Triterpenoids from *Camellia japonica* and Their Cytotoxic Activity

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From the ethyl acetate fraction of the stem bark of *Camellia japonica*, three new triterpenoids, 3β-*O*-acetyl-16β-hydroxy-12-oxoolean (6), 3β-*O*-acetyl-16β-hydroxy-11-oxoolean-12-ene (7), and 3β-*O*-acetyl-16β-hydroxy-olean-12-ene (8), along with seven known compounds, 3α-hydroxy-1-oxofriedelan (1), friedelin (2), 3β-friedelanol (3), canophyllol (4), 3-oxofriedelan-1(2)-ene (5), β-amyrin (9), camellenodiol (10), and camelledionol (11) were isolated. Their structures were established on the basis of spectroscopic analysis and chemical evidence. The isolated compounds were tested *in vitro* for their cytotoxic activities against the A549, LLC, HL-60 and MCF-7 cancer cell lines. Among them, compound 8 showed cytotoxicity against LLC and HL-60 cancer cell lines with IC_{50} values of 25.2 and 21.7 μM, respectively.

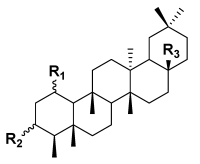
Key words *Camellia japonica*; Theaceae; triterpenoid; cytotoxic activity

Camellia japonica L. (Theaceae) is widely cultivated as an ornamental or garden tree in Korea. The seeds of *C. japonica* have been used as a stomachic and anti-inflammatory in Chinese folk medicine and also as an oil material,[1)] while the flowers and flower buds of this plant have been prescribed in Chinese traditional preparations for treatment of hematemesis and "Oketsu" syndrome (blood stagnation), blood vomiting and bleeding due to internal and external injury, and also as an anti-inflammatory, tonic, and stomachic.[1,2)] Extensive studies have been made on the constituents of *C. japonica*, including those on saponins in fruits[3—5)] and seeds,[1,6)] on flavonol glycosides in leaves,[7)] and on triterpenes in flowers[8)] and seed oil.[9)] Regarding the bioactive constituents in leaves, flavonol glycosides having antioxidant activity,[7)] and saponins having antifungal activity or antifeedant activity toward yellow butterfly larvae[10—12)] have been reported. Furthermore, some of the tannins from leaves, flower buds, and fruits have been shown to have an inhibitory effect on human immunodeficiency virus-1 (HIV-1) protease.[13—18)] In the present paper, we describe the isolation, structural determination and their cytotoxic activity against A549, LLC, HL-60 and MCF-7 cancer cell lines of the isolated compounds.

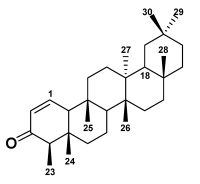
Results and Discussion

Three new and eight known compounds were isolated from the stem barks of *C. japonica*. The known compounds were identified as 3α-hydroxy-1-oxo friedelan (**1**),[19)] friedelin (**2**),[20,21)] 3β-friedelanol (**3**),[21,22)] canophyllol (**4**),[21,23)] 3-oxo friedelan-1(2)-ene (**5**),[21)] β-amyrin (**9**),[21)] camellenodiol (**10**),[8)] and camelledionol (**11**)[8)] on the basis of spectroscopic analysis, chemical evidence and comparison of spectral data with the literature data.

Compound **6**, obtained as a white amorphous powder with $[\alpha]_D^{22}$ +61.8° (c=0.1, $CHCl_3$), gave a positive red coloration in the Liebermann–Burchard reaction. The molecular formula was established as $C_{32}H_{52}O_4$ on the basis of HR-electrospray ionization (ESI)-MS (m/z 501.3922 $[M+H]^+$). The IR spectrum exhibited the presence of hydroxy (3520, 3400 cm^{-1}), and carbonyl (1715 cm^{-1}) groups. The ^{1}H-NMR spectrum shows signals for eight tertiary methyl groups and two axial oxymethine protons at δ 4.48 (1H, dd, J=11.2, 4.8 Hz, H-3) and 4.12 (1H, br d, J=10.0 Hz, H-16).[24)] The ^{13}C-NMR spectrum and distortionless enhancement by polarization transfer (DEPT) spectra revealed a carbonyl carbon at δ 212.2 (C-12), and an acetyl carbonyl carbon at δ 171.2, two oxygenated carbon at δ 80.6 (C-3) and 66.2 (C-16), and nine tertiary methyl carbons. The other carbon signals were observed and assigned to nine methylenes, four methines, and six quaternary carbons. The NMR spectra revealed features of an oleanane-type triterpene compared with that of maniladiol from *Baccharis salicina*.[25)] The full NMR assignments and connectivities were determined by the ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond coherence (HMBC) spectroscopic data analyses. The signals at δ_H 2.05 (3H, s) in ^{1}H-NMR and signals at δ_C 21.5 and 171.2 in ^{13}C-NMR indicated the presence of an acetyl group. In the HMBC spectra, the long-range correlations between the oxy-



	R1	R2	R3
1	=O	α–OH	CH_3
2	H	=O	CH_3
3	H	β–OH	CH_3
4	H	=O	CH_2OH



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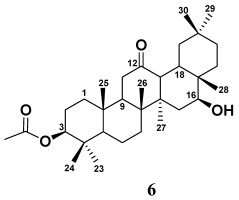

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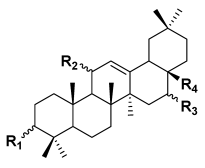

	R1	R2	R3	R4
7	β–OAc	=O	β–OH	CH_3
8	β–OAc	H_2	β–OH	CH_3
9	β–OH	H_2	H_2	CH_3
10	β–OH	H_2	=O	β–OH
11	=O	H_2	=O	β–OH

Fig. 1. Structures of Isolated Compounds (**1**—**11**) from *C. japonica*

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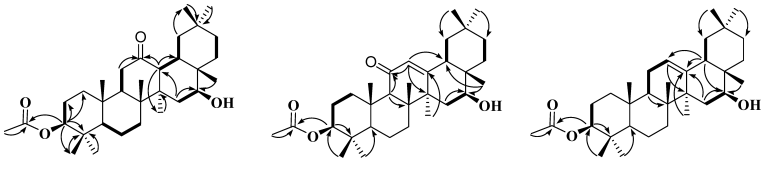
genated proton at δ 4.48 (H-3) and the carbons at δ 16.8 (C-24), 23.6 (C-2), 37.8 (C-4), and the acetyl carbonyl carbon at δ 171.2 inferred the attachment acetyl group to C-3. On the other hand, the correlations from the methyl proton at δ 0.88 (H-28) to the carbon at δ 66.2 (C-16), as well as from the proton at δ 1.67, 1.36 (H-15) to the carbons at δ 37.0 (C-17), 41.9 (C-8), and 66.2 (C-16) confirmed the connectivity of the hydroxy group at C-16. In addition with the coupling constants, the nuclear Overhauser enhancement spectroscopy (NOESY) cross-peaks from signal at δ 4.48 (1H, dd, J=4.8, 11.2 Hz, H-3) to 0.89 (3H, s, H-23) and signal at δ 4.12 (1H, br d, J=10.0 Hz, H-16) to 1.02 (3H, s, H-27), confirmed the α-axial orientation of H-3 and H-16 and consequently the β-equatorial orientation of the two hydroxyl groups. Furthermore, the correlations between the protons at δ 2.83 (H-13), 2.24, 2.18 (H_2-11), 2.14 (H-18) and the ketone carbonyl carbon at δ 212.2 (C-12) in the HMBC spectrum indicated that the ketone carbonyl group was located at C-12. Based on the above evidence analysis, the structure of **6** was determined as 3β-*O*-acetyl-16β-hydroxy-12-oxoolean.

Compound **7** was obtained as a white amorphous powder, with $[\alpha]_D^{22}$ +44.5° (c=0.1, $CHCl_3$). The molecular formula, $C_{32}H_{50}O_4$, was observed from an ion peak at m/z 499.3860 $[M+H]^+$ in the HR-ESI-MS. The IR absorption at 3446, 3395, 1718 and 1656 cm^{-1} indicated the presence of hydroxyl, ketone carbonyl and olefinic groups. The ^{1}H-NMR spectrum displayed the characteristic signals of an olefinic singlet at δ 5.65 (1H, s, H-12), two axial oxymethine protons at δ 4.52 (1H, dd, J=11.6, 4.8 Hz, H-3) and 4.28 (1H, dd, J=11.2, 4.8 Hz, H-16), and nine tertiary methyl signals. The ^{1}H-NMR spectrum further showed a double doublet signal at δ 2.35 (1H, J=4.8, 12.4 Hz) suggested a β-orientation of H-18,[25] which confirmed by cross-peaks from signal at δ 2.35 (H-18) to signal at δ 0.83 (H-28) and δ 0.93 (H-30) in the NOESY spectrum. The ^{13}C-NMR spectrum of **7** revealed 32 carbon signals, which were sorted by DEPT experiment as nine methyls, eight methylenes, six methines, and nine quaternary carbons, of which, two oxygenated methine, two olefinic and two carbonyl carbons were suggested on the basis of the chemical shifts. The above data suggested that compound **7** was an oleanane-type triterpene with the presence of an α,β-unsaturated carbonyl {δ_H 5.65, (1H, s, H-12), δ_C 128.3 (C-12), 168.5 (C-13) and 200.1 (C-11)} in the structure compared with that of 11-keto-β-amyrenyl acetate from *Vellozia compacta*.[26] The full NMR assignments and connectivity were determined by the ^{1}H–^{1}H COSY, HSQC, and HMBC spectroscopic data analyses. In addition, the ^{1}H- and ^{13}C-NMR revealed the presence of an acetyl group (δ_H 2.07, δ_C 171.2 and 21.5). The long-range correlations between the oxygenated proton at δ 4.52 (H-3) and the carbonyl carbon of the acetyl group at δ 171.2, as well as the correlations between the methyl proton at δ 0.89 (H-23, H-24) and the oxygenated carbon at δ 80.8 (C-3) in the HMBC spectrum confirmed the position of the β-acetyl group at C-3. Furthermore, the HMBC spectrum confirmed the connectivites of the β-hydroxy group on C-16 by the correlations from the methyl proton at δ 0.83 (H-28) to the carbon at δ 65.3 (C-16), as well as the correlation between proton at δ 1.50 (H-15) and δ 4.28 (H-16) in the ^{1}H–^{1}H COSY spectrum. In addition with the coupling constants, the NOESY cross-peaks from signal at δ 4.48 (1H, dd, J=4.8, 11.6 Hz, H-3) to 0.89 (3H, s, H-23) and 1.44 (3H, s, H-27), and signal at δ 4.28 (1H, dd, J=4.8, 11.2 Hz, H-16) to 1.44 (3H, s H-27), confirmed the α-axial orientation of H-3 and H-16 and consequently the β-configuration of the two hydroxyl groups at C-3 and C-16. On the basis of the above spectroscopic data, compound **7** was defined as a new triterpenoid, 3β-*O*-acetyl-16β-hydroxy-11-oxoolean-12-en.

Compound **8** was obtained as a white amorphous powder with $[\alpha]_D^{22}$ +39.8° (c=0.1, $CHCl_3$). The IR spectrum exhibited the presence of hydroxyl (3425 cm^{-1}) and olefinic (1660 cm^{-1}) groups. The HR-ESI-MS analysis indicated an ion peak at m/z 531.4073 $[M+HCOOH+H]^+$, which corresponded to the molecular formula $C_{32}H_{52}O_3$. The ^{1}H-NMR spectrum showed signals for nine tertiary methyl groups, one olefinic signal at δ 5.25 (1H, t, J=3.4 Hz, H-12), and two axial oxymethine protons at δ 4.50 (1H, dd, J=4.5, 10.5 Hz, H-3) and 4.20 (1H, dd, J=4.2, 10.8 Hz, H-16).[24] The ^{1}H-NMR spectrum further showed double doublet at δ 2.15 (1H, J=4.6, 13.6 Hz) attributed to H-18, a characteristic signal of the β-oriented H-18.[25] The ^{13}C-NMR and DEPT spectrum revealed 32 carbon signals, including one carbonyl at δ 171.2, two olefinic at δ 122.4 (C-12) and 143.7 (C-13), and two oxygenated carbon at δ 81.1 (C-3) and 66.1 (C-16). The above data suggested that compound **8** was an oleanane-type compared with that of maniladiol from *Baccharis salicina*.[25] The signals at δ 2.05 (3H, s) in ^{1}H-NMR and signals at δ 21.5 and 171.2 in the ^{13}C-NMR indicated the presence of an acetyl group. In the HMBC spectra, the long-range correlations between the oxygenated proton at δ 4.50 (H-3) and the carbons at δ 23.7 (C-2), 37.8 (C-4), and the acetyl carbonyl carbon at δ 171.2 confirmed the attachment β-acetyl group at C-3. Furthermore, the HMBC correlations from the methyl proton at δ 0.80 (H-28) to the carbon at δ 37.5 (C-17), 49.3 (C-18), and 66.1 (C-16), as well as from the proton at δ 1.29, 1.65 (H-15) to the carbons at δ 37.5 (C-17), 43.9 (C-14), and 66.1 (C-16) confirmed the connectivity of the β-hydroxy group at C-16. In addition with the coupling constants, the NOESY cross-peaks from signal at δ 4.50 (1H, dd, J=4.5, 10.5 Hz, H-3) to 0.88 (3H, s, H-23) and 1.21 (3H, s, H-27), and signal at δ 4.20 (1H, dd, J=4.2, 10.8 Hz, H-16) to 1.21 (3H, s, H-27), confirmed the α-axial orientation of H-3 and



6 **7** **8**

Fig. 2. Key HMBC Correlations of Compounds **6**—**8**

H-16 and consequently the β-equatorial orientation of the two hydroxyl groups. On the basis of the above spectroscopic data, compound **8** was established as 3β-*O*-acetyl-16β-hydroxyolean-12-en.

The isolates were evaluated for *in vitro* cytotoxic activity against cancer cell lines A549, LLC, HL-60, and MCF-7 using the MTT assay method.[27,28] As the results in Table 3, compound **8** showed cytotoxicity against LLC and HL-60 cancer cell lines with IC_{50} values of 25.2 and 21.7 μM, respectively. Compound **9** exhibited weak cytotoxic activity against A549 and HL-60 cell lines with IC_{50} values of 46.2 and 38.6 μM, respectively. In the case of compound **4**, it displayed moderate cytotoxic activity against HL-60 cancer cell lines with an IC_{50} value of 17.1 μM and weak inhibitory activity on A549 and LCC cell lines. In addition, compounds **2** and **10** showed weak cytotoxic activity in the inhibition of HL-60 cancer cell lines with IC_{50} value of 48.5 and 65.4 μM, respectively.

Experimental

General Experimental Procedures Optical rotations were measured with a JASCO DIP-370 polarimeter using a 100 mm glass cell. UV spectra were measured with a Thermo 9423AQA2200E UV spectrometer. IR spectra were measured with a Bruker Equinox 55 FT-IR spectrometer. A Waters HPLC was used for purification and isolation with an COSMOSIL HPLC column. NMR spectra were recorded on Varian Plus 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometers. Conventional pulse sequences were used for COSY, HMBC, NOESY and HSQC. All chemical shifts (δ) are given in ppm units with reference to tetramethylsilane (TMS) as an internal standard and the coupling constants (*J*) are in Hz. HR-ESI-MS was measured on a JMS-700 Mstation mass spectrometer. TLC was carried out on precoated silica gel 60 F254 (Merck). Chromatography suppliers were used for isolation: silica-gel (Kieselgel 60, 70—230 mesh and 230—400 mesh, Merck) or reverse phase silica-gel (LiChroprep® RP-18, 40—63 μm, MERCK). Optical density (OD) values in the cytotoxic activity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were read on a TECAN-ELISA Microplate Reader.

Plant Material The stem barks of *C. japonica* were collected in March, 2008 at Korea Research Institute of Chemical Technology, Daejeon, Korea, and identified by Professor Byung Sun Min, College of Pharmacy, Catholic University of Daegu, Gyeongsan, Korea.

Extraction and Isolation The air-dried stem barks of *C. japonica* (5.2 kg) was extracted with MeOH (15 l) at room temperature for 72 h and the process was repeated three times. After the evaporation of the solvent under reduced pressure, the crude MeOH extract (378.0 g) was obtained. This extract was suspended in hot water and partitioned with hexane, EtOAc, and BuOH, successively. The EtOAc soluble fraction (41.1 g) was applied to a silica gel column eluted with $CHCl_3$–MeOH (80 : 1 to 0 : 1) to yielded 13 fractions (E1—E13). Subfraction E1 (840 mg) was subjected to a silica gel column, eluted with hexane–EtOAc (50 : 1 to 20 : 1) to obtain compound **2** (107 mg). Subfractions E4 (2.1 g) was applied to a silica gel column and eluted with hexane–EtOAc (30 : 1 to 5 : 1) to afford six subfractions (E4.1—E4.6). Compounds **3** (96 mg) and **9** (76 mg) were crystallized from subfraction E4.2 (138 mg) and E4.4 (338 mg) with hexane–acetone (5 : 1), respectively. Subfraction E4.6 (965 mg) was passed through a silica gel column using hexane–acetone (30 : 1 to 10 : 1) to furnish compound **8** (28 mg). Subfraction E7 (350 mg) was subjected to a silica gel column, eluted with $CHCl_3$–acetone (30 : 1 to 5 : 1) to yielded three subfractions (E7.1—E7.3). Compounds **4** (2.4 mg) and **6** (7.6 mg) was obtained by applying subfraction E7.2 (213 mg) on a RP-18 column eluting MeOH–H_2O (5 : 1 to 20 : 1). Subfraction E8 (518 mg) was applied on a silica gel column using hexane–acetone (10 : 1 to 0 : 1) gave five subfractions (E8.1—E8.5). Subfraction E8.1 (32.7 mg) was applied on a silica gel column by using $CHCl_3$–acetone (40 : 1) to afford compound **11** (7.6 mg). Subfraction E8.2 (75.0 mg) was subjected to a silica gel column using $CHCl_3$–acetone (40 : 1 to 10 : 1), then purified by HPLC {mobile phase: MeCN–H_2O (20 : 80 to 90 : 10); UV: 254 nm; flow rate: 5 ml/min; column COSMOSIL ϕ20×250 mm, 5 μm} to obtain compound **7** (2.0 mg; t_R: 36 min). Compound **1** (8.3 mg) was yielded by applying subfraction E8.5 (165.0 mg) on a silica gel column using $CHCl_3$–MeOH (30 : 1). Repeated silica gel column chromatography of subfraction E9 (1.5 g) using $CHCl_3$–acetone (50 : 1 to 20 : 1) obtained five subfractions (E9.1—E9.5). Subfraction E9.2 (13 mg), and E9.3 (138 mg) was subjected to a silica gel column using hexane–acetone (5 : 1) to afford compound **5** (2.9 mg), and **10** (10 mg), respectively.

Table 1. ^{1}H-NMR (400 MHz) Spectroscopic Data (δ_H, *J* in Hz) of Compounds **6**—**8** in $CDCl_3$

Position	**6**	**7**	**8**
1	1.56 m	2.79 dt (3.6, 13.2)	1.64 m
	1.01 m	2.87 br s	0.98 m
2	1.65 m	1.03 m	1.62 m
		1.63 m	1.57 m
3	4.48 dd (4.8, 11.2)	4.52 dd (4.8, 11.6)	4.50 dd (4.5, 10.5)
4			
5	0.83 m	0.80 m	0.84 m
6	1.54 m	1.88 m	1.56 m
	1.38 m	1.28m	1.43 m
7	1.49 d-like (12.4)	1.60 m	1.54 m
	1.42 d-like (9.2)	1.50 m	1.33 m
8			
9	1.66 m	2.33 s	1.54 m
10			
11	2.24 dd (4.0, 16.8)		1.92 m
	2.18 m		1.87 m
12		5.65 s	5.25 t (3.4)
13	2.83 d (3.8)		
15	1.67 m	1.50 m	1.65 m
	1.36 m	1.73 m	1.29 m
16	4.12 br d (10.0)	4.28 dd (4.8, 11.2)	4.20 dd (4.2, 10.8)
18	2.14 m	2.35 dd (4.8, 12.4)	2.15 dd (4.6, 13.6)
19	1.78 m	1.66 m	1.69 m
	1.21 d-like (13.2)	1.73 m	1.06 m
21	1.78 m	1.12 m	1.36 m
	1.25 d-like (13.2)	1.69 m	1.15 m
22	1.34 m	1.41 m	1.83 m
	1.16 m	1.12 m	1.17 m
23	0.89 s	0.89 s	0.88 s
24	0.88 s	0.89 s	0.87 s
25	0.91 s	1.16 s	0.96 s
26	1.18 s	1.16 s	0.99 s
27	1.02 s	1.44 s	1.21 s
28	0.88 s	0.83 s	0.80 s
29	0.89 s	0.93 s	0.89 s
30	0.96 s	0.93 s	0.91 s
CH_3CO	2.05 s	2.07 s	2.05 s

3β-*O*-Acetyl-16β-hydroxyl-12-oxoolean (**6**): White amorphous powder; $[\alpha]_D^{22}$ +61.8° (*c*=0.1, $CHCl_3$); IR ν_{max} cm^{-1}: 3520, 3400, 1715; HR-ESI-MS $[M+H]^+$ *m/z* 501.3922 (Calcd for $C_{32}H_{53}O_4$, 501.3944); ^{1}H-NMR data see Table 1 and ^{13}C-NMR data see Table 2.

3β-*O*-Acetyl-16β-hydroxyl-11-oxoolean-12-en (**7**): White amorphous powder; $[\alpha]_D^{22}$ +44.5° (*c*=0.1, $CHCl_3$); IR ν_{max} cm^{-1}: 3446, 3395, 1718, 1656; HR-ESI-MS $[M+H]^+$ *m/z*: 499.3860 (Calcd for $C_{32}H_{51}O_4$, 499.3846); ^{1}H-NMR data see Table 1 and ^{13}C-NMR data see Table 2.

3β-*O*-Acetyl-16β-hydroxyolean-12-en (**8**): White amorphous powder; $[\alpha]_D^{22}$ +39.8° (*c*=0.1, $CHCl_3$); IR ν_{max} cm^{-1}: 3425, 1660; HR-ESI-MS $[M+HCOOH+H]^+$ *m/z*: 531.4073 (Calcd for $C_{33}H_{55}O_5$, 531.4051); ^{1}H-NMR data see Table 1 and ^{13}C-NMR data see Table 2.

Cytotoxic Activity The cancer cell lines (A549, HL-60, LLC and MCF-7) were maintained in RPMI and Dulbecco's modified Eagle Medium (DMEM) that included L-glutamine with 10% FBS and 2% penicillin–streptomycin. Cells were cultured at 37 °C in a 5% CO_2 incubator. Cytotoxic activity was measured using a modified MTT assay.[27,28] Viable cells were seeded in the growth medium (100 μl) into 96 well microtiter plates (1×10^4 cells per well) and incubated at 37 °C in a 5% CO_2 incubator. The test sample was dissolved in DMSO and adjusted to final sample concentrations ranging from 5.0 to 150 μM by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to <0.1%. After standing for 24 h, 10 μl of the test sample was added to each well. The same volume of DMSO was added to the control wells. Removing medium after 48 h of the test sample treatment, MTT 10 μl was also

Table 2. ^{13}C-NMR Spectroscopic Data (100 MHz) of Compounds **6**—**8** in $CDCl_3$

Position	**6**	**7**	**8**
1	37.9	39.0	38.4
2	23.6	23.7	23.7
3	80.6	80.8	81.1
4	37.8	38.2	37.8
5	55.4	55.2	55.4
6	18.4	17.5	18.4
7	32.0	32.9	32.8
8	41.9	45.9	40.1
9	49.4	61.2	46.7
10	38.0	39.0	37.0
11	38.6	200.1	23.7
12	212.2	128.3	122.4
13	50.0	168.5	143.7
14	44.4	45.5	43.9
15	35.6	35.7	35.7
16	66.2	65.3	66.1
17	37.0	37.2	37.5
18	39.0	49.5	49.3
19	37.1	45.1	47.0
20	31.1	31.1	31.1
21	31.5	34.1	34.4
22	34.7	30.2	30.7
23	28.1	28.2	28.2
24	16.8	16.9	16.9
25	15.5	16.6	15.8
26	16.4	18.8	17.0
27	22.6	24.7	27.3
28	21.1	22.1	21.7
29	33.8	33.2	33.4
30	23.6	23.9	24.2
$\underline{CH_3}CO$	21.5	21.5	21.5
$CH_3\underline{CO}$	171.2	171.2	171.2

added to the each well (final concentration, 5 mg/ml). After 4 h incubation, the plates were removed, and the resulting formazan crystals were dissolved in DMSO (150 μl). The O.D. was measured at 570 nm. The IC_{50} value was defined as the concentration of sample which reduced absorbance by 50% relative to the vehicle-treated control.

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