

4-1-2016

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Link to published article: <http://dx.doi.org/10.1142/S0192415X16500166>

APA Citation

Guan, Y., Wang, D., Tan, G., Hung, N., Cuong, N., Pezzuto, J., Fong, H., Soejarto, D., & Zhang, H. (2016). Litsea species as potential antiviral plant sources. *American Journal of Chinese Medicine*, 44 (2), 275-290. <https://doi.org/10.1142/S0192415X16500166>

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

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1

2 Running title: Antiviral *Litsea* Plants

3 Number of pages: 27; Number of figures: 4; Number of tables: 3

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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 IC₅₀ 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 EC₅₀ 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 (δ) were expressed in ppm with reference to the
94 solvent signals (CDCl₃; ¹H: 7.24 ppm; ¹³C: 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 mm²) or a Phenomenex
101 LUNA-C-18 column (120 Å, 12 µm, 250 × 50 mm²). 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 CHCl₃ and prepared
182 as CHCl₃ 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 CHCl₃ to afford a CHCl₃ 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/H₂O 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/H₂O 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/H₂O 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/H₂O 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, CHCl₃); UV (MeOH) λ_{max} (AU) 222.4 (1.15), 316.3 (0.02) nm;
201 IR (film) ν_{max} 3414 (*br*), 2973, 2926, 2854, 1702, 1652, 1452, 1378, 1322, 1185, 1072, 1036 cm⁻¹;
202 ¹H NMR and ¹³C NMR spectroscopic data, see Table 1; CIMS *m/z* (rel. int.) 300 [M+NH₄]⁺, 283
203 [M+H]⁺, 251 [M-OMe+H]⁺ (100), 231 [M-H₂O-OMe+H]⁺; HRCIMS *m/z* 283.1924 [M+H]⁺
204 (Calcd for C₁₆H₂₇O₄: 283.1909); HRTOFMS *m/z* 251.1649 [M-OMe+H]⁺ (Calcd for C₁₅H₂₃O₃:
205 251.1647).

206

207 *Litseasesquibutenolide (3)*

208

209 Colorless gum; $[\alpha]_{\text{D}}^{23}$ -15° (*c* 0.04, CHCl₃); UV (MeOH) λ_{max} (AU) 200.0 (0.75), 280.4 (0.05)
210 nm; IR (film) ν_{max} 2966, 2817, 1769, 1708, 1652, 1459, 1384, 1266, 1153, 1104, 923 cm⁻¹; ¹H
211 NMR and ¹³C NMR spectroscopic data, see Table 1; CIMS *m/z* (rel. int.) 298 [M+NH₄]⁺ (100),
212 281 [M+H]⁺, 249 [M-OMe+H]⁺ (100); HRCIMS *m/z* 281.1737 [M+H]⁺ (Calcd for C₁₆H₂₅O₄:
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 IC₅₀ 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_{IIIB}/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 9th 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 CHCl₃ 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 μm , 250 \times 50 mm; Grom Saphir 110 C18, 12 μm , 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 ^1H 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}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}C NMR and DEPT spectra as four non-oxymethyls (δ
272 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
273 29.4/29.3), a non-oxymethine (δ 56.4/56.3), two oxymethines (δ 76.4 and 76.3/76.0), two olefinic
274 methines (δ 155.0/154.9 and 119.3/118.9), an oxy-quaternary carbon (δ 77.0), two olefinic
275 quaternary carbon (δ 142.8 and 142.0/141.8), and a quaternary carbonyl group (δ 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 ^1H - ^1H
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)-] [δ 1.54

284 (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
285 methoxy group [δ 3.20 (s) and δ 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 ^1H - ^1H COSY couplings between the methylene
287 proton signals of the 1-hydroxy-ethyl group and the methylene proton signals [δ 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 δ 3.45 (br d, $J = 10.0$ Hz) to the ^{13}C signals of the three
290 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
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}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 [δ 3.15 (dd, $J =$
301 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
302 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, $J = 8.8, 1.2$
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 1α ,

307 10 α -dihydroxy-11-methoxy-(*E*)-litse-2, 6-dien-4-one and 1 α ,
308 10 β -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 C₁₆H₂₄O₄ according to the HRCIMS ([M+H]⁺ *m/z*
311 281.1737, calcd. 281.1747), was shown to be composed of an α , β -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 ¹H and ¹³C NMR spectral data (Table 1). Analysis of the ¹H-¹H 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 δ 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 ¹³C signal at δ 111.5 (s) suggested the
319 attachment of **unit B** to a dioxy-substituted carbon (C-4), which was then connected by an α ,
320 β -conjugated ester group evidenced by the presence of HMBC correlations between the proton
321 signal at δ 5.88 (q, *J* = 1.6 Hz) and the ¹³C signal of C-4, and between H-5a and the ¹³C signal at δ
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 δ 3.15 (s) to the ¹³C signal of C-4, and the methyl proton signals at δ 1.94 (d, *J* = 1.6
325 Hz) to the ¹³C signals of C-2 [δ 120.3 (d)], -3 and -4. Five double-bond equivalents were
326 calculated from the molecular formula (C₁₆H₂₄O₄) 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 δ 5.88 (*J* = 1.6 Hz) and 1.94 (*J* = 1.6 Hz) and the

330 ¹³C NMR signals at δ 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 ¹³C
333 NMR chemical shift of C-14 (δ 16.6 of **3** vs δ 25.6) and downfield of the ¹³C NMR chemical shift
334 of C-8 (δ 33.4 of **3** vs δ 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 CHCl₃ extract of *L. verticillata* inhibited
339 HIV-1 replication by 76% at a concentration of 20 μ 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 IC₅₀ value of 49.6 μ M and no toxicity to the host
343 HOG-R5 cells at a concentration of 70 μ 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 μ 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 β , 6 α -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 EC₅₀ 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 EC₅₀ 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 EC₅₀ 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

398 **Acknowledgements**

399

400 The work described in this paper was supported by NIH Grants 3U01TW001015-10S1 and
401 2U01TW001015-11A1 (administered by the Fogarty International Center as part of an
402 International Cooperative Biodiversity Groups program, through funds from NIH, NSF, and
403 Foreign Agricultural Service of the USDA), the Research Grants Council of the Hong Kong
404 Special Administrative Region, China (Project No. HKBU 262912), HKBU Interdisciplinary
405 Research Matching Scheme (RC-IRMS/12-13/03), Faculty Research Grant, Hong Kong Baptist
406 University (FRG1/13-14/029), National Natural Science Foundation of China (Grant No.
407 21402166) and Mr. Kwok Yat Wai and Madam Kwok Chung Bo Fun Graduate School
408 Development Fund. The authors are grateful to the Department of Medicinal Chemistry and
409 Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, the Research Resources
410 Center, University of Illinois at Chicago for support in the acquisition of the NMR, MS, IR and
411 UV data, and to the Field Museum, for permission in the use of the botany resources for this
412 research.

413

414

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416

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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** (CDCl₃).

481

482 Figure 3. Selected ROESY correlations for compound **1/2** (CDCl₃).

483

484 Figure 4. Selected HMBC correlations for compound **3** (CDCl₃).

485

486 **Table 1. ¹H (500 MHz) and ¹³C (125 MHz) NMR Spectroscopic Data of Compounds 1-3 in CDCl₃**

487 *Notes:*

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

489 ^b Multiplicities in parentheses represent: s (quaternary carbon), d (CH), t (CH₂), and q (CH₃).

490 ^c 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**

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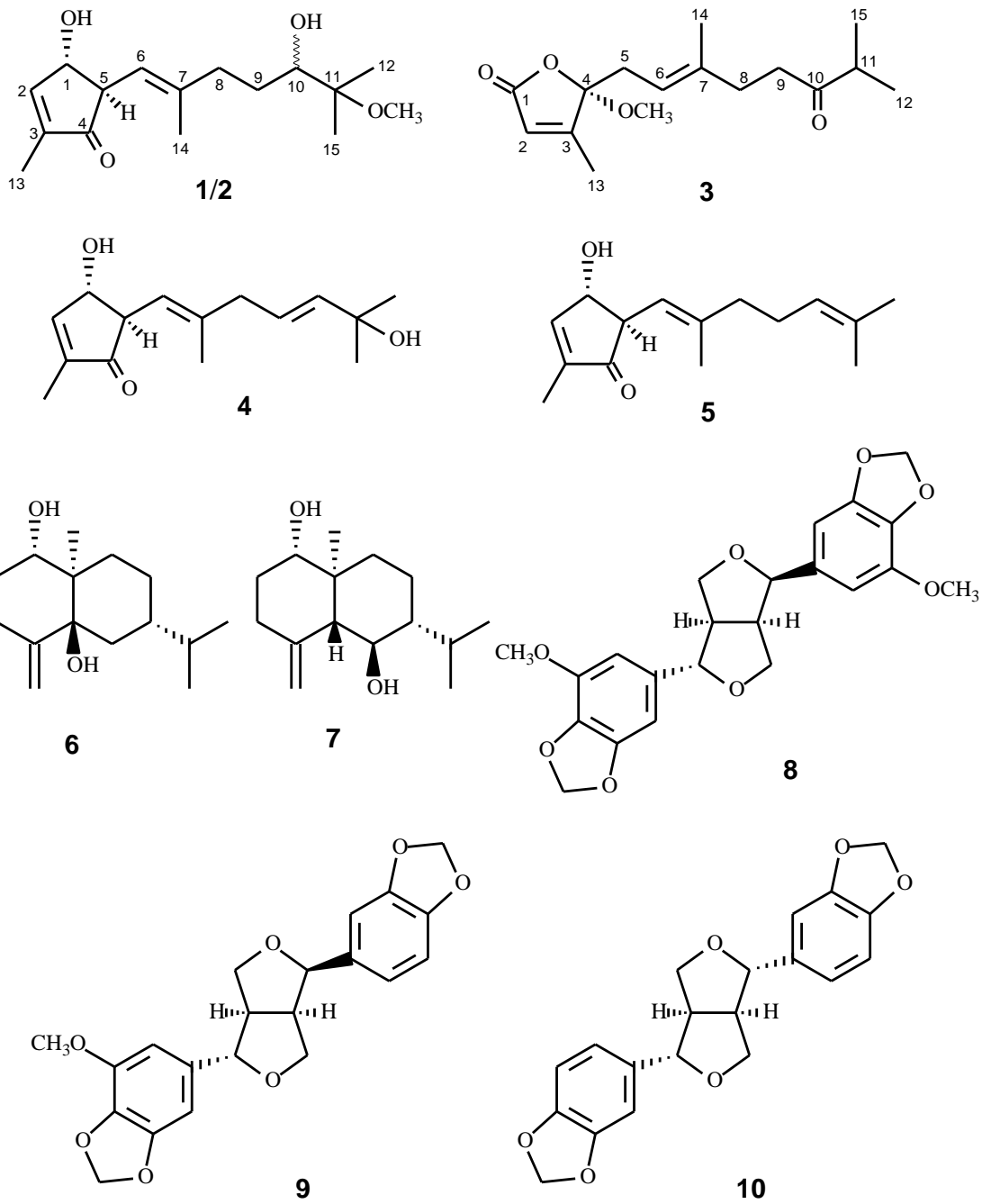
495 **Table 3. Anti-HIV and Cytotoxic Activities of *Litsea* Plant Extracts**

496 *Notes:* Samples were tested for their anti-HIV activities at 20 μ 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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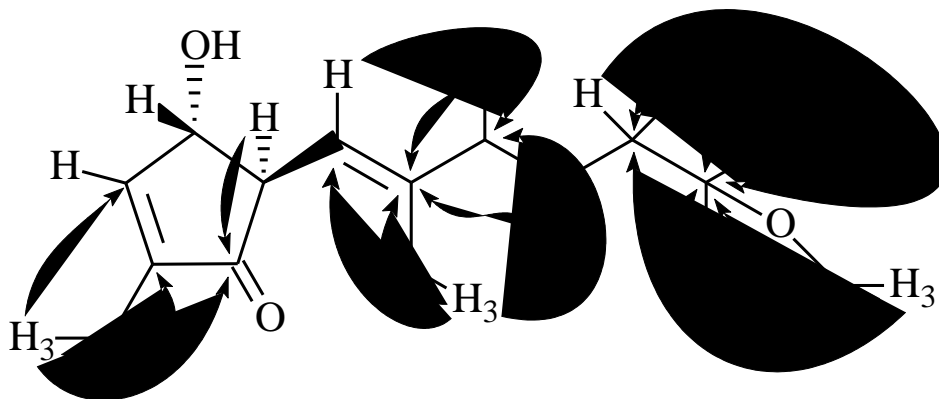


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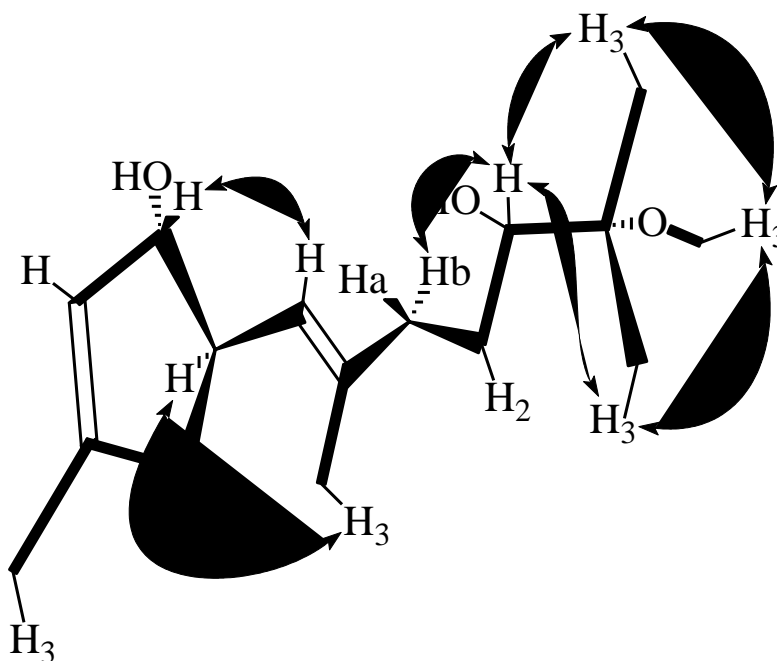
Figure 1.



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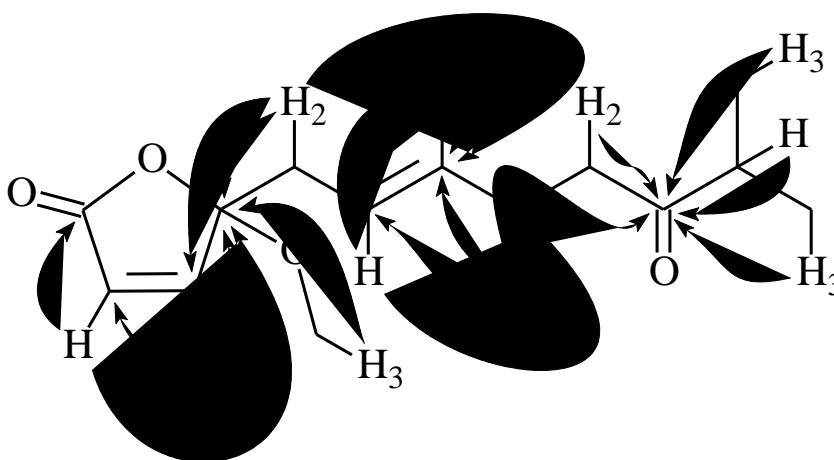
Figure 2.



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Figure 3.



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Figure 4.

512 **Table 1. ¹H (500 MHz) and ¹³C (125 MHz) NMR Spectroscopic Data of Compounds 1-3 in CDCl₃^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) |

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521 **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 | – | – |

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| Plant Species | Code | Plant Part | Anti-HIV Activity | Cytotoxicity (IC ₅₀ : 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 |

