Chemical Constituents of Brazilian Propolis and Their Cytotoxic Activities

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The EtOAc-soluble fraction of the MeOH extract of propolis afforded a new prenylated chromane derivative, 3-hydroxy-2,2-dimethyl-8-prenylchromane-6-propenoic acid **(1)**, along with 22 known compounds, **2**−**23**. Of the known compounds, **4**, **7**, **12**−**19**, and **22** were isolated for the first time from propolis, and the absolute configuration of **23** was established as (2*S*,3*R*). Investigation suggested that *Baccharis* spp. are a significant source of tropical Brazilian propolis, in addition to *Clusia minor*, *Clusia major*, and *Araucaria heterophylla*. All the compounds were tested for their cytotoxicity toward human HT-1080 fibrosarcoma and murine colon 26-L5 carcinoma cells. Among these compounds, **9** and **19**−**21** showed potent cytotoxicity, having ED_{50} values equal to or less than 10 μg/mL.

Propolis is a resinous hive product collected by honeybees from parts of plants, buds, and exudates and has been used as a folk medicine since around 300 BC.[1] Various biological activities, such as anticancer, antioxidant, antiinflammatory, antibiotic, and antifungal effects, have been reported for propolis and its constituents.[2] Recently, propolis has also been extensively used in food and beverages to improve health and prevent diseases such as inflammation, heart disease, diabetes, and even cancer.[3] Because of its wide range of biological activities and its use as a health food, there is a renewed interest in the composition of propolis. Propolis possesses a pleasant aromatic smell and varies in color depending on its source and age. The composition of the propolis depends on the place and time of collection. As a consequence, more than 160 constituents have been identified so far, among which phenolic compounds, including flavonoids, are major constituents (more than 50% of the weight of propolis).[3] We evaluated the quality of propolis collected at different places in Brazil based on free radical scavenging activity,[4] examined the β-cell protective effect against streptozotocin in rats,[5] and isolated four potent antihepatotoxic dicaffeoyl quinic acids.[6] In our continuing study on Brazilian propolis, guided by tumor cell growth inhibition assay, we have now isolated a new prenylated chromane derivative (**1**), together with 22 known compounds (**2**−**23**). The structures of these compounds were elucidated by spectroscopic analysis and chemical transformation. In this paper, we wish to report the isolation of these compounds from propolis and their cytotoxic activity toward human HT-1080 fibrosarcoma and murine colon 26-L5 carcinoma.

Results and Discussion

The methanolic extract of propolis showed comparatively strong cytotoxicity toward HT-1080 and colon 26-L5 tumor cells. It was further partitioned into EtOAc-soluble and -insoluble fractions. The EtOAc-soluble portion had the strongest cytotoxicity and was thus subjected to further investigation. After repeated column chromatography and preparative TLC on Si gel, the fraction afforded a new prenylated chromane derivative (**1**) along with 22 known compounds: 2,2-dimethyl-8-prenylchromene-6-propenoic acid (**2**); 2,2-dimethylchromene-6-propenoic acid (**3**);[7] 2,2-dimethylchromene-6-carboxylic acid (**4**);[8] artepillin (**5**);[9] 4-dihydrocinnamoyloxy-3-prenylcinnamic acid (**6**);[9] 4-hydroxy-3-prenylcinnamic acid (**7**);[10] vanillin (**8**); coniferyl aldehyde (**9**);[11] isocupressic acid (**10**);[12] 15-acetoxyisocupressic acid (**11**);[12] agathic acid (**12**);[13] agathic acid 15-methyl ester (**13**);[13] agathalic acid (**14**);[14] cupressic acid (**15**);[15] tremetone (**16**); viscidone (**17**);[16] 12-acetoxyviscidone (**18**);[17] betuletol (**19**);[18] kaempferide (**20**);[19] ermanin (**21**);[20] 3,5,7-trihydroxy-4′-methoxyflavanol (**22**);[20] and dimeric coniferyl acetate (**23**).[21] To the best of our knowledge, compounds **4**, **7**, **12**−**19**, and **22** were isolated for the first time from propolis.

Dimeric coniferyl acetate **(23)**, which was previously reported from *Lasiolaena morii*,[21] *Baccharis* spp.,[22] and propolis,[23] is a simple diacetate derivative of dihydrodiconiferyl alcohol (DCA), an important intermediate of lignin formation in woody plants. These reports confirmed only the relative configuration (2,3-trans) of **23** on the basis of coupling constant between H-2 and H-3 protons (J = 7.4 Hz) and of NOE experiments. We determined the absolute configuration of **23** through alkaline hydrolysis to DCA. A previous report confirmed the absolute configuration of DCA, according to which the $[\alpha]_D$ value of two possible trans isomers, [i.e., (2*S*,3*R*)−(+)-DCA and (2*R*,3*S*)−(−)-DCA] are +63.3° (*c* 2.1, Me_2CO) and −54.1° (*c* 2.7, Me_2CO), respectively.[24] The $[\alpha]_D$ value +38.74° (*c* 0.12, Me_2CO) of the hydrolyzed product of **23** indicated it should be (2*S*,3*R*)−(+)-

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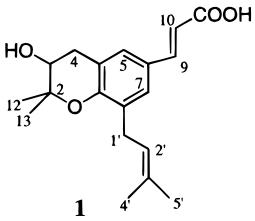

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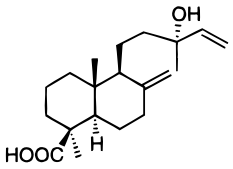

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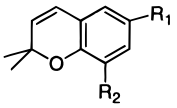
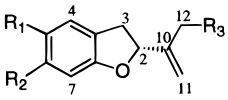
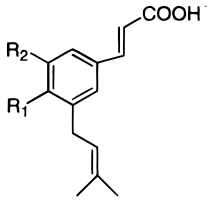
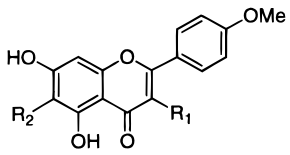


2 R_1 = CH=CHCOOH, R_2 = $CH_2CH=C(Me)_2$
3 R_1 = CH=CHCOOH, R_2 = H
4 R_1 = COOH, R_2 = H

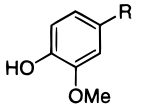




16 R_1 = Ac, R_2 = R_3 = H
17 R_1 = OH, R_2 = Ac, R_3 = OH
18 R_1 = OH, R_2 = Ac, R_3 = OAc

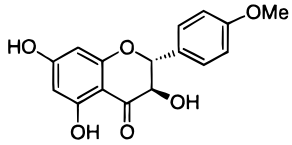


5 R_1 = OH, R_2 = $CH_2CH=C(Me)_2$
6 R_1 = $OCOCH_2CH_2Ph$, R_2 = H
7 R_1 = OH, R_2 = H



19 R_1 = OH, R_2 = OMe
20 R_1 = OH, R_2 = H
21 R_1 = OMe, R_2 = H

8 R = CHO
9 R = CH=CHCHO



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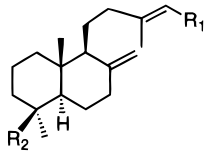

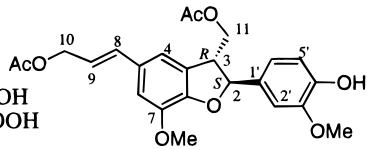

10 R_1 = CH_2OH, R_2 = COOH
11 R_1 = CH_2OAc, R_2 = COOH
12 R_1 = R_2 = COOH
13 R_1 = COOMe, R_2 = COOH
14 R_1 = COOH, R_2 = CHO

23

DCA. The above result suggests the absolute configuration of **23** to be (2*S*,3*R*).

Compound **1** was obtained as a colorless amorphous powder having UV maxima at 320 and 245 nm. It was confirmed to be a racemic mixture because the methyl ester of **1** gave the same mixture of (*R*)- and (*S*)-MTPA esters. The molecular formula of **1** was found to be $C_{19}H_{24}O_4$ by HREIMS measurement, and the IR spectrum showed the presence of hydroxyl and carboxylic acid groups (3400 and 1675 cm^{-1}). The ^1H and ^{13}C NMR spectra of **1** were similar to those of **2** and **3** (Table 1) and showed the signals of meta-coupled benzene protons (δ 7.19, 7.11), two tertiary methyls (δ 1.36, 1.32), a 3-methyl-2-butenyl group, and a propenoic acid group. Protons of one methylene (δ 3.06, 2.77) and one hydroxy methine (δ 3.82) group were observed, however, in the ^1H NMR spectrum, instead of the olefinic protons in **2** and **3**, suggesting a chromane skeleton. The position of the 3-methyl-2-butenyl and propenoic acid groups were determined by 2D NMR including $^1H-^{13}C$ long-range COSY spectrum (Figure 1). Based on these evidences, the structure of **1** was concluded to be 3-hydroxy-2,2-dimethyl-8-prenylchromane-6-propenoic acid.

There are many investigations on the origin of propolis from temperate zones, and almost always poplar buds, especially *Populus nigra*, appeared to be the dominant source of propolis.[25] But little work has been done in connection with the source of tropical propolis (including Brazilian), even though it has biological activities similar to those of propolis found in temperate zones. There are no poplar buds in tropical areas. The resins exuded by the flowers of *Clusia minor* and *Clusia major* (Guttiferae) were previously reported as the dominant sources of tropical Venezuelan propolis, by the occurrence of polyprenylated benzophenones as major components.[26] *Araucaria heterophylla* (Compositae), rich in labdane diterpenes, is also reported as a possible source of Brazilian propolis on consideration of isolated labdane diterpenes.[3] In the present study, we isolated prenylated compounds, labdane diterpenes, and others. Among them, **3**, **5**, **7**, **16**−**18**, **23**, and methyl ester of **6** were previously reported from different *Baccharis* spp. of Compositae grown in the tropical South American zone.[10,27−31] Further, *Baccharis* spp. are also a rich source of various diterpenes, including labdane-type and prenylated compounds.[10,27−31] It thus appears that probable sources of Brazilian propolis would be *Baccharis* spp., in addition to *C. minor*, *C. major* and *A. heterophylla*.

All the isolated compounds were tested against human HT-1080 fibrosarcoma and murine colon 26-L5 carcinoma for their in vitro cytotoxicity, and their ED_{50} values are given in Table 2 along with those of the extracts. Only **9** and **19**−**21**, showed potent cytotoxic activity, having ED_{50} values equal to or less than 10 μg/mL. Compounds **2**, **5**, **17**, and **22** have ED_{50} values less than 50 μg/mL in fibrosarcoma, whereas in carcinoma they showed less cytotoxic activity. Not only these, but the other compounds isolated from propolis, except **10** and **19**, were found to be more active toward human fibrosarcoma than toward murine carcinoma. Neolignan **23** has moderate activity in both cell lines, with ED_{50} values of nearly 40 μg/mL. Compounds **1**, **10**−**13**, and **15**−**16** have ED_{50} values less than 100 μg/mL. The rest of the compounds showed weak cytotoxic activity, having ED_{50} values greater than 100 μg/mL.

According to previous studies, the constituents showing strong anticancer activity from propolis are caffeic acid derivatives, flavonoids, 2,2-dimethyl-8-prenylchromene-6-propenoic acid (**2**), artepillin (**5**), and 17-hydroxycleroda-3,(13*Z*)-dien-15-oic acid.[2,32−34] Even though the clerodane diterpene was reported to have potent cytotoxic activity against human hepatocellular carcinoma HuH13, lung carcinoma HLC-2, HeLa, KB, and rat W3Y cells,[34] the labdane diterpenoids **10**−**15**, showed weak cytotoxicity against HT-1080 and colon 26-L5 cells. These results indicate that the anticancer activity of Brazilian propolis is primarily due to the phenolic compounds, which are also responsible for other biological activities.[1,2]

Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1**−**3** ($CDCl_3$)[a]

	1		**2**		**3**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2		76.5 s		76.2 s		77.2 s
3	3.82 dd (5.5, 5)	69.5 d	5.64 d (10)	131.0 d	5.67 d (10)	131.4 d
4	3.06 dd (17, 5.5)	31.4 t	6.31 d (10)	121.0 d	6.29 d (10)	121.7 d
	2.77 dd (17, 5)					
4a		119.0 s		122.1 s		126.9 s
5	7.11 d (2)	128.6 d	7.03 d (2)	129.8 d	7.13 d (2)	126.4 d
6		126.3 s		126.4 s		121.4 s
7	7.19 d (2)	127.8 d	7.18 d (2)	124.4 d	7.32 dd (9, 2)	129.9 d
8		130.7 s		129.9 s	6.76 d (9)	114.5 d
8a		153.2 s		153.3 s		155.6 s
9	7.67 d (16)	147.2 d	7.67 d (16)	147.1 d	7.69 d (16)	146.8 d
10	6.26 d (16)	114.2 d	6.27 d (16)	114.8 d	6.29 d (16)	116.9 d
11		172.4 s		172.8 s		172.5 s
12	1.36 s	25.1 q	1.43 s	28.2 q	1.45 s	28.3 q
13	1.32 s	22.1 q	1.43 s	28.2 q	1.45 s	28.3 q
1′	3.27 br t (6.5)	28.4 t	3.26 br t (6.5)	28.1 t		
2′	5.25 tqq (7.5, 1.5)	122.0 d	5.26 tqq (7.5, 1.5)	122.1		
3′		132.8 s		132.7 s		
4′	1.74 s	25.8 q	1.74 s	25.8 q		
5′	1.72 s	17.9 q	1.73 s	17.9 q		

[a] Chemical shifts (δ) are in ppm with coupling constants (J in Hz) in parentheses.

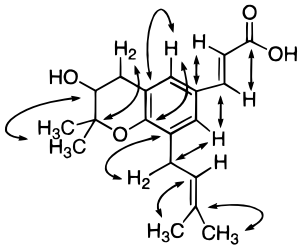

Figure 1. Significant correlations observed in the long-range ^{1}H−^{13}C COSY spectrum of **1**.

Table 2. Cytotoxicity of Propolis Extracts and the Compounds Isolated from the EtOAc-soluble Portion of MeOH Extract (ED_{50} values; μg/mL)[a]

ext./compds.	HT-1080	colon L5-26
H_2O extract	>100	>100
MeOH extract	67.33	62.35
$CHCl_3$ extract	73.12	>100
EtOAc-soluble	53.15	50.74
EtOAc-insoluble	89.55	84.59
1	71.53	77.07
2	46.86	50.22
5	45.47	59.32
6	25.94	77.90
9	4.05	10.44
10	72.91	63.54
11	70.10	73.27
12	75.43	95.92
13	72.82	>100
15	94.86	>100
16	57.40	58.09
17	45.48	57.15
19	5.83	4.95
20	2.91	5.95
21	2.30	7.64
22	26.97	70.98
23	38.92	39.86
doxorubicin HCl	0.034	0.015

[a] Compounds having ED_{50} value >100 μg/mL are not included in table.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-140 digital polarometer. UV spectra were taken in $CHCl_3$ solution on a Shimadzu UV-160A UV−vis spectrophotometer, and IR specta were measured with a Shimadzu IR-408 spectrophotometer in $CHCl_3$ solution. EIMS and HREIMS measurements were performed on JEOL D-300 spectrometer using a direct inlet system at the ionization voltage of 70 eV. ^{1}H, ^{13}C, and 2D NMR were obtained on a JEOL GX-400 spectrometer with tetramethylsilane (TMS) as an internal standard.

Biological Material. Propolis (Yukari propolis) was collected in Brazil in 1995, and a voucher sample (P-2) is preserved in our laboratory.

Extraction and Isolation. Brazilian propolis (1.8 kg) was treated with distilled H_2O (2 L × 2) for 2 h at 80 °C, and the insoluble portion was separated by filtration. The filtrate was partially evaporated and lyophilized to give a H_2O extract (130 g), while the insoluble portion was extracted with MeOH (2 L, reflux, 2 h × 2) and then with $CHCl_3$ (2 L, room temperature, × 2) to yield a MeOH extract (331 g) and a $CHCl_3$ extract (315 g). The MeOH extract was further fractionated into EtOAc-soluble (271 g) and -insoluble fractions (42 g).

The EtOAc-soluble fraction, which showed the greatest cytotoxicity, was subjected to Si gel column chromatography with $CHCl_3$−MeOH gradient system to give seven fractions [fraction 1, 5% MeOH−$CHCl_3$ eluate, 9.6 g; fraction 2, 10% MeOH−$CHCl_3$ eluate, 29.4 g; fraction 3, 10% MeOH−$CHCl_3$ eluate, 8.7 g; fraction 4, 10% MeOH−$CHCl_3$ eluate, 3.0 g; fraction 5, 20% MeOH−$CHCl_3$ eluate, 44.8 g; fraction 6, 20% MeOH−$CHCl_3$ eluate, 49.3 g; fraction 7, 30% MeOH−$CHCl_3$ eluate, 46.9 g].

Fraction 1 contained fatty material, and further Si gel column chromatography and preparative TLC of fractions 2−6 yielded the following compounds: fraction 2 **2** (74.2 mg), **3** (41.0 mg), **4** (6.0 mg), **5** (100.1 mg), **6** (22.8 mg), **9** (14.8 mg), **11** (103.0 mg), **13** (66.8 mg), **14** (8.5 mg), **16** (24.4 mg), **18** (12.3 mg), **19** (45.0 mg), and **23** (24.0 mg); fraction 3 **3** (165.0 mg), **8** (63.8 mg), **9** (90.5 mg), and **12** (14.5 mg); fraction 4 **3** (10.0 mg); fraction 5 **10** (83.0 mg), **15** (28.2 mg), **20** (55.6 mg), **21** (11.0 mg), and **22** (53.5 mg); fraction 6 **1** (53.2 mg), **7** (202.6 mg), **10** (22.2 mg), and **17** (25.9 mg). Compounds **3**−**23** were

identical in all respects (mp, UV, IR, NMR, MS) to respective compounds described in its literature. The detail data of **2** and **16** were not reported yet, so they were included here.

3-Hydroxy-2,2-dimethyl-8-prenylchromane-6-propenoic acid (1): colorless amorphous powder; UV ($CHCl_3$) λ_{max} (log ϵ) 320 (3.5), 245 (3.19) nm; IR ($CHCl_3$) ν_{max} 3400, 1675, 1625, 1440, 1260, 1140 cm^{-1}; EIMS *m/z* 316 $[M]^+$ (100), 283 (20), 246 (20), 245 (30), 189 (20), 105 (40), 44 (58); HREIMS *m/z* 316.1712 (calcd for $C_{19}H_{24}O_4$, 316.1712); ^1H and ^{13}C NMR, Table 1.

Preparation of (*S*)-(−)-MTPA and (*R*)-(+)-MTPA esters of 1. To a solution of **1** (10 mg) in MeOH (0.5 mL), excess CH_2N_2 was added under stirring. After 12 h, the mixture was evaporated under reduced pressure to obtain methyl ester of **1** (11.2 mg). (*S*)−(−)-MTPA-Cl (10 μL) was added to a solution of methyl ester of **1** (5 mg) in $CHCl_3$ (0.5 mL) and pyridine (0.5 mL), and the mixture was stirred for 12 h at room temperature. The reaction mixture was purified by preparative TLC with MeOH−$CHCl_3$ (1:19) to give a mixture of (*R*)- and (*S*)-MTPA esters (3.1 mg). The same product (4.3 mg) was obtained when (*R*)-(+)-MTPA-Cl was reacted with methyl ester of **1** instead of (*S*)-(−)-MTPA-Cl in the above reaction. The ^1H NMR ($CDCl_3$) of the mixture was as follows: δ 7.60, 7.53 (each 1H, d, J = 16.0 Hz, H-9), 7.50−7.31 (m, aromatic proton of MTPA moiety), 7.20, 7.13 (each 1H, d, J = 2.0 Hz, H-7), 7.03, 7.01 (each 1H, d, J = 2.0 Hz, H-5), 6.28, 6.25 (each 1H, d, J = 16.0 Hz, H-10), 5.20, 5.15 (each 1H, tqq, J = 7.5, 1.5 Hz, H-2′), 3.78, 3.79 (each 6H, −OMe), 3.45 (each 2H, br d, J = 8.0 Hz, H-3), 3.24, 3.20 (each 2H, br t, J = 6.0 Hz, H_2-1′), 2.92 (2H, dd J = 17.0, 5.5 Hz, H-4), 2.80 (2H, dd J = 17.0, 5.0 Hz, H-4), 1.72, 1.69 (each 3H, H_3-4′), 1.69, 1.68 (each 3H, H_3-5′), 1.31, 1.28 (each 3H, H_3-12), 1.26, 1.25 (each 3H, H_3-13).

2,2-Dimethyl-8-prenylchromene-6-propenoic acid (2): colorless amorphous powder; UV ($CHCl_3$) λ_{max} (log ϵ) 323 (4.16), 279 (4.29), 248 (4.24) nm; IR ($CHCl_3$) ν_{max} 1675, 1625, 1595, 1375, 1270, 1145, 1120, 980 cm^{-1}; EIMS *m/z* 298 $[M]^+$ (27), 283 (100), 275 (9), 256 (18), 149 (36), 121 (18), 95 (45), 81 (63); HREIMS 298.1558 (calcd for $C_{19}H_{22}O_3$, 298.1559); ^1H and ^{13}C NMR, Table 1.

Tremetone (16): yellow oil; $[\alpha]^{25}_D$ −60.33° (*c* 0.46, EtOH); UV (EtOH) λ_{max} (log ϵ) 279 (3.9), 227 (3.8), and 207 (3.7) nm; IR ($CHCl_3$) ν_{max} 1620, 1600, 1580, 1485, 1355, 1280, 1260, 1120, 910, 810 cm^{-1}; EIMS *m/z* 202 $[M]^+$ (93), 187 (100), 159 (43), 141 (20), 131 (16), 115 (12); ^1H NMR ($CDCl_3$) δ 7.80 (2H, br d, J = 8.0 Hz, H-4 and H-6), 6.81(1H, d, J = 8.0 Hz, H-7), 5.26 (1H, dd, J = 8.0, 9.0 Hz, H-2),5.08 (1H, br s, H-11), 4.93 (1H, br s, H-11), 3.37(1H, dd, J = 16.0, 10.0 Hz, H-3), 3.05 (1H, dd, J = 16.0, 8.0 Hz, H-3), 2.53 (3H, s, H_3-9), 1.75 (3H, s, H_3-12); ^{13}C NMR ($CDCl_3$) δ 196.5 (s, C-8), 164.0 (s, C-7a), 143.3 (s, C-10), 130.8 (s, C-5), 130.5 (d, C-4), 127.4 (s, C-3a), 125.4 (d, C −6), 112.6 (d, C-11), 108.8 (d, C-7), 86.9 (t, C-2), 34.0 (t, C-3), 26.4 (q, C-9), 17.1 (q, C-12).

Hydrolysis of Dimeric Coniferyl Acetate (23). To a solution of **23** (5 mg) in MeOH (0.5 mL), 5% methanolic NaOH solution (0.5 mL) was added, and the mixture was stirred for 4 h at room temperature. Saturated NH_4Cl solution (5 mL) was added to the reaction mixture, and the mixture was then extracted with EtOAc (5 mL × 3). The EtOAc extract was evaporated and separated by preparative TLC to give DCA (1.8 mg), $[\alpha]^{25}_D$ +38.74° (*c* 0.12, Me_2CO).

Cytotoxicity Assay. Human HT-1080 fibrosarcoma and murine colon 26-L5 carcinoma cells were maintained in Eagle's minimum essential medium and RPMI (both Nissui Pharm. Co., Ltd., Tokyo, Japan), respectively. These media were supplemented with 10% fetal calf serum (Gibco BRL Products, Gaithersburg, MD), 0.1% sodium bicarbonate, and 2 mM glutamine (Wako Pure Chemicals Ind., Ltd., Kyoto, Japan).

Cellular viability in the presence and absence of experimental agents was determined using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT, Sigma, St. Louis, MO) assays as described previously.[35,36] In brief, exponentially growing cells were harvested, and a 50-μL suspension containing 2500 cells was plated in 96-well microtiter plates (Falcon, Becton Dickinson, NJ). After 24 h of incubation at 37 °C under 5% CO_2 to allow cell attachment, the cells were treated with varying concentrations of test specimens in their respective medium (100 μL) and incubated for 4 days under the same conditions as above. After adding a solution of MTT for 4 h, the amount of formazan formed was measured spectrophotometrically at 590 nm using Immuno Mini NJ-2300 plate reader.

Test specimens were dissolved in DMSO and then diluted by medium. DMSO less than 0.1% in the test solution had no effect on the all. Doxorubicin HCl (Kyowa Hakko Co., Ltd., Tokyo, Japan) was used as a positive control, and ED_{50} values were calculated from the mean values of data from six wells.

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