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ANTITUMOR AGENTS, 162.[1] CELL-BASED ASSAYS FOR IDENTIFYING NOVEL DNA TOPOISOMERASE INHIBITORS: STUDIES ON THE CONSTITUENTS OF *FATSIA JAPONICA*

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ABSTRACT.—Two pleiotropic multi-drug resistant (PDR) KB cell lines were hypersusceptible to a cytotoxic extract from *Fatsia japonica*. Fractionation of an active extract using a cell-based assay for DNA topoisomerase inhibitors led to the isolation of three known triterpene glycosides, FJ-1–3 [**1–3**]. The structures of **1–3** were identified as 3-*O*-α-L-arabinopyranosyl-oleanolic acid [**1**], 3-*O*-α-L-arabinopyranosyl-hederagenin [**2**], and 3-*O*-[β-D-glucopyranosyl(1→4)-α-L-arabinopyranosyl]-hederagenin [**3**], respectively. However, these isolates were not DNA topoisomerase II inhibitors in vitro and nor were they active when re-tested for differential cytotoxicity. Compounds **1–3** appear to function by interfering selectively with cellular drug accumulation. Other fractions probably contained compounds active against DNA topoisomerase I; however, the enriched preparations were not cytotoxic. The present findings indicate a simple modification to improve the cell-based bioassay procedure employed to guide fractionation.

DNA topoisomerases play crucial roles in several biological processes and these enzymes are proven targets for chemotherapeutic drug development (2,3). The biochemical actions of topoisomerases are well defined (4). Various functional assays have been developed to detect topoisomerase inhibitors and to direct natural product isolation with the purpose of discovering lead structures for drug development (5–7). Our program includes a differential cytotoxicity screen followed by fractionation and isolation guided by a cell-based assay for topoisomerase inhibitors. The cell lines utilized in the pre-screen bear several biochemical alterations and are resistant to toposiomerase II poisons, including the antitumor agents VP-16 (etoposide) and doxorubicin (8). Such inhibitors stabilize a DNA-enzyme covalent reaction intermediate known as the cleavable complex (3,4). This lesion, which can be measured experimentally as protein-linked DNA breaks (PLDB), is the basis of the bioassay strategy employed to guide fractionation. The resistant cells were included in the bioassay protocol with a twofold intent, namely, to prioritize plants for fractionation and to isolate inhibitors with cytotoxic profiles of potential clinical significance.

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[1]For Part 161, see Lee and Wang (1).

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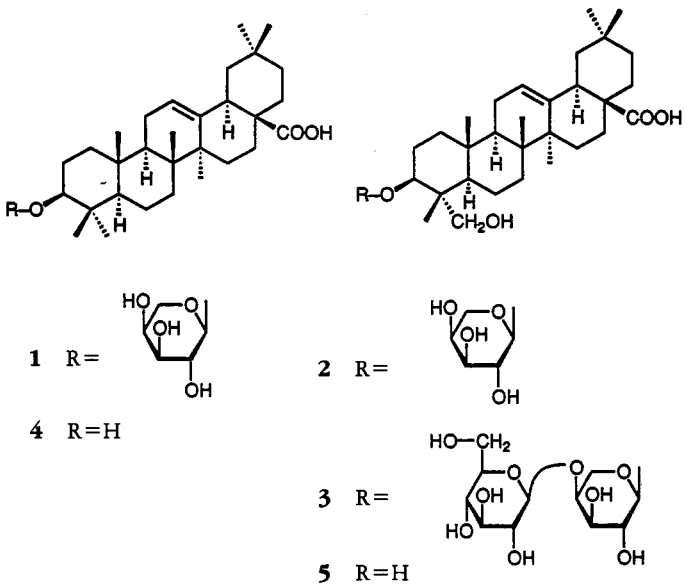
Fatsia japonica Decne. & Planch. (Araliaceae) (Japanese name: "Yatsude"), which contains hemolytic and toxic constituents, is used in various folk remedies (9,10). A number of triterpenoid saponins have been isolated from the flowers, mature fruits, and leaves of this species (11,12). In the present investigation, *F. japonica* was identified using our pre-screen as a potential source of novel DNA topoisomerase inhibitors. Activity-guided fractionation resulted in the isolation of three triterpene glycosides of known structure, FJ-1–3 [**1–3**]. The structures of FJ-1–3 were identified as 3-*O*-α-L-arabinopyranosyl-oleanolic acid [**1**], 3-*O*-α-L-arabinopyranosyl-hederagenin [**2**], and 3-*O*-[β-D-glucopyranosyl (1→4)-α-L-arabinopyranosyl]-hederagenin [**3**], respectively. The activities of these three compounds as DNA topoisomerase II inhibitors have been evaluated. Other constituents identified as potential topoisomerase I inhibitors remain to be isolated, and are contained in fractions which did not inhibit cell growth and were therefore unlikely to be directly responsible for differential cytotoxicity.



RESULTS AND DISCUSSION

The procedure used to fractionate the active extract from *F. japonica* leaves is described in the Experimental. The Me_2CO-soluble fraction exhibited differential cytotoxicity against two pleotrophic multi-drug resistant (PDR) cell lines, KB-1c and -7d (Figure 1). These cells are 29- and 145-fold resistant to VP-16, respectively, compared to non-drug-resistant cells, in part because they contain about twofold lower than normal levels of DNA topoisomerase II (8). The approximate twofold to fourfold differential was observed reproducibly over a narrow concentration range but was not clearly correlated with VP-16 resistance (Figure 1). This type of response could be induced by constituents acting as non-cleavable complex-inducing topoisomerase II inhibitors because resistant cells contain reduced levels of the target enzyme. The active fraction was subjected to Diaion HP-20 cc and eluents were analyzed for their ability to interfere with VP-16-induced PLDB. Additional measurements included PLDB formation and interference with PLDB induced by camptothecin, a topoisomerase I poison (13). These analyses were conducted to detect topoisomerase I actives and were included because increased, possibly compensatory levels of topoisomerase I, were detected in

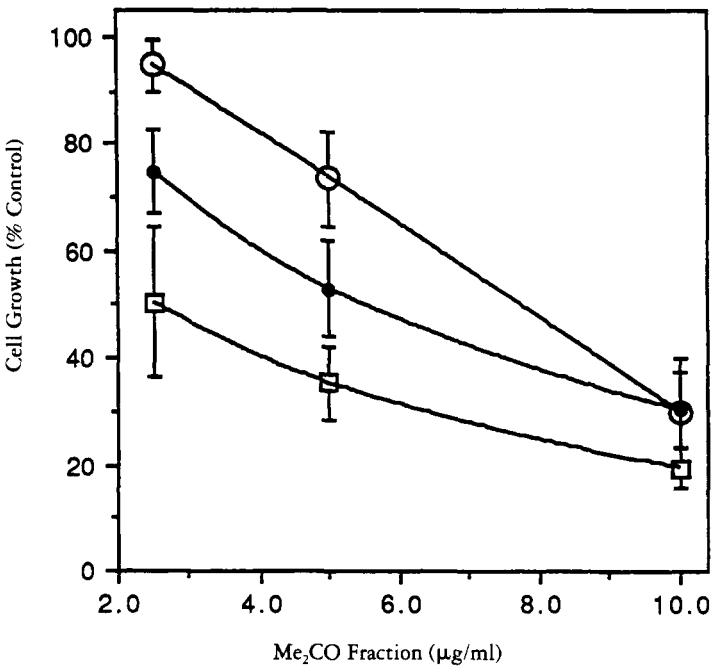


FIGURE 1. Toxicity differential against PDR KB cells displayed by an active Me_2CO-soluble fraction. Cytotoxicity was evaluated using the growth inhibition assay described in the Experimental. Values are mean ±S.D. from triplicate treatments; ○, KB parent; ● KB-7d; □ KB-1c.

resistant cells (8). No samples induced intracellular PLDB significantly above background levels (Table 1). However, other bioassay results highlighted in Table 1 demonstrate that two activities of possible interest were separated. Constituents enriched in fraction 5 appeared to be topoisomerase II actives based on the reduction in VP-16 induced PLDB (about 80% at 50 μg/ml; Table 1). The same bioassay was used

TABLE 1. Detection and Separation of Active *Fatsia japonica* Constituents.

Fraction	Eluent[1]	PLDB[2] (cpm)	PLDB Interference (cpm)[3]		Toxicity[4]
			VP-16	Camptothecin	
Me_2CO	—	564±154	36279±3270	9802±476	−
1	H_2O	620±169	30484±3482	9927±2063	−
2	25% MeOH	679±105	34736±2531	10631±667	−
3	50% MeOH	371±277	35694±3423	**5214±483**	−
4	75% MeOH	646±289	34116±3612	12355±1234	−
5	90% MeOH	711±136	**4529±410**	11864±1122	+
6	100% MeOH	1200±411	29180±1935	10181±1264	−

[1]The Me_2CO-soluble fraction was chromatographed on Diaion HP-20 and eluted successively with the solvents indicated (Figure 1).

[2]Protein-linked DNA breaks were measured in KB cell cultures treated in triplicate for 1 h at 50 μg/ml. Details are given in the Experimental. Background (mock-treated) cpm were 353±159 (n=16).

[3]Interference with VP-16 and camptothecin-induced PLDB was determined following a pre-treatment with extracts as described in the Experimental. Positive (drug-treated) control cpm values were 24441±5214 (n=17) for VP-16 and 11,200±1253 (n=5) for camptothecin.

[4]Cytotoxicity was evaluated at the end of treatments by a microscopical exam; "+"=Cell shrinkage and crenulation, "−"=No apparent morphological change.

to direct isolation of the novel triterpene glycosides **1–3** as active constituents. These isolates were further evaluated as inhibitors. KB cells pre-treated with **1** exhibited a dose-dependent decrease in PLDB upon co-treatment with VP-16 but not with camptothecin (Figure 2). A similar selectivity was displayed by fraction 5 actives (Table 1). However, pretreatment with VP-16 abolished the effect of **1** in co-treatments suggesting pre-formed (intracellular) cleavage complexes were not sensitive to **1** (Figure 2 legend). This interpretation was confirmed using cell nuclei for PLDB measurements (data not shown). No inhibitory effect by **1** (50 μg/ml) on enzyme-catalyzed DNA unknotting was detected using an in vitro assay (data not shown). Furthermore, **1** did not exhibit differential cytotoxicity. The IC_{50} values for cell growth inhibition by **1** were 2.5±0.4 μg/ml against KB and 3.5±0.5 μg/ml against KB-7d. This result suggests that compound **1** was not the active principle detected using the pre-screen (Figure 1). Evaluation of **2** and **3** in similar studies demonstrated that all three isolates shared the same types of bioactivity (data not shown).

Constitutents detected in fraction 3 appeared to be topoisomerase I actives based on the selective inhibition of camptothecin-induced PLDB (about 50% at 50 μg/ml; Table 1). The same bioassay was used to guide fractionation by Si gel cc. Subsequent evaluation demonstrated fractions 3 and 3c could effectively compete with PLDB induced by camptothecin pre-treatment, suggesting the active constituent(s) could antagonize

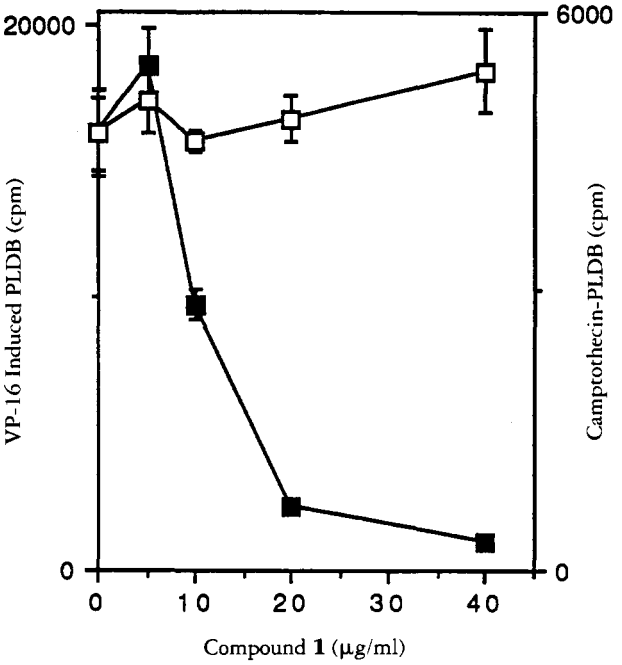


FIGURE 2. Selective interference with drug-induced PLDB's and dependence on **1** pre-treatment. Drug-induced PLDB's were measured using the bioassay method described in the Experimental. Values are means±S.D. from triplicate treatments. Cells pre-incubated with various concentrations of isolate **1** prior to co-treatment with 40 μM VP-16 (■) or 40 μM camptothecin (□). PLDB in cells treated with VP-16 1 h before co-treatment with 40 μg/ml **1** were 15500±674 cpm. Background PLDB values in mock-treated controls were 378±61 cpm.

cleavable complexes formed intracellularly (Figure 3). Fraction 3c (10 μg/ml) inhibited topoisomerase I activity in vitro; however, this fraction did not impact on KB or -7Dd cell growth (two day treatment at 100 μg/ml; data not shown).

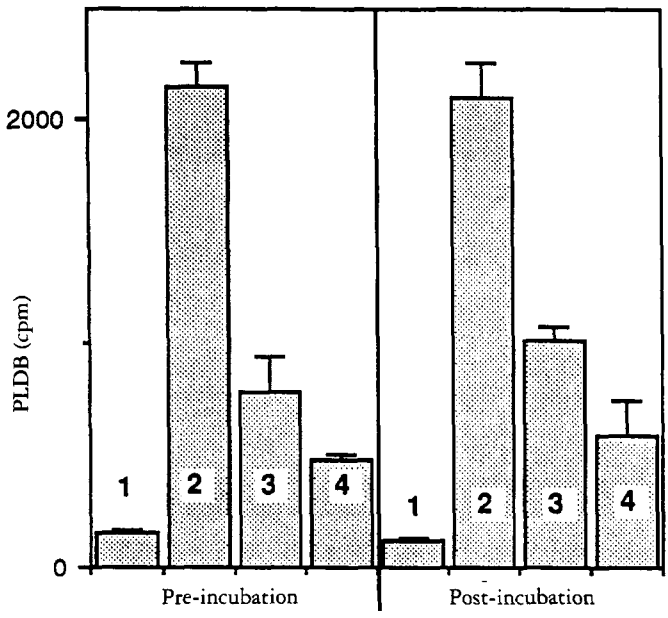


FIGURE 3. Antagonistic effect of *F. japonica* constituents on camptothecin-induced intracellular PLDB's. KB cells were exposed to fractions before adding camptothecin (pre-incubation condition) or after one hour of camptothecin treatment (post-incubation condition); PLDB's were then measured as described in the Experimental. Values are mean±S.D. from the following treatments in triplicate; 1, mock-treated; 2, 30 μM camptothecin; 3, 50 μg/ml fraction 3; 4, 50 μg/ml fraction 3c.

We have employed a differential cytotoxicity assay to detect the presence of novel DNA topoisomerase inhibitors in crude extracts from medicinal plants. In the present work, an active extract from *F. japonica* was subjected to bioassay-directed fractionation and isolation. Compounds **1–3** were identified as active constituents based on their ability to selectively antagonize cellular PLDB induced by VP-16. Additional studies did not corroborate the hypothesis that these isolates were topoisomerase II antagonists. Instead, **1–3** appeared to function as false positives, possibly interfering with VP-16 uptake. This property was dependent upon glycosidic substituents because the aglycones, oleanolic acid [**4**] and hederagenin [**5**], were inactive (Figure 1; unpublished observations). Unlike **1–3**, the active constituent(s) detected in fractions 3 and 3c was found to function intracellularly, possibly as a DNA topoisomerase I antagonist. No inhibitors of this specific type have been discovered to date; however, recent studies indicate tannins as potential candidates. Chebulagic acid and corilagin were isolated from *Erodium stephanianium* using a mechanism-based in vitro screening strategy. These tannins displayed a unique mechanism of action and were potent DNA topoisomerase I inhibitors in vitro (7,14). We have also identified 52 active tannins including chebulagic acid, corilagin, and sanguiin H-6, using both cell-based and in vitro assays for topoisomerase II inhibitors (15). Mechanistic studies demonstrated that sanguiin H-

6, a dimeric ellagitannin isolated from *Sanguisorba officinalis*, inhibited both forms of topoisomerase (I and II), probably by antagonizing enzyme-DNA interactions (16). Structural and biochemical characteristics of the active constituent(s) in fractions 3 and 3c remain to be determined, their direct involvement in differential toxicity appears unlikely based on the present findings.

Identification of **1–3** as false-positives highlights an unexpected flaw in the method employed to guide the isolation of topoisomerase antagonists. The simple modification of treating cells with VP-16 before co-incubation with preparations of interest should minimize the problem. For instance, fraction 5 would not have been prioritized for bioassay-directed isolation had VP-16-induced intracellular cleavable complexes been pre-formed, because no interference activity would have been detected under these assay conditions (Figure 3 and legend). Use of the pre-screen to detect inhibitors bearing novel cytotoxic profiles led to the recent discovery of several synthetic acridone derivatives (17). However, use of the method with phytochemical mixtures remains to be validated. One percent of crude plant extracts screened to date have been found to exhibit a toxicity differential against PDR KB cells. Bioassay-directed fractionation of these extracts to generate isolate(s) for structural and biochemical characterization are ongoing.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mps were measured with a Fisher-Johns melting-point apparatus without correction. Optical rotations were determined with a Rudolph Research Autopol III polarimeter. ^{1}H- and ^{13}C-nmr spectra were obtained on a Bruker AC-300 spectrometer with TMS as internal standard.

PLANT MATERIAL.—The leaves of *Fatsia japonica* Dence et Planch (Araliaceae) were collected in Nanjing, Jiansu Province, People's Republic of China, in June 1992. A voucher specimen was deposited in the Herbarium of the School of Pharmacy, Shanghai Medical University, Shanghai, People's Republic of China.

EXTRACTION AND ISOLATION.—The dried leaves of *F. japonica* (1.0 kg) were extracted with 95% EtOH. After removal of the solvent by evaporation, the residue (55.3 g) was dissolved in MeOH, mixed with Celite 545 (250 g), dried, and packed into a column. Successive elution with hexane, $CHCl_3$, Me_2CO, and MeOH yielded four fractions: a hexane-soluble fraction (5.7 g), a $CHCl_3$-soluble fraction (7.9 g), a Me_2CO-soluble fraction (15.2 g), and a MeOH-soluble fraction (25.4 g). The Me_2CO-soluble fraction was chromatographed over Diaion HP-20 with stepwise elution of H_2O, 25% MeOH, 50% MeOH, 75% MeOH, 90% MeOH, and MeOH furnishing six fractions: fraction 1 (4.4 g), fraction 2 (1.7 g), fraction 3 (2.3 g), fraction 4 (3.1 g), fraction 5 (1.4 g), and fraction 6 (2.6 g). Fraction 5 was subsequently subjected to Si gel cc. Elution with $CHCl_3$-MeOH (19:1) yielded **1** (22.3 mg). Further elution with the same solvent system afforded **2** (57.5 mg). Subsequent elution with $CHCl_3$-MeOH-H_2O (9:1:0.1) yielded **3** (124.6 mg). The structures of **1–3** were assigned as 3-*O*-α-L-arabinopyranosyl-oleanolic acid [**1**], 3-*O*-α-L-arabinopyranosyl-hederagenin [**2**], and 3-*O*-[β-D-glucopyranosyl(1→4)-α-L-arabinopyranosyl]-hederagenin [**3**], respectively, by chemical and spectral examination. The isolation of compounds **1–3** from *F. japonica* has been reported previously (11,12).

Fraction 3, which inhibited camptotechin-induced PLDB, was further chromatographed on Sephadex LH-20 with Me_2CO to afford three fractions: fraction 3a (0.5 g), fraction 3b (1.75 g), and fraction 3c (8 mg).

3-O-α-L-Arabinopyranosyl-oleanolic acid [**1**].—Colorless needles (MeOH); mp 249–252°; $[\alpha]^{16}_D$ +64.3° (c=0.33, MeOH); ^{1}H nmr (pyridine-d_5) δ 0.86, 0.96, 0.97, 1.01, 1.02, 1.29, 1.31 (each 3H, s, *tert*-Me), 3.33 (2H, m, H-3, H-18), 3.85 (1H, dd, J=2.0 and 12.5 Hz, arabinosyl H-5), 4.18 (1H, dd, J=3.5 and 9.0 Hz, arabinosyl H-3), 4.34 (2H, m, arabinosyl H-4 and H-5), 4.44 (1H, dd, J=7.0 and 9.0 Hz, arabinosyl H-2), 4.78 (1H, d, J=7.0 Hz, anomeric-H), 5.49 (1H, br s, H-12); ^{13}C nmr (pyridine-d_5) δ 15.6 (C-25), 17.0 (C-24), 17.5 (C-26), 18.6 (C-6), 23.8 (C-11), 23.9 (C-16), 23.9 (C-30), 26.2 (C-27), 26.7 (C-2), 28.3 (C-23), 28.4 (C-15), 31.0 (C-20), 33.3 (C-22), 33.3 (C-7), 34.3 (C-21), 37.1 (C-10), 38.9 (C-1), 39.6 (C-4), 39.8 (C-8), 42.1 (C-18), 42.2 (C-14), 46.5 (C-19), 46.7 (C-17), 48.1 (C-9), 56.0 (C-5), 66.8 (arabinosyl C-5), 69.6 (arabinosyl C-4), 73.0 (arabinosyl C-2), 74.7 (arabinosyl C-3), 88.7 (C-3), 107.5 (arabinosyl C-1), 122.6 (C-12), 144.9 (C-13), 180.2 (C-28).

ACID HYDROLYSIS OF **1**.—A solution of **1** (10 mg) in 2% H_2SO_4 (0.5 ml) was heated on a waterbath overnight. The reaction mixture was diluted with H_2O, and extracted with $CHCl_3$. Crystallization from

MeOH yielded oleanolic acid (3 mg). Analysis of the aqueous layer by cellulose tlc showed the presence of arabinose.

3-O-α-L-Arabinopyranosyl-hederagenin [**2**].—Colorless needles (MeOH); mp 239.5–242°; $[\alpha]^{16}D$ +64.4° (c=0.75, MeOH); ^{1}H nmr (pyridine-d_5) δ 0.94, 0.95, 0.96, 1.02, 1.04, 1.25 (each 3H, s, *tert*-Me), 3.31 (1H, dd, J=4.0 and 13.5, H-18), 3.74 (2H, m, arabinosyl H-3 and H-5), 4.08 (1H, dd, J=3.0 and 9.0 Hz, arabinosyl H-3), 4.27–4.34 (2H, m, arabinosyl H-4 and H-5), 4.28 (2H, s, H_2-23), 4.44 (1H, dd, J=7.0 and 9.0 Hz, arabinosyl H-2), 5.01 (1H, d, J=7.0 Hz, anomeric H), 5.50 (1H, br s, H-12); ^{13}C nmr (pyridine-d_5) δ 13.7 (C-24), 16.2 (C-25), 17.5 (C-26), 18.2 (C-6), 23.8 (C-11), 23.8 (C-30), 23.9 (C-16), 26.2 (2C) (C-2, C-27), 28.4 (C-15), 31.0 (C-20), 33.0 (C-7), 33.3 (2C) (C-22, C-29), 34.3 (C-21), 37.0 (C-10), 38.9 (C-1), 39.9 (C-8), 42.0 (C-18), 42.2 (C-14), 43.6 (C-4), 46.5 (C-19), 46.7 (C-17), 47.7 (C-5), 48.2 (C-9), 64.6 (C-23), 67.0 (arabinosyl C-5), 69.7 (arabinosyl C-4), 73.2 (arabinosyl C-2), 74.8 (arabinosyl C-3), 82.0 (C-3), 106.7 (arabinosyl C-1), 122.6 (C-12), 144.9 (C-13), 180.2 (C-28).

ACID HYDROLYSIS OF **2**.—A solution of **2** (10 mg) was hydrolyzed as for **1**. Work-up as described above yielded hederagenin (3 mg). Arabinose was detected by cellulose tlc examination of the aqueous layer.

3-O-[β-D-Glucopyranosyl(1→4)-α-L-arabinopyranosyl]-hederagenin [**3**].—Colorless needles (MeOH); mp 245–247°; $[\alpha]^{16}D$ +44.6° (c=0.79, pyridine); ^{1}H nmr (pyridine-d_5) δ 0.84, 0.95, 0.99, 1.00, 1.04, 1.23 (each 3H, s, *tert*-Me), 3.30 (1H, dd, J=4.0 and 13.5 Hz, H-18), 3.65–3.80 (3H, m, H-3 and sugar-H), 4.03–4.30 (6H, m, H_2-23 and sugar-H), 4.36 (1H, dd, J=4.0 and 12.0 Hz, glucosyl H-6), 4.45 (1H, dd, J=2.5 and 12.0 Hz, glucosyl H-6'), 4.56 (1H, t, J=6.5 Hz, arabinosyl H-2), 5.20 (1H, d, J=6.5 Hz, arabinosyl H-1), 5.21 (1H, d, J=7.5 Hz, glucosyl H-1), 5.49 (1H, br s, H-12); ^{13}C nmr (pyridine-d_5) δ 13.5 (C-24), 16.1 (C-25), 17.5 (C-26), 18.3 (C-6), 23.7 (C-11), 23.8 (C-30), 23.9 (C-16), 26.0 (C-2), 26.2 (C-27), 28.4 (C-15), 31.0 (C-20), 33.0 (C-7), 33.3 (C-22), 33.3 (C-29), 34.3 (C-21), 37.0 (C-10), 38.8 (C-1), 39.8 (C-8), 42.0 (C-18), 42.2 (C-14), 43.6 (C-4), 46.5 (C-19), 46.7 (C-17), 48.0 (C-5), 48.2 (C-9), 62.6 (glucosyl C-6), 64.9 (C-23), 65.0 (arabinosyl C-5), 68.3 (arabinosyl C-3), 71.5 (glucosyl C-4), 73.7 (arabinosyl C-2), 76.3 (glucosyl C-2), 78.3 (glucosyl C-5), 78.3 (glucosyl C-3), 81.5 (arabinosyl C-4), 82.3 (C-3), 104.0 (arabinosyl C-1), 105.0 (glucosyl C-1), 122.6 (C-12), 144.9 (C-13), 180.2 (C-28).

ACID HYDROLYSIS OF **3**.—A solution of **3** (15 mg) in 2% H_2SO_4 (2 ml) was hydrolyzed as for **1**. The reaction mixture was worked up as described above and yielded hederagenin (3 mg). Cellulose tlc examination of the aqueous layer showed the presence of arabinose and glucose.

BIOASSAY METHODS FOR FRACTIONATION AND EVALUATION.—The nasopharyngeal carcinoma human KB cell line was provided by M. Fisher (Pharmacology, UNC-CH). Characteristics of the VP-16 resistant KB sub-clones, KB-1c and -7d, obtained from Dr. Y.-C. Cheng, Yale University, have been described in detail in an earlier publication (8). Cultures were kept in a humidified 5% CO_2 atmosphere and were maintained at 37° in RPM1-1640 medium supplemented with 5% (v/v) fetal bovine serum. Over the course of the experiments, cell lines doubled every 24±4 h. Plant extracts were routinely evaluated for differential toxicity using cells cultured in a microtiter plate format by microscopical examination and by measuring cellular reduction of MTT at the end-point (17). Positive extracts were re-tested in a second experiment by direct cell counts following a two-day treatment. Stimulation of intracellular PLDB's and interference with VP-16 or camptothecin-induced topoisomerase-mediated breaks were evaluated using published methods (4,15). Briefly, KB cells were labeled overnight with tritiated thymidine (60–90 Ci/mmol, 0.5 μCi/ml), chased for 2 h and then treated for 1 h with extracts/test compounds. Protein-linked DNA breaks in samples were measured as potassium/SDS-precipitable radioactivity. To measure interference, VP-16 or camptothecin was added following 30 min to 1 h pre-treatment with test samples alone, then co-treated cells were processed 1 h later. In some experiments, topoisomerase poisons were added before co-treating with test samples (see Results and Discussion). The preparation of labeled cell nuclei and buffer used for PLDB analysis was described previously (15). Topoisomerase inhibition in vitro was determined using the standard unknotting and relaxation assay procedures (4,14). Purified enzymes were purchased from Topo Gen (Columbus, OH).

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