ARTICLES

Cytotoxic Constituents from the Fruit of *Diospyros ferrea*

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ABSTRACT

Two naphthoquinones, isodiospyrin (1), and 8′-hydroxyisodiospyrin (2), six triterpenes, friedelin (3), epifriedelinol (4), lupeol (5), lupenone (6), betulin (7) and lup-20(29)-en-3β,30-diol (8), and two sterols, β-sitosterol (9a) and stigmasterol (9b), were isolated from the *n*-hexane extract of the fruit of *Diospyros ferrea*. All of these compounds were evaluated for *in vitro* cytotoxicity in 4 cancer cell lines. Compounds 1 and 2 exhibited strong cytotoxicity against Hep-3B, KB, COLO-205 and HeLa cells (ED_{50} = 0.17, 1.72, 0.16 and 0.21 μg/mL for 1; ED_{50} = 1.31, 1.75, 1.96 and 1.79 μg/mL for 2).

Key words: *Diospyros ferrea*; Ebenaceae; Naphthoquinones; Triterpenes; Sterols.

INTRODUCTION

Although constituents from the stems of *Diospyros ferrea* have been investigated and several naphthoquinones and triterpenoids were found,[1] a study of the chemicals present in the fruit of D. *ferrea* has not been reported. In our continued search for potential antitumor constituents from the plant family Ebenaceae, we previously isolated some active principles from *Diospyros* spp.[2,3] We now report that the *n*-hexane extract of the fruit of *D. ferrea* ex-

hibited potent cytotoxicity against Hep-3B (hepatoma), HeLa (cervix carcinoma), COLO-205 (colon carcinoma) and KB (nasopharyrnx carcinoma) cells, which led to the isolation of naphthoquinones (**1, 2**), triterpenes (**3, 4, 5, 6, 7, 8,**), and sterols (**9**). The structures of **1** and **2** were elucidated by 2D NMR studies, including ^{1}H-^{13}C heteronuclear COSY, and long-range ^{1}H-^{13}C COSY for the complete assignment of the ^{1}H and ^{13}C NMR spectra. The identifies of compounds **1-9** were confirmed by comparison with the literature data and authentic samples. In addition, these isolates were further tested for their cytotoxicities against the 4 cancer cell lines.

RESULTS AND DISCUSSION

The alcoholic extract of the dried fruit of *D. ferrea* was extracted five times with *n*-hexane. Repeated column chromatography of the hexane extract yielded isodiospyrin (**1**), 8′-hydroxyisodiospyrin (**2**), friedelin (**3**), friedelinol (**4**), lupeol (**5**), lupenone (**6**), betulin (**7**), lup-20(29)-en-3β,30-diol (**8**), and a mixture of β-sitosterol (**9a**) and stigmasterol (**9b**).

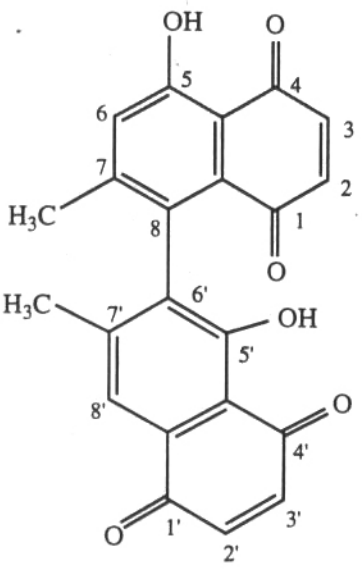
Compounds **1** and **2** were both obtained as a red amorphous powder. Compound **1**, {*m/z* 374 $[M]^+$, mp 228-230 °C, $[\alpha]_D$ -216 ($CHCl_3$; c = 1.0)} has a molecular formula of $C_{22}H_{14}O_6$, based on EIMS. The IR spectrum [υ_{max} cm^{-1}: 1660 (unchelated C=O), 1640 (chelated C=O), 1600 (C=C)] and ^{13}C NMR spectrum (signals at δ 184.3, 184.7, 189.9 and 190.2) revealed the presence of aromatic and conjugated diketone groups. Two aromatic rings (C-5, 6, 7, 8, 9, 10, 5′, 6′, 7′, 8′, 9′ and 10′), and two olefinic (C-2, 3, 2′ and 3′) signals (δ 113-161), as well as the UV spectrum [(UV) λ_{max} nm (log ε): 254 (4.0) and 430 (1.4)] indicated the presence of a dimeric naphthoquinone moiety in **1**. In the ^{1}H NMR spectrum, signals for two methyl, two peri-hydroxyl, two aromatic protons, and four quinonoid protons suggested that **1** contained two 7-methyljuglone monomers. This finding was further supported by the long-range correlation (HMBC) NMR spectrum of **1**. The spectrum showed cross signals between the aromatic carbon signal at δ 158.4 (C-5) and the signal for the isolated aromatic proton at δ 7.24 (H-6), and between the carbonyl carbon signal at 184.7 (C-1′) and the signal of the other isolated aromatic proton at δ 7.55 (H-8′), which indicated that the two monomers are connected by an 8-6′ linkage. From the above corroboration and by comparison with the literature values,[1,4,5] compound **1** was confirmed as isodiospyrin.

Compound **2** showed a molecular ion at *m/z* 390, consistent with a molecular formula of $C_{22}H_{14}O_7$. The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1** except for an additional hydroxyl group and the absence of an isolated aromatic proton. The HMBC spectrum of **2** clearly showed correlations between the quaternary carbon signal at δ 127.0 (C-8) and the proton signal at δ 2.17 (Me-11), between the quaternary carbon signal at δ 186.6 (C-1′) and the proton signal at δ 1.83 (Me-11′), and no correlation between C-8 and Me-11′,

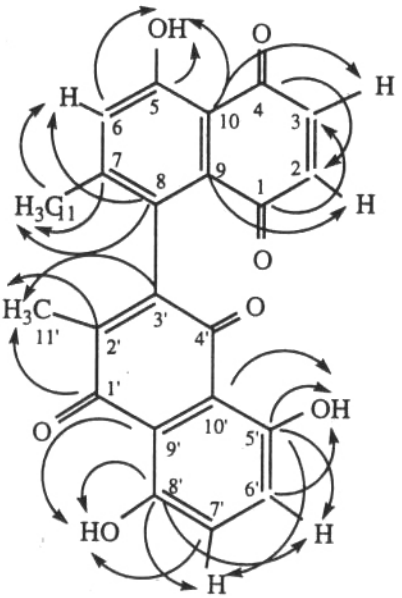
suggesting a connectivity between C-8 and C-3′. By comparison of the ^{1}H and ^{13}C NMR, IR, MS, and UV spectra of **2** with published data[1,6], compound **2** was confirmed as 8′-hydroxyisodiospyrin. Since the ^{13}C NMR chemical shifts for 8′- hydroxyisodiospyrin have not been assigned, an HMBC experiment (Figure 1) was also employed to assign most of the ^{13}C NMR signals of **2** as described in the experimental section.

The presence of eight methyl groups and 30 carbons in the ^{1}H and ^{13}C NMR spectra of **3** and **4** suggested that they were friedelin type triterpenes. The similar chemical shifts except for additional signals at δ_H 3.71 and δ_c 72.75 in **4**, and characteristic peaks at *m/z* 273 for **3** and *m/z* 275 for **4** in the MS spectra indicated that **4** was an analogue of **3** with the carbonyl oxygen at C-3 replaced by an OH group in compound **4**. Moreover, compounds **3** and **4** were verified as friedelin and epi-3β-friedelinol, respectively, by comparing their spectral data with literature values.[7-9]

Compounds **5-7** revealed a positive Liebermann-Buchard tests. The characteristic methylene protons at δ 4.55 and 4.66 (2H, H-29), and six to seven tertiary methyl groups in the ^{1}H NMR spectra, as well as 30 carbons in the ^{13}C NMR spectra revealed that compounds **5-7** possess a lupane skeleton triterpene. The presence of a carbonyl oxygen in **6** and an OH group in **5** at C-3, and a CH_2OH group in **7** at C-17 as determined by ^{1}H and ^{13}C NMR allowed assignment of compounds **5**, **6** and **7** as lupeol, lupenone and betulin, respectively. Compared with lupeol, the methylene protons (H-29) of **8** shifted to the lower field (δ 4.88 and 4.91) due to the replacement of a methyl group (H-30) by a CH_2OH group. Therefore, compound **8** was assigned as lup-20(29)-en-3β,30-diol. All the above lupeol de-



Isodiospyrin (**1**)



8'-hydroxyisodiospyrin (**2**)

rivatives (**5-8**) and compound **9** were further identified by comparison with published data.[10-14]

Compounds **1** to **9** were assayed for cytotoxicity[17] against KB, COLO-205, Hep-3B and HeLa cancer cell lines. Compounds **1** and **2** displayed cytotoxicity against all 4 cell lines: hepatoma (Hep-3B, ED_{50} = 0.17, 1.31 μg/mL, respectively), nasopharynx carcinoma (KB, ED_{50} = 1.72, 1.75 μg/mL, respectively), colon carcinoma (COLO-205, ED_{50} = 0.16, 1.96 μg/mL, respectively) and cervical carcinoma (HeLa, ED_{50} = 0.21, 1.79 μg/mL, respectively). The potent activities exhibited by **1** and **2**, as well as the cytotoxicities displayed by similar naphthoquinones isolated from *Diospyros maritima* and *D. morrisiana*[2,3], suggest that naphthoquinone compounds could play an important role in several cytotoxic plants of the genus *Diospyros*.[2,3,15,16] Additional studies are needed to further evaluate their structure-cytotoxicity relationships.

EXPERIMENTAL

Instrumentation

^1H and ^{13}C NMR spectra were recorded at 300.13 and 75.46 MHz, respectively, on a Bruker 300 AC spectrometer. The spectra of heteronuclear correlation (HMBC) were established employing a coupling constant of 8 Hz. HRMS and EIMS were performed on a JEOL SX-102A instrument. Silica gel (Merck 70-230 mesh) was used for CC, and precoated silica gel (Merck $60F_{254}$) plates were used for TLC. HPLC was performed on a SPD-6AV liquid chromatograph using a semipreparative silica gel column. Melting points were determined on a Fisher-Johns apparatus and are uncorrected.

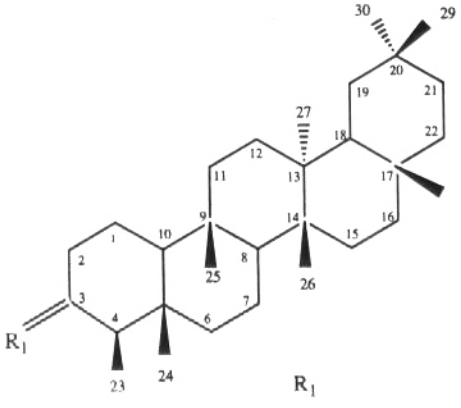
Plant Material

The fruit of *Diospyros ferrea* was collected in Septemper, 1992 in Taipei, Taiwan. A voucher specimen was deposited at the National Research Institute of Chinese Medicine, Taipei, Taiwan.

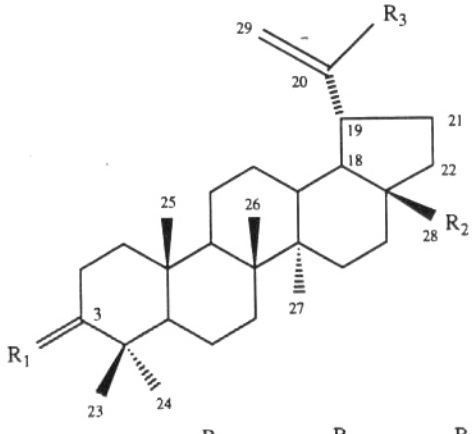
Extraction and Isolation

The dried fruit of *D. ferrea* (4.6 Kg) was extracted three times with ethanol. The crude ethanol syrup was then extracted five times with hexane. The *n*-hexane extract (106 g) was chromatographed on a silica gel column (1.3 kg) with *n*-hexane-EtOAc (5:1) to give 6 fractions (each 21), fr. 1-6. The bioactive fr. 2 was further separated by column chromatography on silica gel (600 g) with *n*-hexane, *n*-hexane-EtOAc (20:1), *n*-hexane-EtOAc (10:1), *n*-hexane-EtOAc (7:1), *n*-hexane-EtOAc (5:1), *n*-hexane-EtOAc (1:1), *n*-hexane-EtOAc (1:2), and EtOAc to yield ten fractions, fr. 2-1 to 2-10 (each 1L). Compound **3** (202 mg)

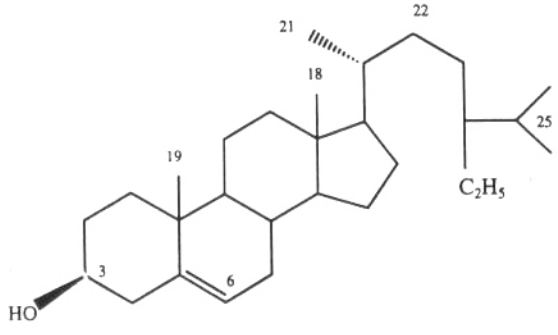
was isolated from fr. 2-2 [1.2 g, *n*-hexane-EtOAc (7:1), and compound **5** (63 mg) was obtained from fr. 2-3 [1.7 g, *n*-hexane-EtOAc (5:1)] by washing with MeOH. Fr. 2-4 was rechromatographed over silica gel (100 g) with an *n*-hexane-EtOAc gradient to yield 12 fractions (fr. 2-4-1 to 2-4-12, each 500 mL). Compound **4** (125 mg) was obtained from fr. 2-4-3 [*n*-hexane-EtOAc = 7:1 (1 L)], compound **7** (37 mg) from fr. 2-4-4 [*n*-hexane-EtOAc = 5:1 (1 L)], and **8** (17mg) from fr. 2-4-4 [*n*-hexane-EtOAc = 3:1 (1 L)]. Fr. 2-5 was further purified by HPLC (Hibar silica-gel, 250 × 10 mm, hexane-EtOAc = 3:1) to afford **1** (t_R = 16 min, 10 mg). Fr. 3 was further chromatographed over silica gel (800 g) with hexane-EtOAc (3:1), followed by CH_2Cl_2-EtOAc (3:1), EtOAc and MeOH to yield 11 fractions (each 1 L),



	R_1
3	O
4	H, HO



	R_1	R_2	R_3
5	HO, H	CH_3	CH_3
6	O	CH_3	CH_3
7	HO, H	CH_2OH	CH_3
8	HO, H	CH_3	CH_2OH



9a

9b Δ^{22} (trans)

fr. 3-1 to 3-11. Compounds **2** (9 mg) and **6** (201 mg) were obtained from fr. 3-2 [hexane-EtOAc = 5:1 (1 L)] and 3-3 [hexane-EtOAc = 3:1 (1 L)], respectively. Phytosterols (**9a & 9b**) (67 mg) were isolated from fr. 3-4 by washing with MeOH and recrystallization in CH_2Cl_2.

Isodiospyrin (1)[1,4,5]

Red amorphous powder, mp 228-230 °C, $C_{22}H_{14}O_6$; EIMS *m/z* (rel. int): 374 $[M]^+$ (100), 359 (70), 357 (11), 331 (24), 331 (23), 189 (15); $[\alpha]_D$-216° ($CHCl_3$; c = 1.0). ^1H NMR ($CDCl_3$): δ 1.96 (3H, *s*, Me-7), 1.98 (3H, *s*, Me-7′), 6.67 (1H, *d*, *J* = 10.3 Hz, H-2), 6.87 (1H, *d*, *J* = 10.3 Hz, H-3), 6.89 (2H, *s*, 2′-H and H-3′), 7.24 (1H, *s*, H-6), 7.55 (1H, *s*, H-8′), 12.00 (1H, *s*, 5-OH), 12.37 (1H, *s*, 5′-OH); ^{13}C NMR ($CDCl_3$): δ 184.27 (*s*, C-1), 184.74 (*s*, C-1′), 139.39 (*d*, C-2), 139.98 (*d*, C-2′), 137.52 (*d*, C-3), 138.58 (*d*, C-3′), 189.92 (*s*, C-4), 190.18 (*s*, C-4′), 158.43 (*s*, C-5), 161.74 (*s*, C-5′), 125.54 (*d*, C-6), 134.96 (*s*, C-6′),145.28 (*s*, C-7), 147.97 (*s*, C-7′), 130.08 (*s*, C-8), 121.18 (*d*, C-8′), 128.42 (*s*, C-9), 128.63 (*s*, C-9′), 113.02 (*s*, C-10), 114.05 (*s*, C-10′), 20.30 (*q*, C-11), 20.48 (*q*, C-11′).

8′-Hydroxyisodiospyrin (2)[1,6]

Red amorphous powder, mp 270-272 °C, $C_{22}H_{14}O_7$; EIMS *m/z* (rel. int): 390 $[M]^+$, (100), 375 (37), 347 (35), 317 (12), 213 (8), 189 (7); ^1H NMR ($CDCl_3$): δ 6.75 (1H, *d*, *J* = 10.3 Hz, H-2), 6.92 (1H, *d*, *J* = 10.3 Hz, H-3), 7.30 (*m*, H-6), 2.17 (3H, *s*, Me-11), 7.30 (2H, *m*, H-6′ and 7′), 1.83 (3H, *s*, Me-11′); ^{13}C NMR ($CDCl_3$): δ 184.82 (*s*, C-1),139.68 (*d*, C-2), 137.96 (*d*, C-3), 190.06 (*s*, C-4), 162.08 (*s*, C-5), 125.69 (*d*, C-6), 145.80 (*s*, C-7), 127.00 (*s*, C-8), 129.14 (*s*, C-9), 114.00 (*s*, C-10), 20.57 (*q*, C-11), 186.60 (*s*, C-1′), 143.00 (*s*, C-2′), 147.22 (*s*, C-3′), 185.22 (*s*, C-4′), 158.76 (*s*, C-5′), 129.63 (*d*, C-6′, 7′), 158.85 (*s*, C-8′), 112.12 (*s*, C-9′, 10′), 13.36 (*q*, C-11′).

Friedelin (3)[7,8]

White powder, mp 258-260 °C; EIMS *m/z*: 426 $[M]^+$; compound **3** was identified by comparsion with an authentic sample.

Epifriedelinol (4)[9]

White powder, mp 276-279 °C; EIMS *m/z* (rel. int): 428 $[M]^+$ (12), 275 (29), 218 (53), 109 (100); ^1H NMR ($CDCl_3$): δ 0.87 (3H, *s*), 0.91 (3H, *s*), 0.92 (3H, *d*, *J* = 5.8 Hz), 0.97 (6H, *s*), 0.98 (6H, *s*), 1.15 (3H, *s*), 3.71 (1H, *m*, H-3).

Lupeol (5)[10,11]

White powder, mp 211-213 °C; EIMS *m/z* (rel. int): 426 $[M]^+$ (31), 411 (13), 218

(100), 203 (54), 189 (42); ^{1}H NMR ($CDCl_3$): δ 0.74 (3H, *s*), 0.77 (3H, *s*), 0.81 (3H, *s*), 0.93 (3H, *s*), 0.95 (3H, *s*), 1.01 (3H, *s*), 1.66 (3H, *s*, 30-Me), 3.16 (1H, *dd*, *J* = 5.5, 10.4 Hz, H-3), 4.54 (1H, *brs*, H-29), 6.66 (1H, *d*, *J* = 2.0 Hz, H-29); ^{13}C NMR ($CDCl_3$): δ 14.55 (*q*, C-27), 15.36 (*q*, C-24), 15.98 (*q*, C-26), 16.11 (*q*, C-25), 18.00 (*q*, C-28), 18.33 (*t*, C-6), 19.31 (*q*, C-30), 20.94 (*t*, C-11), 25.16 (*t*, C-12), 27.44 (*t*, C-2,15), 27.99 (*q*, C-23), 29.86 (*t*, C-21), 34.29 (*t*, C-7), 35.59 (*t*, C-16), 37.17 (*s*, C-10), 38.06 (*d*, C-13),38.72 (*t*, C-1), 38.85 (*s*, C-4), 40.01 (*t*, C-22), 40.84 (*s*, C-8), 42.83 (*s*, C-14), 43.00 (*s*, C-17), 47.98 (*d*, C-19), 48.31 (*d*, C-18), 50.54 (*d*, C-9), 55.31 (*d*, C-5), 78.99 (*d*, C-3), 109.31 (*t*, C-29), 150.94 (*s*, C-20).

Lupenone (6)[12,13]

White powder, mp 166-169 °C; EIMS *m/z* (rel. int): 424 $[M]^+$ (82), 409 (22), 218 (53), 205 (100), 189 (45); ^{1}H NMR ($CDCl_3$): δ 0.77 (3H, *s*, 28-Me), 0.90 (3H, *s*, 25 or 26-Me), 0.93 (3H, *s*, 27-Me), 0.98 (3H, *s*, 24-Me), 1.04 (6H, *s*, 23, 25 or 26-Me), 1.66 (3H, *s*, 30-Me), 4.54 (1H, *brs*, H-29), 4.66 (1H, *d*, *J* = 2.0 Hz, H-29); ^{13}C NMR ($CDCl_3$): δ 14.45 (*q*, C-27), 15.76 (*q*, C-25), 15.94 (*q*, C-26), 17.99 (*q*, C-28), 19.29 (*q*, C-30), 19.66 (*t*, C-6), 21.00 (*q*, C-24), 21.45 (*t*, C-11), 25.13 (*t*, C-12), 26.63 (*q*, C-23), 27.40 (*t*, C-15), 29.81 (*t*, C-21), 33.54 (*t*, C-7), 34.12 (*t*, C-2), 35.50 (*t*, C-16), 36.85 (*s*, C-10), 38.15 (*d*, C-13), 39.59 (*t*, C-1), 39.95 (*t*, C-22), 40.75 (*s*, C-8), 42.87 (*s*, C-14), 42.96 (*s*, C-17), 47.29 (*s*, C-4), 47.93 (*d*, C-19), 48.22 (*d*, C-18), 49.76 (*d*, C-9), 54.90 (*d*, C-5), 109.37 (*t*, C-29), 150.81 (*s*, C-20), 218.15 (*s*, C-3).

Betulin (7)[10]

White powder, mp 250-253 °C; EIMS *m/z* (rel. int): 442 $[M]^+$ (62), 411(80), 234 (35), 207 (51); ^{1}H NMR ($CDCl_3$): δ 0.74 (3H, *s*), 0.80 (3H, *s*), 0.94 (3H, *s*), 0.96 (3H, *s*), 1.00 (3H, *s*), 1.66 (3H, *s*, C = C-Me), 2.36 (1H, *m*, H-19), 3.16 (1H, *dd*, *J* = 5.01, 10.9 Hz, H-3), 3.31, 3.77 (2H, *d*, *J* = 10.8 Hz, H-28), 4.56, 4.66 (2H, *d*, *J* = 2.3 Hz, H-29); ^{13}C NMR ($CDCl_3$): δ 14.55 (*q*, C-27), 15.34 (*q*, C-24), 15.97 (*q*, C-26), 16.09 (*q*, C-25), 18.29 (*t*, C-6), 19.07 (*q*, C-30), 20.82 (*t*, C-11), 25.21 (*t*, C-12), 27.03 (*t*, C-15), 27.36 (*t*, C-2), 27.97 (*q*, C-23), 29.17 (*t*, C-16), 29.74 (*t*, C-21) 33.96 (*t*, C-22), 34.22 (*t*, C-7), 37.15 (*s*, C-10), 37.30 (*d*, C-13), 38.70 (*t*, C-1), 38.85 (*s*, C-4), 40.92 (*s*, C-8), 42.70 (*s*, C-14), 47.66 (*s*, C-17), 47.73 (*d*, C-19), 48.75 (*d*, C-18), 50.40 (*d*, C-9), 55.28 (*d*, C-5), 60.51 (*t*, C-28), 78.95 (*d*, C-3), 109.67 (*t*, C-29), 150.45 (*s*, C-20).

Lup-20(29)-en-3β,30-diol (8)[9,14]

White powder, mp 189-192 °C; EIMS *m/z* (rel. int): 442 $[M]^+$ (61), 424 $[M\text{-}H_2O]^+$ (59), 411 (21), 409 (69), 234 (45), 220 (85), 207 (5), 189 (100); ^{1}H NMR ($CDCl_3$): δ 0.73 (3H, *s*), 0.75 (3H, *s*), 0.80 (3H, *s*), 0,92 (3H, *s*), 0.94 (3H, *s*), 1.00 (3H, *s*), 3.16 (1H, *m*, H-3

α), 4.09 (2H, *br s*, CH_2-30), 4.90 (2H, *br s*, $W_{1/2}$ = 5 Hz, H-29).

Phytosterols [β-sitosterol (9a) and stigmasterol (9b)]

White needles, mp 138-139 °C; EIMS *m/z*: 414 $[M]^+$ (100), 412 $[M]^+$ (35); this compound was confirmed by comparsion with an authentic sample.

Cytotoxicity Assay

The assay against KB (nasal pharynegeal carcinoma), Hep-3B (hepatoma), HeLa (cervix carcinoma) and COLO-205 (colon carcinoma) tumor cells was based on reported method[18-20]. These cells, except for KB, were kindly provided by the Cell Bank of the Veterans General Hospital, Taipei, R.O.C. The KB cells were purchased from the American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium supplemented with a 5 % CO_2 incubator at 37 °C. The cytotoxicity assay depends on the binding the methylene blue to fixed monolayers of cells at pH 8.5, washing the monolayer, and releasing the dye by lowering the pH. In brief, test samples and control standard drug were prepared at a concentration of 1, 5, 10, 40 and 100 μg/mL. After seeding 2880 cells/well in a 96-well microplate for 3 hours, 20 μL of sample or standard agent was placed in each well and incubated at 37 °C for 3 days. After removing the medium from the microplates, the cells were fixed with 10 % formal saline for 30 min, then dyed with 1 % (w/v) methylene blue in 0.01 M borate-buffer (100 μL/well) for 30 min. The 96 well tray was dipped into a 0.01 M borate-buffer solution 4 times in order to remove the dye. Then, 100 μL/well ethanol-0.1 M HCl (1:1 v/v) was added as a dye eluting solvent, and the absorbance was measured on a microtiter plate reader (Dynatech, MR 7000) at a wavelength of 650 nm. The ED_{50} was defined by a comparison with the untreated cells as the concentration of test sample resulting in 50 % reduction of absorbance.

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