Phytochemical study of *Illicium angustisepalum* and its biological activities

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**KEY WORDS**

*Illicium angustisepalum*; Chemical ingredients; Antibacterial; Neuroprotection; Anti-acetylcholinesterase

**Abstract** Sixteen compounds, including two new natural products (1 and 2), were obtained from the twigs of *Illicium angustisepalum*. The structures were elucidated based on NMR, MS, IR data and optical rotation values. Compounds 4, 5, 6 and 8 displayed moderate antibacterial activities against clinical isolates; compounds 4, 5, 8, 9 and 15 protected neural cells against oxidative stress; and compounds 10 and 14 exhibited anti-acetylcholinesterase activity.

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1. Introduction

The genus *Illicium* L., commonly known as star anise or anise tree, comprises of over thirty species to form one of the earliest evolutionary branches of the angiosperms. The genus was previously classified as the only member of the Illiciaceae family; but later, the Angiosperm Phylogeny Group (APG) III system of 2009 included *Illicium* in the Schisandraceae without recognizing Illiciaceae as a distinct family. Represented by evergreen trees and shrubs disjunctly distributed in North America, Mexico, Peru, the West Indies and Eastern Asia, with the highest concentration of species in Northern Myanmar and Southern China, the genus is known to contain unique secondary metabolites, such as terpenoids, phenylpropanoids, lignans and benzoquinones. The fruit part of several *Illicium* species is particularly rich in sesquiterpenes of the secoprezizaene, anisalactone, and allocedrene types.

While plants, such as *Illicium verum* (the common star anise) which is well known for use as a spice and medicinal plant, and *Illicium parviflorum* as a garden plant, *Illicium angustisepalum* A.C. Smith is a lesser known species endemic to Southern China. Until now, only two reports concerning its chemical composition (abietane diterpenes and a prezizaene sesquiterpene) and one published paper describing anti-inflammatory and analgesic effects of the crude extract can be found in literature. Herein, we report the isolation of sixteen compounds from the twigs of *I. angustisepalum* (1–16, Fig. 1), including the new structures of 3-O-benzoyl-myrccenediol (1) and 3,6-dimethyl-3-hydroxy-tetrahydro-2H-pyran-2-one (2). The isolated compounds were evaluated in a battery of in vitro bioassay models for cytotoxic, antimicrobial, neuroprotective and anti-inflammatory activities.

2. Results and discussion

Compound 1 was obtained as a colorless amorphous powder. The HR-ESI-MS of 1 displayed a quasi-molecular ion [M+H]+ at m/z 275.1569 (for C17H23O3; Calcd. 275.1647), corresponding to a hydroxyl group was assignable to C-2 (δC 168.0). This piece of evidence was helpful in locating an ester group between the aromatic ring and the monoterpenic portion of the molecule. The hydroxyl group was assignable to C-2 (δC 72.9) based on HMBC correlations with H1-1, H-3 and H-10. Finally, the location of the two terminal double bonds was assigned based on the HMBC long-range correlations between H2-8a/8b and C-6/C-7, as well as between H2-9 and C-5/C-6/C-7. Comparison of the NMR data with literature values suggested close similarity of the aliphatic portion of 1 to myrcene-2,3-diol. Taking all evidence together, the planar structure of 1 was determined to be 3-O-benzoyl-2-methyl-6-methyleneoct-7-ene-2,3-diol and given a trivial name of 3-O-benzoyl-myrccenediol.

The small value of optical rotation (δ[a]20 + 1.4°; c 0.07, MeOH) suggested it was a racemic mixture of the 3R and 3S enantiomers. To the best of our knowledge, compound 1 is a new natural product.

Compound 2 was obtained as an oil. The HR-ESI-MS quasi-molecular ion [M+H]+ at m/z 145.0866 (Calcd. for C7H12O3, 145.0861) indicated a molecular formula of C7H12O3 with two indices of hydrogen deficiency. The IR absorption at 1779 cm−1 suggested the presence of a δ-lactone. The 13C NMR and DEPT-135 NMR spectra (in methanol-d4, Table 2) displayed seven carbons, including two CH3 (C-7, δC 21.8; C-8, δC 29.8), two CH2 (C-4, δC 44.4; C-5, δC 43.8), a CH (C-6, δC 75.6), a tertiary oxygenated carbon (C-3, δC 68.5) and a carbonyl carbon (C-2, δC 174.0). The 1H–13C COSY spectrum revealed correlations between H-6 and H-5, suggesting that the two methylene groups are adjacent to each other. The COSY data also correlated H-6 (δH 4.77, m) with H-3 and H-5. It is noteworthy that the carbonyl carbon at δC 174.0 (C-2) displayed HMBC correlation with H-6 at δH 4.77. Compound 2 was thus elucidated to be 3,6-dimethyl-3-hydroxy-tetrahydro-2H-pyran-2-one based on available evidence. Its stereochemistry has not been determined. Nevertheless, it is noted that, in a similar structure [3-hydroxy-3-methyl-6-(3-methylbut-3-enyl)tetrahydro-2H-pyran-2-one], the 3β-methyl group resonates at δH 2.1, whereas the methyl group in 2 resonates δH 1.3. Such a discrepancy may be due to a reversed stereochromy at C-3 or C-6. To the best of our knowledge, compound 2 is a new natural product.

The structures of other compounds isolated from *I. angustisepalum* were determined by detailed examination of their spectroscopic data and comparison with published values. They were identified to be magaricins A (3) and C (4), angustanicos acids E (5), F (6), G (8), angustanol (7), (−)-r-muurolol (9), cloven-2-furoic acid (10), angustisepalin (11), majunic (12), 6β-hydroxy-4-stigmasten-3-one (13), thymol (14), 2,6-dimethoxychavicol (6-methoxy Eugenol, 15) and (+)-taxifolin (dihydroquercetin, 16). With the exception of 3–8 and 11, other compounds are reported from *I. angustisepalum* for the first time.

In order to establish the biological profile of *I. angustisepalum* and the isolates, they were evaluated in a battery of testing models as follows.

2.1. Cytotoxicity study

All compounds were tested in three cell lines, *i.e.* MDA-MB-435 human melanoma cancer cells, MDA-MB-231 human breast cancer cells and OVCAR3 human ovarian cancer cells, using vinblastine as positive control. None of the compound displayed cytotoxic activity (IC50 > 25 μg/mL). These results were consistent with the non-
cytotoxic property previously reported for angustanoic acids B and E, angustanol and other similar compounds.\textsuperscript{12,20}

\subsection*{2.2. Antimicrobial activity evaluation}

Isolated compounds from \textit{I. angustisepalum} were tested against clinical isolates of antibiotic resistant strains. Among them, angustanoic acid E (5) was the most active against \textit{Escherichia coli} BW25113 ΔTolC (MIC 21 μmol/L), \textit{Staphylococcus aureus} USA 300 (MIC 42 μmol/L), and \textit{S. aureus} MSSA 476 (MIC 42 μmol/L). It was also active against \textit{Bacillus anthracis} sterne and \textit{Bacillus cereus} 14579 with MIC of 21 μmol/L, but was inactive against \textit{Acinetobacter calcoaceticus}. On the other hand, majusanic acid C (4) and angustanoic acids F (6) and G (8) displayed MIC of 75, 75, and 150 μmol/L, respectively, against \textit{E. coli} BW25113 ΔTolC. In the literature, angustanoic acids (e.g. angustanoic acid F)\textsuperscript{21} and majusanic acids (e.g. majusanic acids B, D, E and F)\textsuperscript{22} have shown to possess antiviral activity against Coxackievirus B viruses.

\subsection*{2.3. Neuroprotection activity study}

Investigation of bioprotection was evaluated in pheochromocytoma PC12 cells against damages induced by 1-methyl-4-phenylpyridinium (MPP\textsuperscript{+}). The most significant protection against

\begin{table}[h!]
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\begin{tabular}{|l|l|l|}
\hline
 \textbf{Position} & \textbf{δ\textsubscript{H}} & \textbf{δ\textsubscript{C}} \\
\hline
1 & 1.22, s & 25.5, CH\textsubscript{3} \\
2 & – & 72.9, C \\
3 & 5.09, dd (J = 2.3, 10.1) & 81.3, CH \\
4 & 4a, 2.02, m (overlapped) & 29.3, CH\textsubscript{2} \textsubscript{4b}, 1.87, m (overlapped) & 29.6, CH\textsubscript{2} \\
5 & 2.23, m (overlapped) & 139.7, CH \\
6 & – & 113.7, CH\textsubscript{2} \\
7 & 6.34, dd (J = 11.0, 17.7) & 29.6, CH\textsubscript{2} \\
8 & 8a, 5.16, d (J = 17.7) & 116.7, CH\textsubscript{2} \\
9 & 5.0, m (overlapped) & 26.4, CH\textsubscript{3} \\
10 & 1.22, s & 168.0, C \\
1' & – & 131.6, C \\
2' & – & 130.6, CH \\
3' & 8.07, dd (J = 7.5, 1.3) & 129.6, CH \\
4' & 7.49, t (J = 7.5) & 129.6, CH \\
5' & 7.61, t (J = 7.3) & 134.3, CH \\
6' & 7.49, t (J = 7.5) & 130.6, CH \\
7' & 8.07, dd (J = 7.5, 1.3) & 130.6, CH \\
\hline
\end{tabular}
\caption{\textsuperscript{1}H NMR and \textsuperscript{13}C NMR spectral data for compound 1.}
\end{table}

\begin{table}[h!]
\centering
\begin{tabular}{|l|l|l|}
\hline
 \textbf{Position} & \textbf{δ\textsubscript{H}} & \textbf{δ\textsubscript{C}} \\
\hline
2 & – & 174.0, C \\
3 & – & 68.5, C \\
4 & 2.50–2.57, m & 44.4, CH\textsubscript{2} \\
5 & 5a, 1.64, dd (J = 7.1, 11.8) & 43.8, CH\textsubscript{2} \\
6 & 5b, 1.90, dd (J = 1.8, 11.8) & 21.8, CH\textsubscript{2} \\
7 & 4.77, m & 113.7, CH\textsubscript{2} \\
8 & 1.37, d (J = 6.4) & 139.7, CH\textsubscript{2} \\
9 & 1.30, s & 75.6, CH \\
\hline
\end{tabular}
\caption{\textsuperscript{1}H NMR and \textsuperscript{13}C NMR spectral data for compound 2.}
\end{table}

\textsuperscript{\textsuperscript{a}}Data were measured in MeOD-d\textsubscript{4} at 400 MHz for \textsuperscript{1}H NMR and 100 MHz for \textsuperscript{13}C NMR. The δ values are expressed in ppm and the coupling constants (J) in Hz. The assignments were made on the basis of DEPT, \textsuperscript{1}H–\textsuperscript{1}H COSY, HSQC and HMBC results.

\textsuperscript{\textsuperscript{b}}Data were measured in MeOD-d\textsubscript{4} at 400 MHz for \textsuperscript{1}H NMR and 100 MHz for \textsuperscript{13}C NMR. The δ values are expressed in ppm and the coupling constants (J) in Hz. The assignments were made on the basis of DEPT, \textsuperscript{1}H–\textsuperscript{1}H COSY, HSQC and HMBC results.
MPP+ damage was exerted by angustanoic acid G (8) at 1.3 μmol/L, comparable to the positive control AOP-6A (at a concentration of 16 μmol/L). Moderate protective effect was also observed for majusanic acid C (4), angustanoic acid E (5), (-)-τ-murolol (9) and 2,6-dimethoxychavicol (15), at concentration range between 5–30 μmol/L. The neuroprotection activity may be related to their antioxidant property. It is interesting to note that, while majucin itself was not tested, its 13C NMR spectrum showed chemical shifts (ν) of 77.23 ppm for 13C NMR; methanol-2,6-dimethoxychavicol (15) was a potent neuroprotectant against MPP+ damage at 5 μmol/L. The neuroprotection activity may be related to their antioxidant property. It is interesting to note that, while majucin itself was not tested, its 13C NMR spectrum showed chemical shifts (ν) of 77.23 ppm for 13C NMR; methanol-2,6-dimethoxychavicol (15) was a potent neuroprotectant against MPP+ damage at 5 μmol/L.

2.4. Acetylcholinesterase inhibitory activity evaluation

Using TLC bioautographic screening, both the volatile oil fraction and the EtOAc fraction of *I. angustisepalum* were active in inhibiting acetylcholinesterase activity. Thymol (14) was an active ingredient in the volatile fraction. The compound has been reported to exhibit anti-acetylcholinesterase activity (IC50 0.2 mg/mL). When the isolated compounds were tested in the same system, 3,6-dimethyl-3-hydroxy-tetrahydro-2H-pyran-2-one (2), clovane-2,6-diol (10), majusanic acid (11), 2,6-dimethoxychavicol (15), and taxifolin (16) displayed inhibitory activity. Among these compounds, taxifolin has been shown to strongly inhibit acetylcholinesterase at an IC50 of 30 nmol/L. The inhibitory activity was further demonstrated using the Ellman’s method, in which 10 and 14 exhibited the best results with IC50 values of 45 and 90 μmol/L, respectively.

3. Conclusions

The twigs of *I. angustisepalum* afforded sixteen compounds in the present phytochemical study. Several of these secondary metabolites displayed in vitro activities in anti-microbial, neuroprotection and anti-acetylcholinesterase test models. As a whole, *I. angustisepalum* is an under-exploited plant species; more in-depth studies are warranted in order to fully evaluate its potentials.

4. Experimental

4.1. General experimental procedures

All solvents used were analytical or HPLC grade. TLC: Merck aluminium backed sheets coated with 60F254 silica gel or 60F254 RP-silica gel; visualization by using a UV lamp (λmax 254 nm), and spraying with Komarowsky reagent (a mixture of 2% 4-hydroxybenzaldehyde MeOH and 5% H2SO4/EtOH, 10:1 (ν/v)), followed by heating. Open column chromatography: silica gel (SiO2), MCI gel CPH20P (Supelco, Sigma–Aldrich, USA) or Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden). For HPLC purification, a C18 semi-preparative HPLC column (Phenomenex C18 column, 250 mm × 10 mm, 5 mm) and a Shimadzu UFLC system were used; the UV detection wavelength and flow rate were set at 254 nm and 4 mL/min, respectively. Optical rotations: at Na D line; Perkin-Elmer 241 digital polarimeter using quartz cell with a path length of 100 mm. NMR Spectra: Bruker DPX-400 spectrometer; chemical shifts (δ) in ppm using residual solvent as the internal standard (DMSO-d6: 2.50 ppm for 1H NMR and 39.51 ppm for 13C NMR; CDCl3: 7.24 ppm for 1H NMR and 77.23 ppm for 13C NMR; methanol-d4: 3.31, 4.78 ppm for 1H NMR and 49.2 ppm for 13C NMR); coupling constants (J) in Hz. HR-ESI-MS: Shimadzu LC–MS-IT-TOF mass spectrometer.

4.2. Plant material

Twigs of *I. angustisepalum* were collected in spring 2011 from Lantau Island, Hong Kong (China) by one of the author (Ming Zhao) and authenticated by Miss Yuying Zong of the School of Chinese Medicine, the Chinese University of Hong Kong. Voucher specimens were deposited at the same institute. Fresh twigs were cut into small pieces, dried under sun and milled into fine powder.

4.3. Extraction and isolation

The pulverized twigs (1 kg) were exhaustively extracted by percolation with 90% EtOH at room temperature and dried under reduced pressure. The dried extract (232 g) was partitioned into petroleum ether-soluble (39 g), ethyl acetate-soluble (50 g), butanol-soluble (63 g) and water-soluble (78 g) fractions. The ethyl acetate-soluble part was separated into 47 fractions by a flash column of SiO2 eluted by mixtures of petroleum ether and EtOAc (100:0 to 0:100). Sixteen compounds (1–16) were purified following repeated column chromatography and semi-preparative HPLC.

4.3.1. 3-O-Benzoyl-myrcenediol (1)

Colorless amorphous powder, 2 mg; δ(H) 1H 25.4 + 1.4 (ν 0.07, MeOH); IR (film) νmax 3429, 2971, 2936, 2342, 1713, 1275, 1119, 716 cm−1; 1H NMR and 13C NMR data, see Table 1; HR-ESI-MS m/z 275.1659 ([M+H]+), Calcd. for C17H20O4, 275.1647.

4.3.2. 3,6-Dimethyl-3-hydroxy-tetrahydro-2H-pyran-2-one (2)

Yellow oil, 1.5 mg; δ(H) 1H 25.4 −2.2 (ν 0.28, MeOH); IR (film) νmax 3366, 2970, 2933, 1779, 1705, 1378, 1122 cm−1; 1H NMR and 13C NMR data, see Table 2; HR-ESI-MS m/z 145.0866 ([M+H]+), Calcd. for C9H10O4, 145.0861.

4.3.3. Majusanic acid B (3)

Colorless amorphous powder, 8 mg; δ(H) 1H 25.4 + 75.75 (ν 0.8, MeOH); IR (film) νmax 3400, 2958, 2934, 1701, 1046, 1025, 1000 cm−1; HR-ESI-MS m/z 332.1988 ([M+H]+), Calcd. for C20H23O3, 332.1980.

4.3.4. Majusanic acid C (4)

Colorless amorphous powder, 11 mg; δ(H) 1H 25.4 + 88.0 (ν 0.1, MeOH); IR (film) νmax 2964, 2931, 1692, 1471, 1256, 1171, 1147, 1071, 755 cm−1; HR-ESI-MS m/z 330.2195 ([M+H]+), Calcd. for C21H22O5, 330.2187.

4.3.5. Angustanoic acid E (5)

Colorless amorphous powder, 9 mg; δ(H) 1H 25.4 + 22.1 (ν 0.19, MeOH); IR (film) νmax 2956, 2931, 2850, 1695, 1437, 1231, 108, 950, 888 cm−1; HR-ESI-MS m/z 299.2004 ([M+H]+), Calcd. for C20H20O5, 299.2004.

4.3.6. Angustanoic acid F (6)

Colorless crystal, 10 mg; δ(H) 1H 25.4 + 88.0 (ν 0.1, MeOH); IR (film) νmax 3400, 2962, 2930, 1706, 1436, 1230, 1155, 1017, 951 cm−1; HR-ESI-MS m/z 315.1966 ([M–H]−), Calcd. for C20H22O5, 315.1953.
4.3.7. **Angustanol (7)**

Colorless amorphous powder, 24 mg; [α]_D^25 + 9.0° (c 0.1, acetone); IR (film) ν_max 3357, 2960, 2931, 2906, 2871, 1697, 1455, 1375, 1139, 1041 cm⁻¹; HR-ESI-MS m/z 302.2244 ([M+H]⁺, Calcd. for C_{13}H_{20}O_{2}, 302.2318).

4.3.8. **Angustanoic acid G (8)**

Colorless crystal, 27 mg; [α]_D^25 + 135.3° (c 0.17, MeOH); IR (film) ν_max 2932, 1706, 1677, 1271, 1017, 951 cm⁻¹; HR-ESI-MS m/z 301.1786 ([M+H]⁺, Calcd. for C_{19}H_{25}O_{3}, 301.1797).

4.3.9. (−)-α-Murolol (9)

Yellow oil, 6 mg; [α]_D^25 −70.0° (c 0.1, DCM); IR (film) ν_max 3345, 2957, 2932, 2907, 2870, 1713, 1668, 1454, 1369, 1019, 952 cm⁻¹; HR-ESI-MS m/z 205.1948 ([M−H₂O+H]⁺, Calcd. for C_{13}H_{25}, 205.1950).

4.3.10. **Clovane-2,9-diol (10)**

Colorless amorphous powder, 58 mg; [α]_D^25 −5.0° (c 0.08, CHCl₃); IR (film) ν_max 3480, 2955, 1785, 1754, 1371, 1372, 1250, 1178, 1091, 711 cm⁻¹; HR-ESI-MS m/z 417.1532 ([M+H]⁺, Calcd. for C_{15}H_{26}O_{2}, 417.1542).

4.3.11. **Angustisepalin (11)**

Colorless amorphous powder, 30 mg; [α]_D^25 −33.3° (c 0.06, CHCl₃); IR (film) ν_max 3480, 2955, 1785, 1754, 1373, 1372, 1250, 1178, 1091, 711 cm⁻¹; HR-ESI-MS m/z 328.1158 ([M+H]⁺, Calcd. for C_{13}H_{25}O_{2}, 328.1152).

4.3.12. **Majucin (12)**

Colorless crystal, 9 mg; [α]_D^25 −71.4° (c 0.22, MeOH); IR (film) ν_max 3453, 2939, 2874, 1770, 1731, 1510, 1453, 1372, 1211, 1123, 1008 cm⁻¹; HR-ESI-MS m/z 328.1158 ([M+H]⁺, Calcd. for C_{13}H_{25}O_{2}, 328.1152).

4.3.13. **6p-Hydroxy-4-stigmasten-3-one (13)**

Colorless crystal, 3 mg; [α]_D^25 + 1.42° (c 0.07, MeOH); IR (film) ν_max 3400, 2918, 2849, 2359, 1341 cm⁻¹; HR-ESI-MS m/z 429.3586 ([M+H]⁺, Calcd. for C_{19}H_{29}O_{2}, 429.3720).

4.3.14. **Thymol (14)**

Colorless powder, 9 mg. The identity of thymol was confirmed by means of co-TLC with a reference standard (Sigma–Aldrich) and also by analysis of GC–MS data. In the TLC analysis, the R_T values of 14 and the reference standard were both 0.9 after elution with petroleum ether–ethyl acetate (6:4). The GC chromatogram displayed signal at 12.67 and 12.66 min, respectively, for 14 and the reference standard. GC–MS revealed the presence of [M+H]⁺ at m/z 150.00 for both compounds.

4.3.15. **2,6-Dimethoxycouvacol (15)**

Yellow oil, 30 mg; [α]_D^25 −0.3° (c 0.69, MeOH); IR (film) ν_max 3443, 2937, 1613, 1515, 1459, 1428, 1328, 1239, 1213, 1199 cm⁻¹; HR-ESI-MS m/z 194.0943 ([M+H]⁺, Calcd. for C_{11}H_{14}O_{3}, 194.0939).

4.3.16. **(+)-Taxifolin (16)**

Yellow amorphous powder, 57 mg; [α]_D^25 + 18.0° (c 0.5, acetone); IR (film) ν_max 3344, 1638, 1469, 1283, 1162, 1086 cm⁻¹; HR-ESI-MS m/z 303.0502 ([M+H]⁺, Calcd. for C_{13}H_{14}O_{7}, 303.0501).

4.4. **Cytotoxicity assay**

Cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA), and the assay procedures previously described were adopted³⁵.

4.5. **Antibacterial assay**

The antibacterial activity against strains of *A. calcoaceticus, B. anthracis sterne, B. cereus 14579, Enterococcus faecalis V583, E. coli MG1655, E. coli BW25113 ΔTolC*, *S. aureus USA 300,* and *S. aureus MSSA 476* were performed according to established procedures²⁷,²⁸.

4.6. **Neuroprotection assay**

PC12 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and treated as previously described²⁶. The cells were seeded onto 96-well culture plates at a density of 2 × 10⁴ cells/well, cultured in serum-free medium and incubated for 24 h in the presence or absence of MPP⁺ (500 μM/L) and test compounds. Cell viability was measured using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA).

4.7. **Acetylcholinesterase inhibition assays**

Two assays were performed to test for anti-acetylcholinesterase activity. The TLC-autobiographic assay was performed according to the modified Marston’s method²⁸,³⁰. For detection, Fast Blue B salt solution was sprayed onto the TLC plate to give a purple coloration. Acetylcholinesterase inhibitors produced white spots on the purple background. A microplate assay was also adopted from the Ellman’s method³¹. Enzyme inhibition was expressed as pIC₅₀ (negative logarithms of molar concentrations of inhibitor required to decrease acetylcholinesterase activity by 50%). The percent of inhibition was calculated relative to a blank.

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