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Phytochemical study of *Illicium angustisepalum* and its biological activities

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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[1]. 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[2]. Represented by evergreen trees and shrubs disjunctively 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[3]. The fruit part of several *Illicium* species is particularly rich in sesquiterpenes of the secoprezizaane, anislactone, and allocedrane types[3].

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 prezizaane sesquiterpene)[4,5] and one paper describing anti-inflammatory and analgesic effects of the crude extract[6] 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-myrcenediol (**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-acetylcholinesterase 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 $C_{17}H_{23}O_3$; Calcd. 275.1647), corresponding to the molecular formula of $C_{17}H_{22}O_3$ with seven indices of hydrogen deficiency. The IR broad stretch at 3429 cm^{-1} suggested the presence of hydroxyl and a broad stretch at 1713 cm^{-1} was consistent with the presence of benzoyl carbonyl moiety. The ^{1}H NMR spectrum (Table 1) displayed signals for olefinic protons at δ_H 6.34 (dd, *J* = 11.0, 17.7, H-7), 5.16 (*d*, *J* = 17.7, H-8a), 5.02 (br. s, H-8b), 5.0, (br. s, H-9) and two methyl groups at δ_H 1.22 (s, H_3-1/10). The ^{13}C NMR and DEPT-135 NMR spectra of **1** (Table 1) exhibited 17 carbons signals corresponding to two methyl, four methylenes (two olefinic and two aliphatic), seven methines (five aromatic, an olefinic and an aliphatic carbons), an oxygenated tertiary carbon, a carbonyl carbon and two quaternary carbons. The presence of two double bonds accounted for two degrees of unsaturation; the remaining five were ascribed to an aromatic ring and a carbonyl group. In the ^{1}H–^{1}H COSY spectrum, signal at δ_H 7.61 (t, *J* = 7.3 Hz, H-5′) displayed correlations with the overlapped signal at δ_H 7.49 (H-4′/6′), which further exhibited correlation with an overlapped signal at δ_H 8.07 (dd, *J* = 7.5, 1.3 Hz, H-3′/7′), suggesting the presence of an unsubstituted aromatic ring. Correlations between H_2-4 (δ_H 1.87/2.02, m) and H-3 (δ_H 5.09, dd, *J* = 2.3, 10.1 Hz) and H_2-5 (δ_H 2.23, m) were observed. In addition, observation of COSY correlations between δ_H 6.34 (H-7), δ_H 5.16 (H-8a) and δ_H 5.02 (H-8b) led to establishing the connectivity of the skeleton. Aided by HMBC data, structure **1** was proposed as depicted. It is

noteworthy that both the methine proton at δ_H 5.09 (H-3) and aromatic protons at δ_H 8.07 (H-3′/7′) displayed long-range correlations with C-1′ carbonyl carbon (δ_C 168.0). This piece of evidence was helpful in locating an ester group between the aromatic ring and the monoterpene portion of the molecule. The hydroxyl group was assignable to C-2 (δ_C 72.9) based on HMBC correlations with H_3-1, H-3 and H_3-10. Finally, the location of the two terminal double bonds was assigned based on the HMBC long-range correlations between H_2-8a/8b and C-6/C-7, as well as between H_2-9 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[7–10]. 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-myrcenediol. The small value of optical rotation ($[\alpha]_D^{25}$ + 1.4°; *c* 0.07, MeOH) suggested it was a racemic mixture of the 3*R* and 3*S* 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 $C_7H_{13}O_3$, 145.0861) indicated a molecular formula of $C_7H_{12}O_3$ with two indices of hydrogen deficiency. The IR absorption at 1779 cm^{-1} suggested the presence of a δ-lactone. The ^{13}C NMR and DEPT-135 NMR spectra (in methanol-d_4, Table 2) displayed seven carbons, including two CH$_3$ (C-7, δ_C 21.8; C-8, δ_C 29.8), two CH$_2$ (C-4, δ_C 44.4; C-5, δ_C 43.8), a CH (C-6, δ_C 75.6), a tertiary oxygenated carbon (C-3, δ_C 68.5) and a carbonyl carbon (C-2, δ_C 174.0). The ^{1}H–^{1}H COSY spectrum revealed correlations between H_2-4 and H_2-5, suggesting that the two methylene groups are adjacent to each other. The COSY data also correlated H-6 (δ_H 4.77, m) with H_3-7 and H_2-5. It is noteworthy that the carbonyl carbon at δ_C 174.0 (C-2) displayed HMBC correlation with H-6 at δ_H 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][11], the 3β-methyl group resonates at δ_H 2.1, whereas the methyl group in **2** resonates δ_H 1.3. Such a discrepancy may be due to a reversed stereochemistry 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 majusanic acids B (**3**) and C (**4**)[12], angustanoic acids E (**5**), F (**6**), G (**8**)[4], angustanol (**7**)[4], (–)-τ-muurolol (**9**)[13], clovane-2β,9α-diol (**10**)[14], angustisepalin (**11**)[5], majucin (**12**)[15], 6β-hydroxy-4-stigmasten-3-one (**13**)[16], thymol (**14**)[17], 2,6-dimethoxychavicol (6-methoxyeugenol, **15**)[18] and (+)-taxifolin (dihydroquercetin, **16**)[19]. 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 (IC$_{50}$ > 25 µg/mL). These results were consistent with the non-
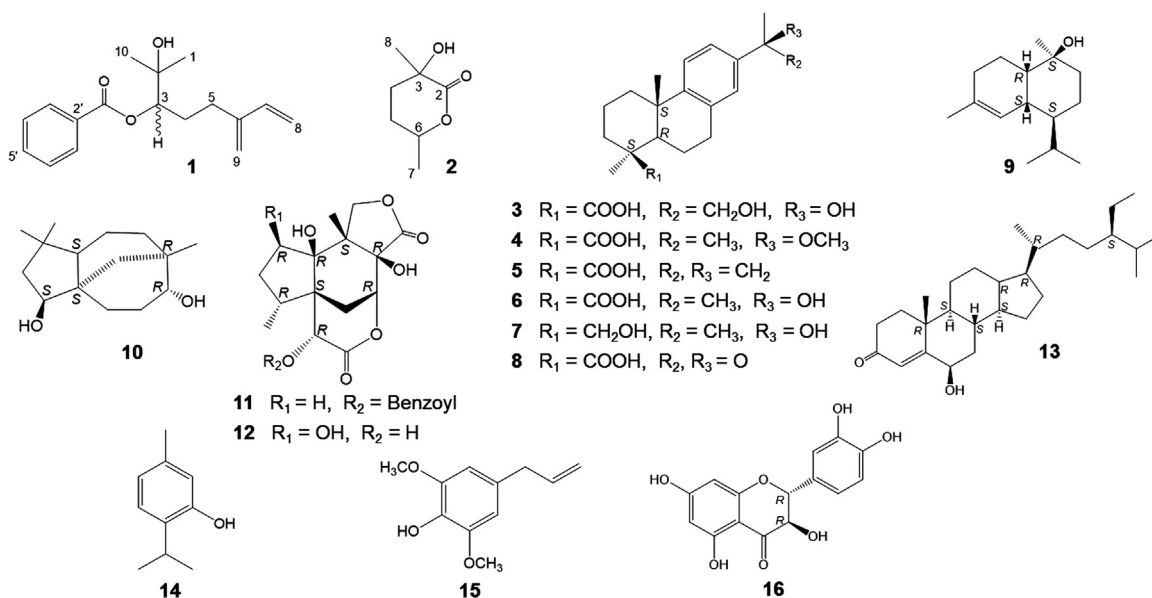
Figure 1 Sixteen compounds isolated from the twigs of *I. angustisepalum* (**1–16**), including the new structures of 3-*O*-benzoyl-myrcenediol (**1**) and 3,6-dimethyl-3-hydroxy-tetrahydro-2*H*-pyran-2-one (**2**).

Table 1 ^{1}H NMR and ^{13}C NMR spectral data for compound **1**.

Position	δ_H	δ_C
1	1.22, s	25.5, CH$_3$
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$_2$
	4b, 1.87, m (overlapped)	
5	2.23, m (overlapped)	29.6, CH$_2$
6	–	147.2, C
7	6.34, dd (J = 11.0, 17.7)	139.7, CH
8	8a, 5.16, d (J = 17.7)	113.7, CH$_2$
	8b, 5.02, m (overlapped)	
9	5.0, m (overlapped)	116.7, CH$_2$
10	1.22, s	26.4, CH$_3$
1′	–	168.0, C
2′	–	131.6, C
3′	8.07, dd (J = 7.5, 1.3)	130.6, 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)	129.6, CH
7′	8.07, dd (J = 7.5, 1.3)	130.6, CH

^{a}Data were measured in MeOD-d_4 at 400 MHz for ^{1}H NMR and 100 MHz for ^{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, ^{1}H–^{1}H COSY, HSQC and HMBC results.

Table 2 ^{1}H NMR and ^{13}C NMR spectral data for compound **2**.

Position	δ_H	δ_C
2	–	174.0, C
3	–	68.5, C
4	2.50–2.57, m	44.4, CH$_2$
5	5a, 1.64, dd (J = 7.1, 11.8)	43.8, CH$_2$
	5b, 1.90, dd (J = 1.8, 11.8)	
6	4.77, m	75.6, CH
7	1.37, d (J = 6.4)	21.8, CH$_3$
8	1.30, s	29.8, CH$_3$

^{a}Data were measured in MeOD-d_4 at 400 MHz for ^{1}H NMR and 100 MHz for ^{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, ^{1}H–^{1}H COSY, HSQC and HMBC results.

cytotoxic property previously reported for angustanoic acids B and E, angustanol and other similar compounds[12,20].

2.2. Antimicrobial activity evaluation

Isolated compounds from *I. angustisepalum* were tested against clinical isolates of antibiotic resistant strains. Among them, angustanoic acid E (**5**) was the most active against *Escherichia*

coli BW25113 ΔTolC (MIC 21 μmol/L), *Staphylococcus aureus* USA 300 (MIC 42 μmol/L), and *S. aureus* MSSA 476 (MIC 42 μmol/L). It was also active against *Bacillus anthracis sterne* and *Bacillus cereus* 14579 with MIC of 21 μmol/L, but was inactive against *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 *E. coli* BW25113 ΔTolC. In the literature, angustanoic acids (*e.g.* angustanoic acid F)[21] and majusanic acids (*e.g.* majusanic acids B, D, E and F)[22] have shown to possess antiviral activity against Coxsackie B viruses.

2.3. Neuroprotection activity study

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

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-muurolol (**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 did not show significant neurotropic effect, some of the related *seco*-prezi-zaanes (such as jiadifenolide) were found to promote neurite outgrowth in PC12 cells[23].

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 (IC$_{50}$ 0.2 mg/mL)[24]. When the isolated compounds were tested in the same system, 3,6-dimethyl-3-hydroxy-tetrahydro-2H-pyran-2-one (**2**), clovane-2β,9α-diol (**10**), angustisepalin (**11**), 2,6-dimethoxychavicol (**15**), and taxifolin (**16**) displayed inhibitory activity. Among these compounds, taxifolin has been shown to strongly inhibit acetylcholinesterase at an IC$_{50}$ of 30 nmol/L[25]. The inhibitory activity was further demonstrated using the Ellman's method, in which **10** and **14** exhibited the best results with IC$_{50}$ 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 (λ_{max} 254 nm), and spraying with Komarowsky reagent (a mixture of 2% 4-hydroxybenzaldehyde MeOH and 5% H$_2$SO$_4$/EtOH, 10:1 (*v/v*)), followed by heating. Open column chromatography: silica gel (SiO$_2$), 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-d_6: 2.50 ppm for ^{1}H NMR and 39.51 ppm for ^{13}C NMR; CDCl$_3$: 7.24 ppm for ^{1}H NMR and 77.23 ppm for ^{13}C NMR; methanol-d_4: 3.31, 4.78 ppm for ^{1}H NMR and 49.2 ppm for ^{13}C 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 SiO$_2$ 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; [α]$_D^{25}$ + 1.4° (*c* 0.07, MeOH); IR (film) ν_{max} 3429, 2971, 2936, 2366, 2342, 1713, 1275, 1119, 716 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; HR-ESI-MS *m/z* 275.1569 ([M+H]$^+$, Calcd. for C$_{17}$H$_{23}$O$_3$, 275.1647).

4.3.2. 3,6-Dimethyl-3-hydroxy-tetrahydro-2H-pyran-2-one (2)
Yellow oil, 1.5 mg; [α]$_D^{25}$ –2.2° (*c* 0.28, MeOH); IR (film) ν_{max} 3366, 2970, 2933, 1779, 1705, 1378, 1247, 1122 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 2; HR-ESI-MS *m/z* 145.0866 ([M+H]$^+$, Calcd. for C$_7$H$_{13}$O$_3$, 145.0861).

4.3.3. Majusanic acid B (3)
Colorless amorphous powder, 8 mg; [α]$_D^{25}$ + 75.75° (*c* 0.8, MeOH); IR (film) ν_{max} 3400, 2958, 2934, 1701, 1046, 1025, 1000 cm^{-1}; HR-ESI-MS *m/z* 332.1988 ([M+H]$^+$, Calcd. for C$_{20}$H$_{28}$O$_4$, 332.1980).

4.3.4. Majusanic acid C (4)
Colorless amorphous powder, 11 mg; [α]$_D^{25}$ + 88.0° (*c* 0.1, MeOH); IR ν_{max} 2964, 2931, 1692, 1471, 1256, 1171, 1147, 1071, 755 cm^{-1}; HR-ESI-MS *m/z* 330.2195 ([M+H]$^+$; Calcd. for C$_{21}$H$_{30}$O$_3$, 330.2187).

4.3.5. Angustanoic acid E (5)
Colorless amorphous powder, 9 mg; [α]$_D^{25}$ + 22.1° (*c* 0.19, MeOH); IR (film) ν_{max} 2956, 2931, 2850, 1695, 1437, 1231, 108, 950, 888 cm^{-1}; HR-ESI-MS *m/z* 299.2004 ([M+H]$^+$, Calcd. for C$_{20}$H$_{27}$O$_2$, 299.2004).

4.3.6. Angustanoic acid F (6)
Colorless crystal, 10 mg; [α]$_D^{25}$ + 88.0° (*c* 0.1, MeOH); IR (film) ν_{max} 3400, 2962, 2930, 1706, 1436, 1230, 1155, 1017, 951 cm^{-1}; HR-ESI-MS *m/z* 315.1966 ([M–H]$^-$, Calcd. for C$_{20}$H$_{27}$O$_3$, 315.1953).

4.3.7. Angustanol (7)

Colorless amorphous powder, 24 mg; $[\alpha]_D^{25}$ + 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 $C_{20}H_{30}O_2$, 302.2238).

4.3.8. Angustanoic acid G (8)

Colorless crystal, 27 mg; $[\alpha]_D^{25}$ + 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. (−)-τ-Muurolol (9)

Yellow oil, 6 mg; $[\alpha]_D^{25}$ −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–H$_2$O+H]$^+$, Calcd. for $C_{15}H_{25}$, 205.1950).

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

Colorless amorphous powder, 58 mg; $[\alpha]_D^{25}$ −5.0° (*c* 0.08, CHCl$_3$); IR (film) ν_{max} 3366, 2920, 2851, 1744, 1718, 1463, 1367, 1245, 1073 cm^{-1}; HR-ESI-MS *m/z* 238.1933 ([M+H]$^+$, Calcd. for $C_{15}H_{26}O_2$, 238.1926).

4.3.11. Angustisepalin (11)

Colorless amorphous powder, 30 mg; $[\alpha]_D^{25}$ −33.3° (*c* 0.06, CHCl$_3$); IR (film) ν_{max} 3480, 2955, 1785, 1754, 1731, 1451, 1372, 1250, 1178, 1091, 711 cm^{-1}; HR-ESI-MS *m/z* 417.1532 ([M+H]$^+$, Calcd. for $C_{22}H_{25}O_8$, 417.1542).

4.3.12. Majucin (12)

Colorless crystal, 9 mg; $[\alpha]_D^{25}$ −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 $C_{15}H_{20}O_8$, 328.1152).

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

Colorless crystal, 3 mg; $[\alpha]_D^{25}$ + 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 $C_{29}H_{49}O_2$, 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_f 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-Dimethoxychavicol (15)

Yellow oil, 30 mg; $[\alpha]_D^{25}$ −0.3° (*c* 0.69, MeOH); IR (film) ν_{max} 3443, 2937, 1613, 1515, 1459, 1428, 1328, 1239, 1213, 1119 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; $[\alpha]_D^{25}$ + 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 $C_{15}H_{11}O_7$, 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 adopted[26].

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 procedures[27].

4.6. Neuroprotection assay

PC12 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and treated as previously described[28]. The cells were seeded onto 96-well culture plates at a density of 2×10^4 cells/well, cultured in serum-free medium and incubated for 24 h in the presence or absence of MPP$^+$ (500 μmol/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 method[29,30]. 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 method[31]. Enzyme inhibition was expressed as pIC$_{50}$ (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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