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Cytotoxic Constituents from the Leaves of *Zanthoxylum schinifolium*

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Several coumarins, methylschimilenol, schimitrienin, schinallylone, isoschinilenol, hydroxyepoxy-collinin, umbelliferone,[1] alkaloid, benzenoid, chlorophylls, triterpenoid, diterpenoid, sesquiterpenoid, steroid,[2] lignan, collinin,[3] epoxyaurapten, hydrangetin, umbelliferone, acetoxycollinin, dictamine, norchelerythrine, skimmianine, β-amyrin,[4] scoparone, schinifoline, scopoletin[5] and xanthoxylin[6] have been isolated from *Zanthoxylum schinifolium* (Rutaceae). Biological activities such as anti-diabetes,[7] monoamine oxidase inhibition,[8] anti-platelet,[4] lipid peroxidation inhibition,[9] anti-HBV DNA replication,[3] antioxidant,[5] insecticides, and nitrite-scavenging[10] have been reported from this plant.

The roots, stems, pericarps, and seeds of *Z. schinifolium* were each extracted with MeOH, and the leaves were extracted with 80% MeOH and concentrated. These extracts were examined on MTT for cytotoxicity against Jurkat T cell clone E6.1. The results showed that the leaves extract had the strongest MTT cytotoxicity. The MeOH extract of *Z. schinifolium* leaves was subsequently fractionated into four parts: methylene chloride, ethyl acetate, *n*-butanol and water. These fractions were examined on MTT for cytotoxicity. The results showed that the methylene chloride fraction exhibited the strongest MTT cytotoxicity. Chromatographic separation of the methylene chloride and butanol fractions had yielded a quinolin (**1**), three phenylpropanoids (**2**, **3**, **12**), four coumarins (**4** ~ **7**), three triterpenoids (**8** ~ **10**), an alkaloid (**11**), an alcohol glucoside (**13**) and three monoterpene glucosides (**14**, **15**, **16**). One of these compounds were identified as new *threo*-6-amino-5-hydroxy-5-methyl-1,3-oxazinan-4-one (**11**) together with fifteen known, 3-heptyl-2-methylisoquinolin-1(2*H*)-one (**1**), integrifoliodiol (**2**), cuspidiol (**3**), bergapten (**4**), aurapten (**5**), 8-hydroxy-7-methoxychromen-2-one (**6**), 6,7-dimethoxy-2*H*-naphthalen-1-one (**7**), lupeol (**8**), lupeone (**9**), β-sitosterol (**10**), syringin (**12**), 2-propyl alchol β-D-glucopyranoside (**13**), vomifoliol-9-*O*-β-D-glucopyranoside (**14**), betulalbuside A (**15**) and cnidioside C (**16**) on the basis of spectroscopic and chemical evidences. All of the compounds were isolated for the first time from this plant except **5** and **7**. In the MTT cytotoxicity assay against Jurkat T cell clone E6.1, IC_{50} values of cuspidiol (**3**) and auraptene (**5**) were obtained at 7.3 μg/mL and 16.5 μg/mL, respectively.

Experimental Section

Plant material. The leaves of *Zanthoxylum schinifolium* were collected in July 2004 from Gyeongsan, Gyeongbuk, Korea.

General experimental procedures. Mps were determined on a Yanaco micro melting point apparatus and were uncorrected. Optical rotations were taken on a Jasco DIP-370 digital polarimeter. IR spectra were measured on a Jasco FT/IR 300E spectrophotometer. UV spectra were obtained on a Shimadzu UV-160A spectrophotometer. ^1H, ^{13}C and 2D NMR spectra were performed on a Varian OXFORD AS400 MHz instrument. Spectrometry was measured in $CDCl_3$ or CD_3OD using TMS as an internal standard. Low- and high-resolution FABMS data were collected on a JMS-700 spectrometer. EIMS were recorded on a Quattro II spectrometer. For TLC, silica gel 60 F-254 (EM 5717) glass plates (0.25 mm) were used and visualized by spraying with 10% sulfuric acid in H_2O and heating.

MTT cytotoxicity assay. The cytotoxic effect of compounds **1** ~ **16** on Jurkat T cells clone E6.1 was analyzed by a 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay reflecting cell viability, as described elsewhere.[11] For the MTT assay, Jurkat T cells clone E6.1 (5×10^4 per well) were added to a serial dilution of compounds in 96-well plates. After incubation for 44 h, 50 μL of the MTT solution (1.1 mg/mL) was added to each well and incubated for an additional 4 h. The colored formazan crystal produced from the MTT was dissolved in 150 μL of dimethyl sulfoxide (DMSO). The optical density (OD) values of the solutions were measured at 540 nm using a plate reader.

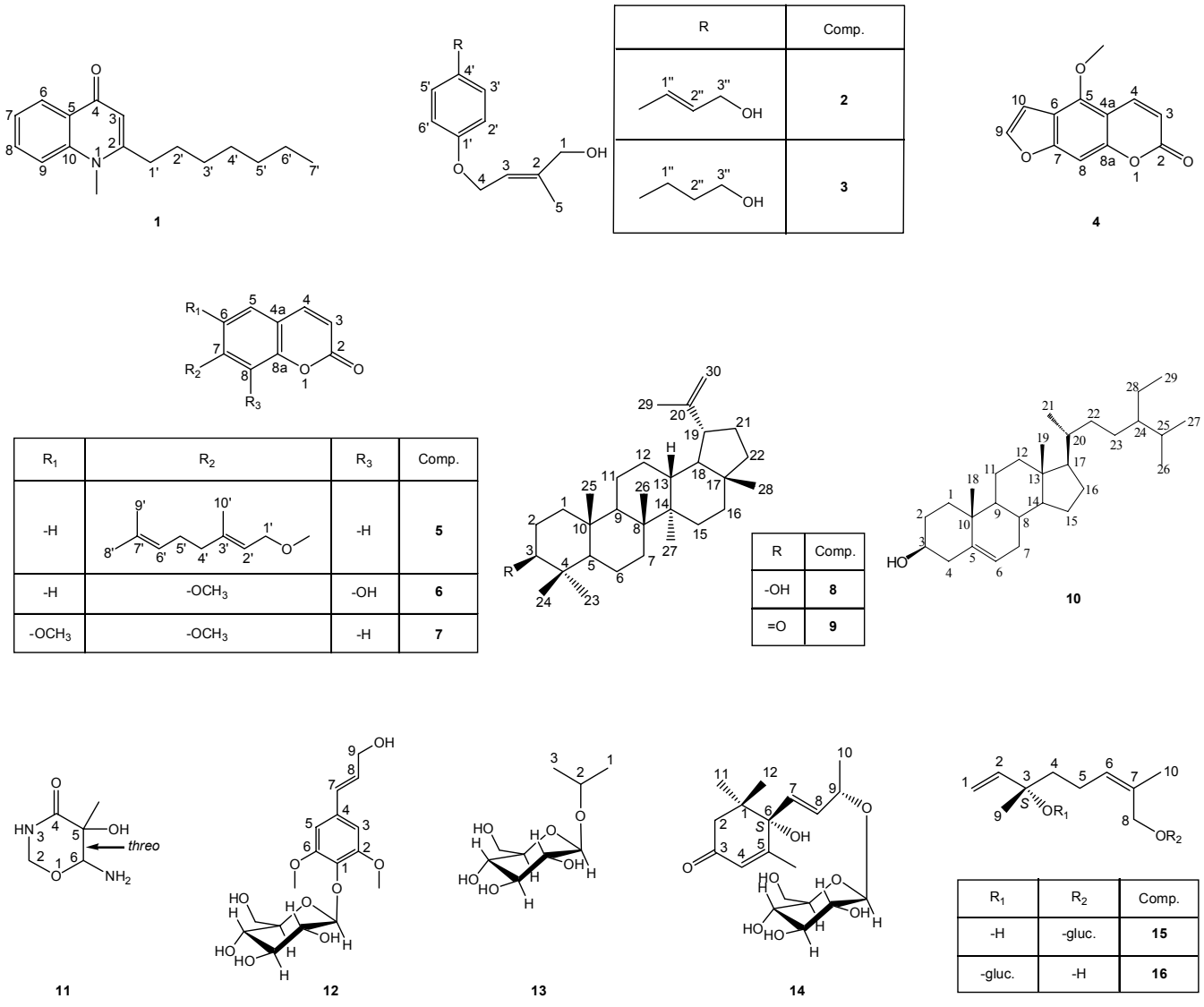
Extraction and isolation. The leaves of *Z. schinifolium* (30 kg) were freeze-dried and powdered to yield the leaves powder 10 kg. The powder was extracted with 80% MeOH to yield 1,206 g of an extract upon removal of the solvent. It was partitioned between CH_2Cl_2-H_2O (1:1) to yield the CH_2Cl_2-soluble fraction (311 g) and H_2O solvent. The H_2O solvent was partitioned with *n*-BuOH-H_2O (1:1) to yield the *n*-BuOH-soluble fraction (385 g) and the H_2O soluble fraction (492 g). In the all fractions, CH_2Cl_2 fraction showed the strongest MTT cytotoxicity. The CH_2Cl_2-soluble fraction (150 g) was subjected to open flash column chromatography over silica gel (200 g) eluted with hexane-EtOAc and CH_2Cl_2-MeOH gradient. Fractions (SL-MC-A to SL-MC-W) were collected and pooled according to their similar TLC patterns. Fraction SL-MC-R was chromatographed on a reverse-phase column (60 × 3.0 cm, RP-C18) with MeOH-H_2O (gradient from 60:40 to 80:20) to afford compounds **1**, **2** and **3**. Fractions SL-MC-J, -D and -H were recrystallized to afford three compounds, **4**, **8** and **9**, respectively. Fraction

Table 1. Cytotoxicity (IC_{50} value) of **1** ~ **16** on Jurkat T cells clone E6.1

Compound	IC_{50} (μg/mL)
1	> 50.0
2	> 50.0
3	7.3
4	> 50.0
5	16.5
6	> 50.0
7	> 50.0
11	> 50.0
12	> 50.0
13	> 50.0
14	> 50.0
15	> 50.0
16	> 50.0

SL-MC-I was recrystallized to afford two compounds, **5** and **10**. Fraction SL-MC-O was chromatographed on a reverse-phase column (60 × 3.0 cm, Sephadex LH-20) with MeOH-H_2O (gradient from 65:35 to 95:5) to fractionate seven parts: SL-MC-O-1 ~ SL-MC-O-7. Fraction SL-MC-O-7 was chromatographed on a silica-phase column (60 × 3.0 cm) with $CHCl_3$-MeOH-H_2O (90:10:0.1) to afford compound **6**. Fraction SL-MC-O-4 was chromatographed on a reverse-phase column (60 × 3.0 cm, RP-C18) with MeOH-H_2O (65:35) to afford compound **7**.

The butanol fraction (385 g) was subjected to open flash column chromatography over silica gel (400 g) eluted with hexane-CH_2Cl_2 and CH_2Cl_2-MeOH gradient. Fractions, (SL-Bu-A to SL-Bu-W) were collected and pooled according to their similar TLC patterns. Fraction SL-Bu-C was chromatographed on a reverse-phase column (50 × 2.5 cm, RP-C18) with MeOH-H_2O (gradient from 18:82 to 90:10) to afford SL-Bu-C-A and SL-Bu-C-B. SL-Bu-C-A was chromatographed



R_1	R_2	R_3	Comp.
-H		-H	**5**
-H	$-OCH_3$	-OH	**6**
$-OCH_3$	$-OCH_3$	-H	**7**

R	Comp.
-OH	**8**
=O	**9**

R_1	R_2	Comp.
-H	-gluc.	**15**
-gluc.	-H	**16**

Figure 1. Structures of compounds isolated from methylene chloride (**1** ~ **10**) and butanol (**11** ~ **16**) fractions of *Z. schinifolium*.

on a silica-phase column (45 × 2.0 cm) with $CHCl_3$-MeOH (15:1) to afford compound **11**. SL-Bu-C-B was chromatographed on a silica-phase column (40 × 1.8 cm) with $CHCl_3$-MeOH (20:1) to afford compound **12**. Fraction SL-Bu-F was chromatographed on a reverse-phase column (60 × 3.0 cm, RP-C18) with MeOH-H_2O (gradient from 1:100 to 85:15) to fractionate six parts: SL-Bu-F-A ~ SL-Bu-F-F. SL-Bu-F-A was chromatographed on a silica-phase column (40 × 2.0 cm) with $CHCl_3$-MeOH (18:1) to afford compound **13**. SL-Bu-F-C was chromatographed on a silica-phase column (38 × 1.5 cm) with $CHCl_3$-MeOH (20:1) to afford compound **14**. SL-Bu-F-E was chromatographed on a silica-phase column (40 × 1.8 cm) with $CHCl_3$-MeOH (25:1) to afford compound **15**. SL-Bu-F-F was chromatographed on a silica-phase column (40 × 2.0 cm) with $CHCl_3$-MeOH (15:1) to afford compound **16**.

Integrifoliodiol (2): $[\alpha]_D^{25}$ −7.0 (*c* 0.2, CH_2Cl_2); UV (MC) λ_{max} (log ε) 266.5 (4.04) nm; IR (KBr) ν_{max} 3293, 2921, 2854, 1606, 1457, 1396, 1022, 802 cm^{-1}; EIMS *m/z* 234 $[M]^+$; FABMS *m/z*: 235.1 $[M+H]^+$; HRFABMS *m/z* 235.1333 (calc. 233.2334 for $C_{14}H_{18}O_3$); ^{1}H-NMR (400 MHz, $CDCl_3$, δ_H) 7.32 (2H, d, *J* = 8.4 Hz, H-3′, 5′), 6.87 (2H, d, *J* = 8.4 Hz, H-2′, 6′), 6.55 (1H, d, *J* = 16.0 Hz, H-1″), 6.24 (1H, dt, *J* = 16.0, 6.0 Hz, H-2″), 5.77 (1H, dt, *J* = 7.2, 1.6 Hz, H-3), 4.60 (2H, d, *J* = 7.2 Hz, H-4), 4.30 (2H, dd, *J* = 6.0, 1.2 Hz, H-3″), 4.09 (2H, s, H-1), 1.77 (3H, s, H-5); ^{13}C-NMR (100 MHz, $CDCl_3$, δ_C) 158.6 (C-1′), 140.3 (C-2), 131.2 (C-1″), 129.7 (C-4′), 127.9 (C-3′, 5′), 126.5 (C-2″), 120.1 (C-3), 114.7 (C-2′, 6′), 68.0 (C-1), 64.6 (C-4), 64.1 (C-3″), 14.3 (C-5).

***threo*-6-Amino-5-hydroxy-5-methyl-1,3-oxazinan-4-one (11):** $[\alpha]_D^{25}$ 20.1 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 206.5 (3.86) nm; IR (KBr) 3427, 2928, 2982, 1775, 1638, 1474, 1210, 1108, 1033 cm^{-1}; FABMS *m/z* 147.1 $[M+H]^+$, 73.2, 55.3; HRFABMS *m/z* 147.0770 (calc. 147.0765 for $C_5H_{11}O_3N_2$); ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 4.37 (1H, dd, *J* = 10.8, 3.6 Hz, H-2a), 4.33 (1H, dd, *J* = 10.8, 1.2 Hz, H-2b), 4.15 (1H, dd, *J* = 3.6, 1.2 Hz, H-6), 1.48 (3H, s, H-CH_3); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 178.3 (C-4), 73.8 (C-5), 73.4 (C-6), 72.3 (C-2), 21.8 (C-CH_3).

Results and Discussion

The leaves of *Z. schinifolium* were extracted with 80% MeOH. The MeOH extract was subsequently fractionated into four parts: CH_2Cl_2, EtOAc, *n*-BuOH and H_2O. From the MTT cytotoxicity-directed fractionation, we have isolated ten known compounds (**1** ~ **10**) from the CH_2Cl_2 fraction and a new (**11**) and five known compounds (**12** ~ **16**) from butanol fraction by SiO_2 flash, RP-C18 and SiO_2 open, and Sephadex LH-20 column chromatographies.

Compound **11** was found to be new. Known compounds **1** ~ **10** and **12** ~ **16** were determined by direct comparison with authentic samples or by comparing their physical and spectral data with those in the literature: 3-heptyl-2-methylisoquinolin-1(2*H*)-one[12] (**1**), 4-[4-(3-hydroxy-propenyl)-phenoxy]-2-methylbut-2-en-1-ol (integrifoliodiol,[13] **2**), 4-[4-(3-hydroxy-propyl)-phenoxy]-2-methylbut-2-en-1-ol (cuspidiol,[14] **3**), bergapten[15] (**4**), 7-[(3,7-dimethyl-2,6-octa-dienyl)oxy]-coumarin (auraptene,[4] **5**), 8-hydroxy-7-methoxy-chromen-2-one[16] (**6**), 6,7-dimethoxy-2*H*-naphthalen-1-one[17] (**7**), 20(29)-lupen-3-ol (lupeol,[18] **8**), 20(29)-lupen-3-one (lupeone,[19] **9**), β-sitosterol[20] (**10**), syringin[21] (**12**), 2-propyl alchol β-D-glucopyranoside (**13**), vomifoliol-9-*O*-β-D-glucopyranoside[22] (**14**), betulalbuside A[23] (**15**) and cnidioside C[24] (**16**). All of the compounds were isolated for the first time from this plant except **5**, **7** and **10**.

FABMS spectral analysis of compound **11** showed a $[M+H]^+$ at *m/z* 147.1. HRFABMS spectral analysis of **11** showed a $[M+H]^+$ at *m/z* 147.0770 (calc. 147.0765) which corresponded to the molecular formula $C_5H_{11}O_3N_2$. The IR spectrum showed absorption bands at 3426 cm^{-1} (OH), 1775 (C=O), and 1108 cm^{-1} (C-O). The ^{1}H-NMR spectrum of **11** afforded signals for a methyl proton as singlet at δ 1.48 (3H). Two doublet of doublet proton peaks were present at δ 4.37 (1H, dd, *J* = 10.8, 3.6 Hz) and 4.33 (1H, dd, *J* = 10.8, 1.2 Hz) integrating for two protons H-2a and H-2b. That spectrum also indicated a signal at δ 4.15 (1H, dd, *J* = 3.6, 1.2 Hz) which could be assigned to H-6. The ^{13}C-NMR spectrum of **11** indicated the presence of a ketone carbon and a methyl carbon signals at δ 178.3 (C-4) and 21.8 (C-CH_3), respectively. A hydroxylated carbon was exhibited at δ 73.8 (C-5). Two oxygen connected carbons, C-2 and C-6, were exhibited at δ 72.3 and 73.4, respectively. The proton and carbon signals were assigned with the help of HSQC, HMBC and COSY experiments. In the HMBC spectrum of **11**, the correlation displayed connectivities between C-4 and H-2, H-6 and H-C$\underline{H}_3$, between C-6 and H-2ab and H-C$\underline{H}_3$, between C-2 and H-6, and between C-5 and H-6 and H-C$\underline{H}_3$. The correlation in the COSY spectrum displayed connectivities between H-2 and H-6, between H-6 and H-C$\underline{H}_3$. Irradiation of the proton H-C$\underline{H}_3$ at δ 1.48 showed a positive NOE effect with δ 4.15 (H-6), thus the relative stereochemistry between C-5 and C-6 was determined as *threo*. Therefore, the structure of compound **11** was exhibited as *threo*-6-amino-5-hydroxy-5-methyl-1,3-oxazinan-4-one.

In the MTT cytotoxicity assay against Jurkat T cell clone E6.1, IC_{50} values of cuspidiol (**3**) and auraptene (**5**) were obtained at 7.3 μg/mL and 16.5 μg/mL, respectively. Cytotoxicity for **8** ~ **10** could not be available because of their insolubility in DMSO.

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