**  
283 differ from **4** by the presence of a 1-hydroxy-ethyl group at C-9 and C-10 [-CH $_2$ CH(OH)-] [ $\delta$  1.54

284 (m), 1.44 (m), and 3.45/3.40 (br d,  $J = 10.0/10.1$  Hz);  $\delta$  29.4/29.3 (t) and 76.3/76.0 (d)] and a  
285 methoxy group [ $\delta$  3.20 (s) and  $\delta$  49.1 (q)]. There is no second double bond, as in the case of **4**,  
286 being observed in the NMR spectra of **1/2**. The  $^1\text{H}$ - $^1\text{H}$  COSY couplings between the methylene  
287 proton signals of the 1-hydroxy-ethyl group and the methylene proton signals [ $\delta$  2.35 (m) and  
288 2.14 (m)] of **unit A** connected the two methylene carbons in **1/2**, while the presence of the HMBC  
289 correlations of the proton signal at  $\delta$  3.45 (br d,  $J = 10.0$  Hz) to the  $^{13}\text{C}$  signals of the three  
290 carbons of the 1-oxy-1-methyl-ethyl group [ $\delta$  77.0 (s), 20.7 or 20.6 (q), and 19.0 (q)] bonded the  
291 oxy-methine carbon of the 1-hydroxy-ethyl group to the oxy-quaternary carbon (C-11) in the  
292 1-oxy-1-methyl-ethyl group. The methoxy group is also bonded to C-11 due to the presence of the  
293 HMBC correlation between the methoxy proton signal and the  $^{13}\text{C}$  signal of C-11. The planar  
294 structures of **1/2** were then elucidated to be 4-hydroxy-5-(5-hydroxy-6-methoxy-2,  
295 6-dimethyl-hept-1-enyl)-2-methyl-cyclopent-2-enone, which has a molecular formula of  $\text{C}_{16}\text{H}_{26}\text{O}_4$   
296 as determined by the HRCIMS ( $[\text{M}+\text{H}]^+$  ( $m/z$  283.1924, calcd. 283.1909) and HRTOFMS  
297 ( $[\text{M}-\text{OMe}+\text{H}]^+$  ( $m/z$  251.1649, calcd. 251.1647).

298 The geometric and chiral (C-1 and C-5) configurations of **1/2** were determined to be the  
299 same as those in **4** due to their similar chemical shifts and coupling patterns at these carbon  
300 positions, and on the basis of the observation of an ROE correlation between H-5 [ $\delta$  3.15 (dd,  $J =$   
301 9.0, 2.5 Hz)/3.14 (dd,  $J = 9.1, 2.4$  Hz)] and H-14 [ $\delta$  1.75/1.74 (3H, s)], as well as the ROE  
302 correlation between H-1 [ $\delta$  4.59 (br s)] and H-6 [ $\delta$  5.07 (dq,  $J = 8.9, 1.2$  Hz)/5.05 (dq,  $J = 8.8, 1.2$   
303 Hz)] (Fig. 3). Based on the above data, **1** and **2** were determined to be epimeric at C-10, which  
304 mirrors the structural relationship of litseaverticillols F and G (Zhang *et al.*, 2003a). The presence  
305 of this very flexible side chain at C-5 contributed significantly to the compounds being unable to  
306 be separated. The structures for the two compounds (**1/2**) were thus determined as  $1\alpha$ ,



307 10 $\alpha$ -dihydroxy-11-methoxy-(*E*)-litse-2, 6-dien-4-one and 1 $\alpha$ ,  
308 10 $\beta$ -dihydroxy-11-methoxy-(*E*)-litse-2, 6-dien-4-one, and given the trivial names of  
309 litseaverticillols L and M, respectively.

310 Compound **3** with a molecular formula of C<sub>16</sub>H<sub>24</sub>O<sub>4</sub> according to the HRCIMS ([M+H]<sup>+</sup> *m/z*  
311 281.1737, calcd. 281.1747), was shown to be composed of an  $\alpha$ ,  $\beta$ -conjugated ester group, a  
312 second C-C double bond, two tertiary methyls, two secondary methyls, an oxy-methyl, three  
313 methylenes, a methine, a dioxy-substituted carbon, and a keto carbonyl carbon as evidenced by  
314 the <sup>1</sup>H and <sup>13</sup>C NMR spectral data (Table 1). Analysis of the <sup>1</sup>H-<sup>1</sup>H COSY, HMQC and HMBC  
315 (Fig. 4) spectral data in the same manner employed for the structure determination of **1/2** led to  
316 the elucidation of a sub-structural unit of 3, 7-dimethyl-6-oxo-oct-2-enyl group (**unit B**) for **3**.

317 The presence of HMBC correlations of the proton signals at  $\delta$  2.72 (dd, *J* = 15.2, 7.4 Hz,  
318 H-5a) and 2.46 (dd, *J* = 15.8, 7.2 Hz, H-5b) to the <sup>13</sup>C signal at  $\delta$  111.5 (s) suggested the  
319 attachment of **unit B** to a dioxy-substituted carbon (C-4), which was then connected by an  $\alpha$ ,  
320  $\beta$ -conjugated ester group evidenced by the presence of HMBC correlations between the proton  
321 signal at  $\delta$  5.88 (q, *J* = 1.6 Hz) and the <sup>13</sup>C signal of C-4, and between H-5a and the <sup>13</sup>C signal at  $\delta$   
322 164.8 (s, C-3). The methoxy group and the second methyl group in **3** were assigned to C-4 and  
323 C-3 respectively, as supported by the presence of HMBC correlations of the methoxy proton  
324 signals at  $\delta$  3.15 (s) to the <sup>13</sup>C signal of C-4, and the methyl proton signals at  $\delta$  1.94 (d, *J* = 1.6  
325 Hz) to the <sup>13</sup>C signals of C-2 [ $\delta$  120.3 (d)], -3 and -4. Five double-bond equivalents were  
326 calculated from the molecular formula (C<sub>16</sub>H<sub>24</sub>O<sub>4</sub>) of **3**, four of which were accounted for by the  
327 presence of two carbon-carbon double bonds and two carbonyl double bonds. The remaining  
328 unassigned unsaturated bond equivalent must be involved in the formation of a butenolide ring  
329 structure in **3**. The two proton doublet signals at  $\delta$  5.88 (*J* = 1.6 Hz) and 1.94 (*J* = 1.6 Hz) and the

330 <sup>13</sup>C NMR signals at  $\delta$  168.7, 164.8, 120.3, 111.1, and 12.7 assignable to the butenolide moiety are  
331 very similar to those of the known compound, actinolide A (Kim *et al.*, 2002). The double bond  
332 on the side chain was assigned as *E* configuration, which resulted in significant upfield of the <sup>13</sup>C  
333 NMR chemical shift of C-14 ( $\delta$  16.6 of **3** vs  $\delta$  25.6) and downfield of the <sup>13</sup>C NMR chemical shift  
334 of C-8 ( $\delta$  33.4 of **3** vs  $\delta$  39.7) in comparison with the sesquiterpene butenolides with a *Z*  
335 configured double bond (Vassilikogiannakis *et al.*, 2005). Compound **3** was thus elucidated as  
336 5-(2*E*-3, 7-dimethyl-6-oxo-oct-2-enyl)-5-methoxy-4-methyl-5*H*-furan-2-one, and was given the  
337 trivial name of litseasesquibutenolide.

338 During the initial bioactivity evaluation, the total CHCl<sub>3</sub> extract of *L. verticillata* inhibited  
339 HIV-1 replication by 76% at a concentration of 20  $\mu$ g/mL with no apparent toxicity at the same  
340 concentration. Follow-up bioassay-guided fractionation of the recollected plant materials yielded  
341 two active fractions (F-17 and F-18), from which compounds **1-3** were isolated. Litseaverticillols  
342 L/M (**1/2**) exhibited anti-HIV activity with an IC<sub>50</sub> value of 49.6  $\mu$ M and no toxicity to the host  
343 HOG-R5 cells at a concentration of 70  $\mu$ M, which is at a similar level of potency as  
344 litseaverticillol D (**4**) and other litseanes (Zhang *et al.*, 2003a). Compound **3** lacked inhibitory  
345 activity against HIV-1 replication at a concentration of 70  $\mu$ M .

346 Including litseaverticillols L/M (**1/2**) and litseasesquibutenolide (**3**), we have now identified  
347 a total of 39 natural compounds from *L. verticillata*. While lignans are the most abundant  
348 compounds produced in this plant, litseanes are considered as minor components with  
349 litseaverticillol A as the most abundant with a yield of 0.0016%. In cooperation with NIH NIAID  
350 (National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA) under a  
351 collaborative agreement, six compounds obtained from this plant, including litseaverticillol A (**5**),  
352 two eudesmane sesquiterpenes [verticillatol (**6**) and eudesm-4(15)-ene-1 $\beta$ , 6 $\alpha$ -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<sub>50</sub> 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<sub>50</sub> 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<sub>50</sub> 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. lancifolia*, *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 *lancifolia* 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*

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

378 In summary, with the isolation of litseaverticillols L/M (**1/2**), a total of 13 litseane  
379 sesquiterpenes have now been identified. Among these, litseaverticillols A-H and L/M were found  
380 to be naturally occurring molecules, while litseaverticillols I-K were compounds prepared through  
381 total synthesis. The sesquiterpene butenolides such as litseasesquibutenolide (**3**) may be  
382 considered as biosynthetic precursors of the litseane sesquiterpenes, as suggested from the  
383 biomimetic synthesis of litseaverticillols A-H using sesquiterpene butenolides as precursors  
384 (Vassilikogiannakis and Stratakis, 2003; Vassilikogiannakis *et al.*, 2004, 2005; Margaros *et al.*,  
385 2006; Montagnon *et al.*, 2008). Although all of our litseanes showed inhibitory activity against  
386 HIV replication, their selectivity indices of 2-3 are in the sub-optimal range for drug development.  
387 Our laboratory is committed to synthesizing a library of litseane analogues in an attempt to  
388 enhance the anti-HIV potency of the compounds while reducing their toxicity. Analogues with  
389 improved selective indices may be considered potential anti-HIV drug candidates for further  
390 development. The lignan epicexcelcin (**8**) is the most abundant compound found in *L. verticillata*.  
391 Its antiviral activity against several viral targets including EBV and HCMV renders it a potential  
392 lead compound for the synthesis of additional analogues for bioactivity evaluation and potential  
393 drug development. After evaluating 41 extracts made from various plant parts from 10 *Litsea*  
394 plant species, we have determined *L. balansae*, *L. lancifolia* and *L. monopetala* as three additional  
395 anti-HIV plant leads, which are worthy for further phytochemical exploration to discover other  
396 potential novel anti-HIV compounds.

397

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399

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413

414

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416

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475



476 **Legends and Notes**

477

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

479

480 Figure 2. Selected HMBC correlations for compound **1/2** (CDCl<sub>3</sub>).

481

482 Figure 3. Selected ROESY correlations for compound **1/2** (CDCl<sub>3</sub>).

483

484 Figure 4. Selected HMBC correlations for compound **3** (CDCl<sub>3</sub>).

485

486 **Table 1. <sup>1</sup>H (500 MHz) and <sup>13</sup>C (125 MHz) NMR Spectroscopic Data of Compounds 1-3 in CDCl<sub>3</sub>**

487 *Notes:*

488 <sup>a</sup> Coupling constants *J* in Hz are shown in parentheses, and  $\delta$  values are given in ppm.

489 <sup>b</sup> Multiplicities in parentheses represent: s (quaternary carbon), d (CH), t (CH<sub>2</sub>), and q (CH<sub>3</sub>).

490 <sup>c</sup> Multiplicities in parentheses represent: s (singlet), d (doublet), t (triplet), br s (broad singlet), br d, (broad  
491 doublet), dd (doublet of doublet), dt (doublet of triplet), and tq (triplet of quartet), qu (quintet), se (septet).

492

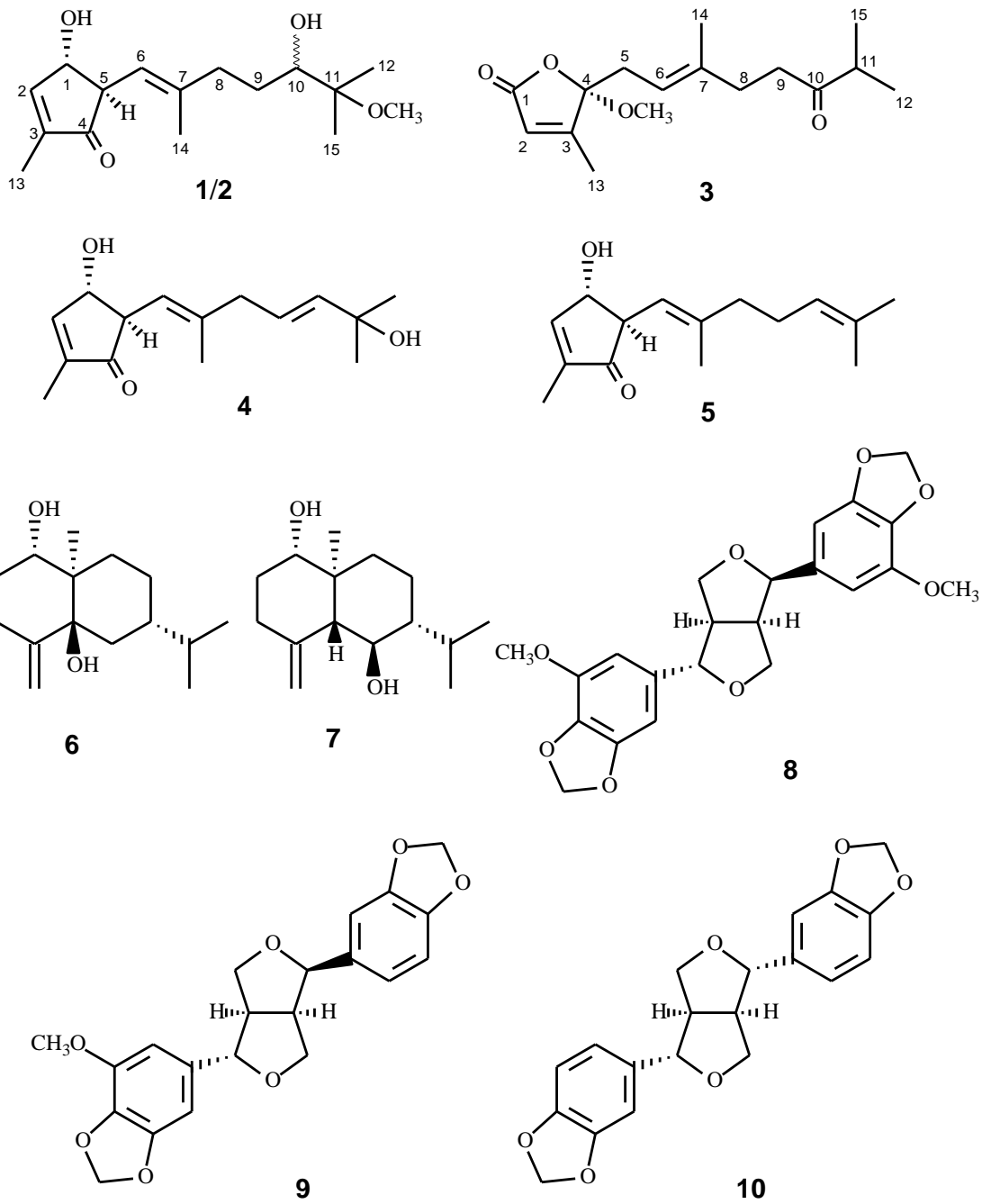
493 **Table 2. Antiviral Activities of Compounds 5-10**

494

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

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

499 prostate carcinoma cells; Col2: human colon carcinoma cells; hTERT: human telomerase-immortalized  
500 normal cells; HUVEC: human umbilical vein endothelial; Lu1: human lung carcinoma cells.  
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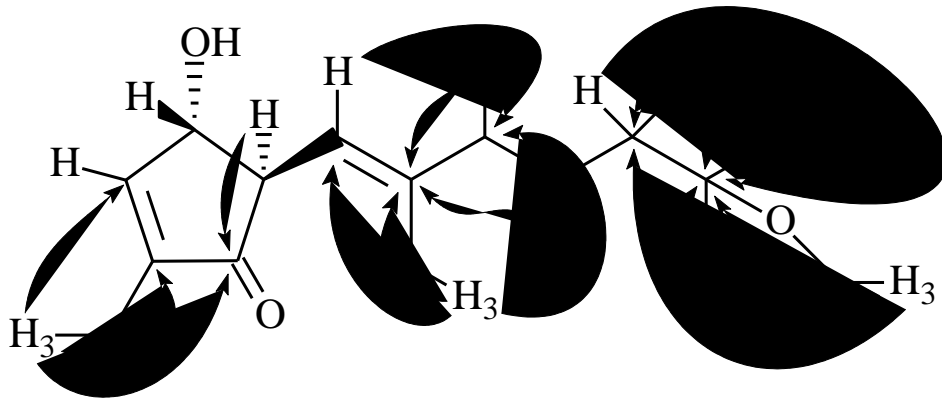


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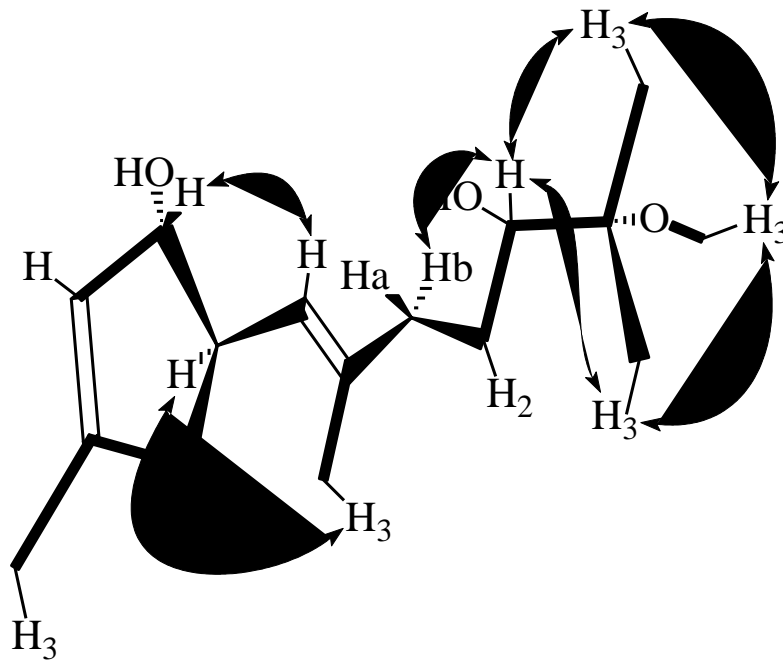
Figure 1.



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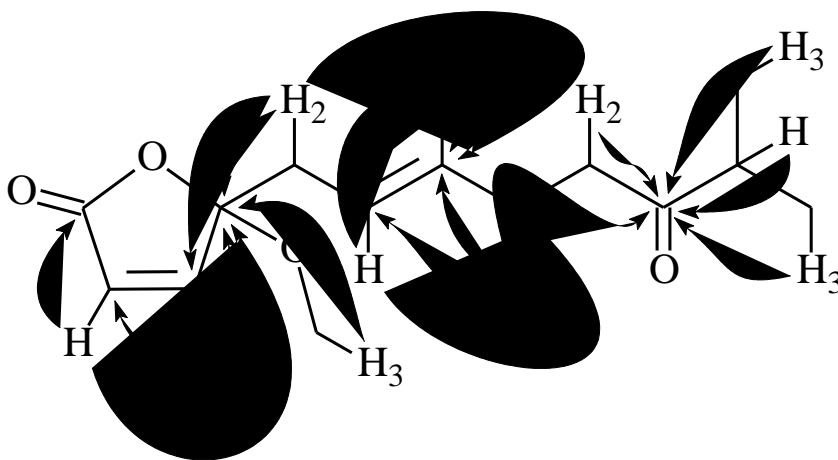
Figure 2.



507

508

Figure 3.



509

510

511

Figure 4.

512 **Table 1. <sup>1</sup>H (500 MHz) and <sup>13</sup>C (125 MHz) NMR Spectroscopic Data of Compounds 1-3 in CDCl<sub>3</sub><sup>a</sup>**

| Position         | 1/2                |  | 3          |                           |
|------------------|--------------------|--|------------|---------------------------|
|                  | $\delta_C^b$       | $\delta_H^c$                               | $\delta_C$ | $\delta_H$                |
| 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,<br>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,<br>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,<br>7.7) |
| 9b               |                    | 1.44, m                                    |            | 2.45 (ABdt, 17.0,<br>6.9) |
| 10               | 76.3 or 76.0 (d)   | 3.45 or 3.40 (br d,<br>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 <sub>3</sub> | 49.1 (q)           | 3.20 (s)                                   | 50.5 (q)   | 3.15 (s)                  |

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521 **Table 2. Antiviral Activities of Compounds 5-10**

| Virus             | Antiviral Activity (EC <sub>50</sub> : 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       | –          | –    |

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| Plant Species          | Code   | Plant Part | Anti-HIV Activity | Cytotoxicity (IC <sub>50</sub> : µg/mL) |       |      |       |      |       |
|------------------------|--------|------------|-------------------|---|-------|------|-------|------|-------|
|                        |        |            |                   | KB                                      | LNCaP | Col2 | hTERT | Lu1  | HUVEC |
| <i>L. balansae</i>     | 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   |
| <i>L. baviensis</i>    | 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   |
| <i>L. chartacea</i>    | SV0382 | LF+TW      | 0                 | >20                                     | >20   | >20  | >20   | >20  | >20   |
|                        | SV0383 | BR         | 17                | >20                                     | >20   | >20  | >20   | >20  | >20   |
| <i>L. cubeba</i>       | 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   |
| <i>L. garrettii</i>    | SV5040 | SB         | 37                | >20                                     | >20   | >20  | >20   | >20  | >20   |
| <i>L. griffithii</i>   | 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  |
| <i>L. lancifolia</i>   | 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   |
| <i>L. monopetala</i>   | 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   | –    | –     | –    | –     |
| <i>L. robusta</i>      | 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   |
| <i>L. verticillata</i> | 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   |

