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Cytotoxicity and inhibition of DNA topoisomerase I of polyhydroxylated triterpenoids and triterpenoid glycosides

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ABSTRACT

Cytotoxicity and inhibition on human DNA topoisomerase I (TOP1) and II (TOP2) of 74 plant-originated triterpenoids and triterpenoid glycosides were investigated. The cytotoxic compounds are primarily polyhydroxylated oleananes (GI_{50} of A549: 1.0–10.19 μM). Sixteen cytotoxic aesculiosides isolated from *Aesculus pavia* inhibited TOP1 catalytic activity by interacting directly with the free enzyme and preventing the formation of the DNA–TOP1 complex. Interestingly, hydrolysis of six active aesculiosides (**1**, **4**, **6**, **8**, **10**, and **23**) lost their TOP1 activities but enhanced their cytotoxicities. None of the test compounds showed any activity against TOP2. Structure–activity relationship (SAR) investigations indicated that cytotoxic oleananes required at least one angeloyl moiety at either C-21 or C-22 but the sugar moiety at C-3 may decrease their cytotoxicities. An angeloyl or tigeloyl group at C-21 is required for oleananes to bind the free TOP1 enzyme although the type and length of acyl moiety at C-22 also affects their activity. However, sugar moiety at C-3 is necessary for their TOP1 activities.

Triterpenoid or steroidal glycosides, known as saponins, are widely distributed in nature, particularly in plants.[1] Research interests on saponins arose rapidly in the last two decades. Several recent reviews of saponins had reported their occurrence in nature, classification, elucidation, and bioactivities.[1,2] The existing studies have been primarily focused on isolation, elucidation, and initial bioactivity screening of saponins particularly from the plants in Asia, and few reports in other regions.[3] Saponins have shown hemolytic, molluscicidal, anti-inflammatory, antibacterial, antifungal, and antiviral activities.[1]

Anti-tumor potential of saponins has been reported in both chemoprevention and chemotherapy. Chemopreventive activities of saponins include inhibition of mutagenesis and inhibition of the tumor promotion/progession.[4] QS-21, a mixture of saponins from *Quillaja saponaria* Molina has shown great promise in clinical trials as vaccine adjuvants.[5] GPI-0100, a semi-synthetic derivative of a saponin isolated from the same tree, is also used as an adjuvant in various cancer vaccines.[5] The cytotoxic effects of saponins were explored as early as in the 1930s but high toxicity precludes any practical applications.[6] To date, most saponins tested in in vitro assays showed only moderate cytotoxicity with few compounds having potent activity.[7] Some saponins showed promising anti-tumor activity in vivo.[4] The mechanism of action of cytotoxic saponins is still poorly understood. Several reports indicated that saponin-induced apoptosis is primarily caused by stimulating the cytochrome c-caspase 9-caspase 3 pathway in human cancers.[8]

To date, there is no systematic study on structure–activity relationship (SAR) of triterpenoid saponins with a large number of active compounds. Although activity reports of triterpenoid saponins are numerous, the authors rarely compared the activities of their compounds with similar compounds in other reports. In some reports, conclusions were not carefully drawn and some SAR speculations were not clearly supported by their own data.[9] Further, there is no report about the inhibition of cytotoxic saponins on DNA topoisomerases. In the last few years, we have isolated over 100 triterpenoids and triterpenoid glycosides from primarily native plants in North America.[3f,g,7b,10] Several aesculiosides from *Aesculus pavia* L. have shown promising activity against 60 cell lines from nine different human cancers including leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate, and breast.[7b] In this study, we report that cytotoxic aesculiosides inhibited human DNA TOP1 catalytic activity by interacting directly with the free enzyme, thereby preventing the binding of TOP1 to the DNA cleavage site. Unlike CPTs however, these TOP1 inhibitors do not stabilize the intermediate DNA–TOP1 covalent complex. Although some triterpenoids have been identified as TOP1 suppressors,[11] the inhibitory effect of triterpenoid glycosides on the TOP1 enzyme has not been previously reported.

Seventy four triterpenoids and triterpenoid glycosides used in this study were pure compounds (>95% in purity, by HPLC) isolated from various plants and structures were elucidated by NMR and MS spectral analyses. Triterpenoid glycosides **1**, **3**, **4**, **6**, **8**, **10**, **12**, **13**, **17**–**20**, **22**–**24**, **28**–**32**, and **34**–**41** were isolated from fruits of *A. pavia*;[3f,7b] **14**–**16**, **21**, **26**, **27**, **42**–**44** from whole plants of *Eryngium yuccifolium* Michx.;[10b] **45** and **51**–**57** from stems and root

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barks of *Cephalanthus occidentalis* L.;[10a] **46**–**48** from seeds of *Sesbania vesicaria* Elliot; sapogenins **2**, **5**, **7**, **9**, **11**, **25**, and **33** resulting from the hydrolysis of **1**, **4**, **6**, **8**, **10**, **24**, and **32**; **49**, **50**, and **60**–**61** from aerial parts of *Cnidoscolus texanus* Small;[3g] **58** and **59** from leaves of *Camptotheca acuminata* Decaisne; and **62**–**74** from leaves and stems of *Panax quinquefolius* L. Pure compounds were evaluated for their ability to inhibit TOP1 inhibitory activity following the protocol described by Webb and Ebeler.[12] Luteolin was used as an enzyme inhibition control, and ethidium bromide was used as a DNA intercalation control. For the quantitative determination of TOP1 inhibitory activity, photographic negatives were densitometrically scanned by Molecular Imager® Gel Doc™ XR System (Bio-Rad, CA). The IC_{50} values were determined by extrapolating the concentration of the inhibitor at which it prevented 50% of the substrate (supercoiled DNA) from being converted into the reaction product (relaxed DNA) by plotting the data. Mean IC_{50} values were determined in this manner from three independent experiments. The compounds with negative result at 125 μM were considered inactive. Relaxation activity of TOP2 was analyzed in the same manner described above except that the reaction was performed in 20 μL of reaction mixtures containing 17 μL of either the relaxed, linear, or supercoiled DNA (0.75 ng), 2 μL of the test compound, and four times of TOP2 amount which gave the full relaxation.

Cell growth and viability of PANC-1, A549, PC-3, HL-60, and MRC-5 were measured by WST-8 assays following a standard protocol (CCK-8, Dojindo Molecular, MD). A control WST reading (T_0) before treatment was measured. Cells were continuously exposed to isolates for 48 h. WST was added and incubated for 2 h, and the amount of formazan was measured by using a microplate reader. T_C or T_D represents the readings of the untreated control or with tested compounds. Percent cell-growth (G) and percent cell-growth inhibition (GI) was calculated as: $G = (T_D - T_0/T_C - T_0) \times 100$; $GI = [1 - (T_D - T_0/T_C - T_0)] \times 100$ for which $T_D \geqslant T_0$; and $G = (T_D - T_0/T_0) \times 100$; $GI = [1 - (T_D - T_0/T_0)] \times 100$ for which $T_D < T_0$. To determine the inhibitory potency of active compounds (GI_{50}, 50% cell-growth inhibition), tests were expanded to additional concentrations (varying from 0.01 to 100 μM). Doxorubicin was used as the positive controls in the cell-based assays. CCK-8 was used to measure the proliferation response of a cytotoxic sapogenin (**25**), according to the manufacturer's instructions.

The test 74 triterpenoids and triterpenoid glycosides belong to four different types: Oleanane-type (**1**–**50**, Table 1), ursane-type (**51**–**59**, Table S1), lupine-type (**60** and **61**, Table S2), and ginsenosides (**62**–**74**, Table S3). The cytotoxic compounds are basically restricted to oleanane type triterpenoid glycosides or their sapogenins (oleananes) except one ursane-type triterpenoid, α-amyrin (**58**) with weak activities. The cytotoxic oleananes include three compounds (Table 1, entries **2**, **9**, and **25**) showed potent cytotoxicities against tumor cells (e.g., with GI_{50} of A549: 1.94, 1.7 and 2.45 μM, respectively), 15 compounds (**1**, **3**–**8**, **10**, **11**, **14**–**16**, **19**, **21**, and **22**) demonstrated moderate activities (e.g., with GI_{50} of A549 varying from 3.81 μM to 10.19 μM), three (**12**, **18**, and **24**) had weak activities (e.g., with GI_{50} of A549: 11.23, 15.11, and 12.92 μM, respectively), and six (**13**, **34**–**36**, **39**, and **41**) displayed very weak cytotoxicities (e.g., with GI_{50} of A549 varying from 17.6 to >25 μM). None of the test compounds showed any activity against human DNA TOP2. Of the cytotoxic oleananes, 16 aesculiosides (compounds **1**, **3**, **4**, **6**, **8**, **10**, **12**, **19**, **20**, **22**–**24**, **34**–**36**, and **41**, Table 1) inhibited human DNA TOP1. Sapogenins (**2**, **5**, **7**, **9**, **11**, and **25**) of six active aesculiosides (**1**, **4**, **6**, **8**, **10**, and **23**, respectively) lost their TOP1 activity but their cytotoxicities against tumor cells were enhanced. Another sapogenin and two natural triterpenoids (**49**–**50**) had no any activities. None of eryngiosides isolated from *E. yuccifolium* demonstrated any TOP1 inhibitory activities, however, four eryngiosides (**14**–**16**, and **21**) showed moderate cytotoxicity against various tumor cells.

It was reported that some saponins showed interesting selective toxicity on tumor cells.[3d] Triterpenoid glycosides from *Acacia victoriae* selectively inhibited growth of human breast cancer cells and apoptosis in leukemia.[3d] The cytotoxic aesculiosides did not demonstrate any selectivity among tumor lines or normal cells. For examples, active aesculiosides showed similar toxicity in both non-small cell lung tumor cells (A549) and normal lung cells (MRC-5). For aesculiosides, hydrolysis increased the toxicity against A549 but had no significant impact on their toxicity against MRC-5. Therefore, some sapogenins of active aesculiosides are more desirable than saponins in identification of cytotoxic compounds with high selectivity. For example, compound **25** is the triterpenoid aglycone of aesculioside IId (**24**). This sapogenin (**25**) was more toxic to A549 (GI_{50} = 2.45 μM) (Fig. 1) than the aesculioside IId (**24**) (GI_{50} = 12.92 μM) but had even no toxicity to normal cells at the concentration to completely inhibit A549.

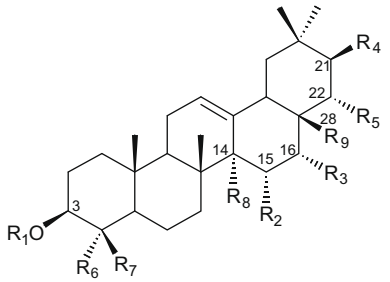
Ginsenosides are one of the most extensively studied groups of saponins. The primary source plants ginsengs (*Panax ginseng* L. and *P. quinquefolius*) have been used in traditional Chinese medicine and are often used as a nutritional supplement. Several in vitro and animal experiments have shown that ginsenosides Rb_2, Rh_2, and Rg_3 (**71**) are primarily responsible for the anti-tumor activity of ginsengs.[13] In fact, Rg_3 is currently marketed as an anti-cancer agent in China.[13] A recent study involving 12 human cancer cell lines demonstrated that Rh_2 is an active ginsenoside (IC_{50}: 20–70 μM) and the compounds Rg_3 (**71**), Re (**62**), Rg_2 (**65**), Rb_1 (**67**), and Rd (**69**) are inactive (**28**). Although our study did not include Rb_2 and Rh_2, all 13 ginsenosides are inactive against human tumor cells and TOP1 and TOP2 activities (Table S3).

Some cytotoxic aesculiosides (**1**, **3**, **4**, **6**, **8**, **10**, **12**, **19**, **22**, and **24**) inhibited human TOP1 catalytic activity by interacting directly with the free enzyme (IC_{50} values varying from 23.8 μM to >100 μM) and preventing formation of the DNA–TOP1 complex. None of these compounds were observed to stabilize the human DNA–TOP1 covalent complex, thereby 'poisoning' the reaction. However, several triterpenoid glycosides (**12**, **19**, **22**–**24**, **34**, **36**, and **41**) were observed to intercalate into the DNA as illustrated by a shift in topoisomer bands at the concentration of 312 μM, as well as partially or completely inhibiting the relaxation of supercoiled DNA. Figure 2 shows the inhibitory activity of aesculioside IV-23D1 (**4**) without poisoning or intercalation effect.

Among the pentacyclic triterpenoid saponins evaluated in this study, 16 of the 50 oleananes (Table 1) exhibited activity against TOP1 while all tested nine ursanes (Table S1) and two lupanes (Table S2) did not show any activity. These results are consistent with our previous cytotoxicity data[7b] and published human TOP1 inhibition of certain triterpenoids.[11]

The oleanane skeleton is the most common among saponins and is present in most orders of the plant kingdom.[2] Among the tested oleananes, 10 compounds including aesculiosides **1**, **3**, **4**, **6**, **8**, **10**, **24**, and **34**–**36** demonstrated activity against human TOP1 enzyme (IC_{50}: 23.8–53.7 μM) (Table 1). Six oleananes including aesculiosides **12**, **19**, **20**, **22**, **23**, and **41** exhibited weaker activity against TOP1 (IC_{50}: 109.3–160.0 μM). Aesculiosides **4**, **24**, and **35** are potent TOP1 inhibitors tested in the present study (Figs. 3 and 4). Compounds **24** and **4** inhibited TOP1 with IC_{50} values of 23.8 and 27.1 μM, respectively. These two aesculiosides completely inhibited the relaxation of supercoiled DNA at less than 63 μM. Compound **35** also demonstrated potent inhibitory activity with an IC_{50} of 53.7 μM, but can completely inhibit TOP1 activity at concentrations greater than 125 μM. However, compounds **18** and **37** showed negligible activity (IC_{50} values of 317.3 and 283.3 μM, respectively) and all other tested oleananes did not show any activity at 312 μM. Additionally, the six triterpenoid sapogenins

Table 1
Inhibitory activity against DNA topoisomerases and cytotoxicity against human tumors of some oleanane-type triterpenoids and triterpenoid glycosides



Compounds **1**-**50**

No.	Name	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	Activity (IC_{50}) (μM)		Cytotoxicity (GI_{50}) (μM)				
											TOP1	TOP2	A549	PC-3	HL-60	PANC-1	MRC-5
1	Aesculioside IV-23C1	AFU	H	OH	O-Ang	O-Ang	CH_3	CH_2OH	CH_3	CH_2OH	53.0 ± 3.6d	(−)	6.36 ± 1.47	1.6 ± 0.47	1.94 ± 0.96	11.14 ± 1.56	5.50 ± 1.92
2	21,22-*O*-Diangeloy-3β,16α,24,28-tetrahydroxyolean-12-ene	H	H	OH	O-Ang	O-Ang	CH_3	CH_2OH	CH_3	CH_2OH	(−)	(−)	1.94 ± 0.47	2.39 ± 0.72	2.72 ± 0.29	4.63 ± 1.62	1.65 ± 0.07
3	Aesculioside IV-23E	AFA	OH	OH	O-Ang	O-Ang	CH_3	CH_3	CH_3	CH_2OH	32.7 ± 8.1bc	(−)	4.58 ± 1.58	14.3 ± 0.31	16.92 ± 2.34	21.48 ± 2.70	5.26 ± 0.79
4	Aesculioside IV-23D1	AFA	H	OH	O-Ang	O-Ang	CH_3	CH_3	CH_3	CH_2OH	27.1 ± 4.5bc	(−)	6.03 ± 0.76	3.26 ± 0.43	1.97 ± 0.66	11.89 ± 1.34	7.43 ± 0.23
5	21,22-*O*-Diangeloyl-3β,16α,28-trihydroxyolean-12-ene	H	H	OH	O-Ang	O-Ang	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	5.48 ± 1.67	6.84 ± 0.21	5.46 ± 1.00	11.21 ± 2.75	7.18 ± 0.33
6	Aesculioside IV_a	AFA	OH	OH	O-Ang	O-MB	CH_3	CH_3	CH_3	CH_2OH	51.8 ± 9.7d	(−)	9.91 ± 2.50	9.22 ± 0.23	7.26 ± 0.40	17.64 ± 3.29	4.78 ± 0.73
7	21-*O*-Angeloyl-22-*O*-(2-methylbutanoyl)-3β,15α,16α,28-tetrahydroxyolean-12-ene	H	OH	OH	O-Ang	O-MB	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	5.37 ± 0.85	12.49 ± 0.53	4.21 ± 0.88	12.8 ± 1.94	6.53 ± 1.22
8	Aesculioside IV_c	AFA	H	OH	O-Ang	O-MB	CH_3	CH_3	CH_3	CH_2OH	49.7 ± 6.4d	(−)	10.19 ± 2.29	7.70 ± 1.86	8.02 ± 1.56	15.64 ± 2.43	4.34 ± 1.88
9	21-*O*-Angeloyl-22-*O*-(2-methylbutanoyl)-3β,16α,28-trihydroxyolean-12-ene	H	H	OH	O-Ang	O-MB	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	1.7 ± 0.21	4.11 ± 0.62	4.60 ± 0.59	9.6 ± 2.18	3.67 ± 0.56
10	Aesculioside IV_b	AFU	H	OH	O-Ang	O-MB	CH_3	CH_2OH	CH_3	CH_2OH	36.0 ± 5.3c	(−)	4.55 ± 0.35	8.37 ± 2.07	6.85 ± 1.09	11.58 ± 1.74	4.23 ± 0.11
11	21-*O*-Angeloyl-22-*O*-(2-methylbutanoyl)-3β,16α,24,28-tetrahydroxyolean-12-ene	H	H	OH	O-Ang	O-MB	CH_3	CH_2OH	CH_3	CH_2OH	(−)	(−)	3.81 ± 0.71	2.34 ± 0.08	2.53 ± 0.93	8.0 ± 2.17	2.71 ± 0.94
12	Aesculioside III_b	AFU	OH	OH	O-Ang	O-Mp	CH_3	CH_2OH	CH_3	CH_2OH	160.0 ± 30.9d	(−)	11.23 ± 1.63	9.93 ± 1.26	10.88 ± 1.35	(−)	N/A
13	Aesculioside II_i	AFU	H	OH	O-Ang	O-Ac	CH_3	CH_2OH	CH_3	CH_2OH	(−)	(−)	20.08 ± 0.62	9.54 ± 0.08	8.37 ± 0.31	14.85 ± 3.79	12.83 ± 0.81
14	Eryngiioside J	GA	OH	OH	O-Ang	O-Ac	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	4.3 ± 0.34	5.08 ± 0.38	7.36 ± 0.28	11.45 ± 0.75	5.61 ± 1.27
15	Saniculasaponin III	IGA	OH	OH	O-Ang	O-Ac	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	4.73 ± 0.21	8.51 ± 0.71	7.11 ± 0.55	16.02 ± 0.61	3.54 ± 0.34
16	Eryngiioside L	IGA	H	OH	O-Ang	O-Ac	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	8.35 ± 2.89	11.32 ± 1.60	8.41 ± 1.84	13.0 ± 1.27	8.71 ± 1.45
17	Aesculioside II_g	AFA	OH	OH	O-Ang	O-Ac	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
18	Aesculioside II_k	AFA	H	OH	O-Ang	O-Ac	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	15.11 ± 3.26	8.35 ± 1.33	8.75 ± 0.26	21.48 ± 1.44	9.89 ± 0.40
19	Aesculioside III_a	AFU	H	OH	O-Ang	OH	CH_3	CH_2OH	CH_3	CH_2OAc	118.7 ± 10.6d	(−)	9.48 ± 0.25	10.09 ± 1.04	7.2 ± 0.68	16.7 ± 3.53	8.65 ± 1.96
20	Aesculioside II_a	AFA	OH	OH	O-Ang	OH	CH_3	CH_3	CH_3	CH_2OH	109.3 ± 29.1d	(−)	(−)	(−)	(−)	(−)	(−)
21	Eryngioside 94A	GA	OH	OH	O-Ang	OH	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	7.34 ± 0.46	7.99 ± 0.29	9.38 ± 1.48	(−)	8.51 ± 1.27
22	Aesculioside II_c	AFA	H	OH	O-Ang	OH	CH_3	CH_3	CH_3	CH_2OH	110.5 ± 26.3d	(−)	8.67 ± 1.29	16.31 ± 2.96	5.75 ± 0.42	(−)	11.24 ± 3.40
23	Aesculioside II_b	AFU	H	OH	O-Ang	OH	CH_3	CH_2OH	CH_3	CH_2OH	118.3 ± 2.9d	(−)	(−)	(−)	(−)	(−)	(−)
24	Aesculioside II_d	AF	H	OH	O-Ang	OH	CH_3	CH_2OH	CH_3	CH_2OH	23.8 ± 4.8b	(−)	12.92 ± 1.14	5.51 ± 1.72	7.50 ± 0.34	10.35 ± 1.12	15.38 ± 2.04
25	21-*O*-Angeloy-3β,16α,22α,24,28-pentahydroxyolean-12-ene	H	H	OH	O-Ang	OH	CH_3	CH_2OH	CH_3	CH_2OH	(−)	(−)	2.45 ± 0.65	2.23 ± 0.21	(−)	11.86 ± 1.32	18.11 ± 1.53
26	Eryngioside F	GA	H	OH	OH	O-Ang	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
27	Eryngioside E	GA	OH	OH	OH	O-Ang	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)

28	Aesculioside I_d	AFU	H	OH	OH	OH	CH_3	CH_2OH	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
29	Aesculioside I_b	AFU	OH	OH	OH	OH	CH_3	CH_2OH	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
30	Aesculioside Ie	AF	H	OH	OH	OH	CH_3	CH_2OH	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
31	Aesculioside I_a	AFA	OH	OH	OH	OH	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
32	Aesculioside I_c	AFA	H	OH	OH	OH	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
33	3β,16α,21β,22α,28-Pentahydroxyolean-12-ene	H	H	OH	OH	OH	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
34	Aesculioside III_d	AFU	OH	OH	O-Tig	O-Tig	CH_3	CH_2OH	CH_3	CH_2OH	48.6 ± 14.0 cd	(−)	23.66 ± 1.90	14.63 ± 3.05	15.25 ± 0.39	21.66 ± 2.97	9.01 ± 2.37
35	Aesculioside III_f	AFU	OH	OH	O-Tig	O-Tig	CH_3	CH_3	CH_3	CH_2OH	53.7 ± 14.5d	(−)	>25	11.65 ± 1.63	14.18 ± 1.97	23.48 ± 3.18	>25
36	Aesculioside III_e	AFU	OH	OH	O-Tig	O-Ang	CH_3	CH_2OH	CH_3	CH_2OH	45.1 ± 14.5 cd	(−)	21.33 ± 0.35	12.8 ± 3.04	14.02 ± 2.02	21.45 ± 2.72	21.52 ± 1.89
37	Aesculioside II_e	AFU	OH	OH	O-Tig	O-Ac	CH_3	CH_2OH	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
38	Aesculioside II_h	AFU	H	OH	O-Tig	O-Ac	CH_3	CH_2OH	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
39	Aesculioside II_j	AFA	H	OH	O-Tig	O-Ac	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	21.77 ± 2.43	13.77 ± 1.82	(−)	21.87 ± 2.11	17.28 ± 0.09
40	Aesculioside II_f	AFA	OH	OH	O-Tig	O-Ac	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
41	Aesculioside III_c	AFA	OH	OH	O-Tig	O-Mp	CH_3	CH_3	CH_3	CH_2OH	131.7 ± 35.9d	(−)	17.6 ± 1.42	12.46 ± 2.03	13.59 ± 2.31	18.5 ± 3.41	N/A
42	Eryngioside A	TG	H	OH	H	O-Glc	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
43	Eryngioside B	TGA	H	OH	H	O-Glc	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
44	Eryngioside C	TG	H	O	H	O-Glc	CH_3	CH_3	CH_3	CH_2OH	(−)	(−)	(−)	(−)	(−)	(−)	(−)
45	3-*O*-β-6-Deoxy-D-glucopyranosyl cincholic acid 28-*O*-β-D-glucopyranosyl ester	Qui	H	H	H	H	CH_3	CH_3	COOH	COO-Glc	(−)	(−)	(−)	(−)	(−)	(−)	(−)
46	3-*O*-β-Glucronopyranosyl-3 β-hydroxyolean-12-en-23,28-dioic acid	GlcA	H	H	H	H	COOH	CH_3	CH_3	COO-Glc	(−)	(−)	(−)	(−)	(−)	(−)	(−)
47	3-*O*-[α-Rhamnopyranosyl-(1→3)-β-glucronopyranosyl]-28-*O*-β-glucopyranosyl-3β-hydroxyolean-12-en-oate	AR	H	H	H	H	CH_3	CH_3	CH_3	COO-Glc	(−)	(−)	(−)	(−)	(−)	(−)	(−)
48	3-*O*-β-Glucronopyranosyl-28-*O*-β-glucopyranosyl-3β-hydroxyolean-12-en-oate	GlcA	H	H	H	H	CH_3	CH_3	CH_3	COO-Glc	(−)	(−)	(−)	(−)	(−)	(−)	(−)
49	Germanicol 3-acetate	Ac	H	H	H	H	CH_3	CH_3	CH_3	CH_3	(−)	(−)	(−)	(−)	(−)	(−)	(−)
50	Germanicol	H	H	H	H	H	CH_3	CH_3	CH_3	CH_3	(−)	(−)	(−)	(−)	(−)	(−)	(−)

Notes:

1. Plant sources of compounds: **1**, **3**, **4**, **6**, **8**, **10**, **12**, **13**, **17**–**20**, **22**–**24**, **28**–**32**, and **34**–**41** were isolated from *Aesculus pavia*; **14**–**16**, **21**, **26**, **27**, and **42**–**44** were isolated from *Eryngium yuccifolium*; **45** was isolated from *Cephalanthus occidentalis*; **46**–**48** were isolated from *Sesbania vesicaria*; **2**, **5**, **7**, **9**, **11**, **25**, and **33** are triterpenoid aglycones of hydrolysis of compounds **1**, **4**, **6**, **8**, **10**, **23**, and **32**; **49** and **50** were triterpenoids isolated from *Cnidoscolus texanus*.

2. R_1, R_4, R_5 and R_9:

TG: Glc^{1}—^{2}Glc^{1}—^{2}Glc

TGA: Gal^{1}—^{2}Glc^{1}—^{2}Glc

AF: Ara*f* ^{1}—^{3}GlcA

AR: Rha^{1}—^{3}GlcA

GA: Xyl^{1}—^{3}GlcA
^{2}|
^{1}Glc

IGA: Ara*p* ^{1}—^{3}GlcA
^{2}|
^{1}Glc

AFU: Ara*f* ^{1}—^{3}GlcA
^{2}|
^{1}Glc

AFA: Ara*f* ^{1}—^{3}GlcA
^{2}|
^{1}Gal

GlcA: β-D-glucuronopyranosyl acid; Gla: β-D-galactopyranosyl; Glc: β-D-glucopyranosyl; Qui: β-L-quinovose; Xyl: β-D-xylopyranosyl; Arap: α-L-arabinopyranosyl; Araf: α-L-arabinofuranosyl; Ang: angeloyl; Tig: tigeloyl; Ac: acetyl; MP: 2-methylpropanoyl; MB: 2-methylbutanoyl.

3. Bioactivities: *Topoisomerase Inhibitory Activity:* For active compounds, IC_{50} (mean ± S.D.) refers to the concentration required to inhibit 50% of TOP1 activity. Numbers followed by different letters are significantly different at $P < 0.05$ (protected LSD test). (−) indicates that the compound is inactive (negative at 312 μM or with IC_{50} >250 μM). *Cytotoxicity*: GI_{50} (mean ± S.D.) refers to the concentration required to have 50% cell-growth inhibition; (−) indicates that the compound is inactive at 25 μM; N/A: no data available.

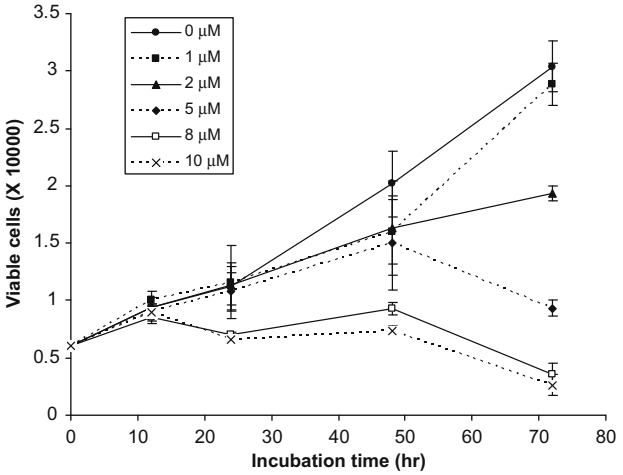


Figure 1. Effects of sapogenin **25** on the proliferation of human non-small cell lung cells (A549).

of active oleananes (**2**, **5**, **7**, **9**, **11**, and **25**) and two natural triterpenoids (**49** and **50**) did not show any activity or exerted no significant inhibition at 312 μM. These results are consistent with published reports describing the inactivity of oleanane-type triterpenoids against TOP1.[11]

It shall be emphasized that the measurement for TOP1 inhibitory potency of a compound (e.g., IC_{50} value) can vary considerably with different experimental methods, particularly with different enzymes and incubation methods. Therefore, it is not reasonable to compare the inhibitory potency of TOP1 inhibitors under different experimental conditions. Because of structural differences, different TOP1 enzymes from human, calf thymus, rat liver, yeast (*Candida albicans*), bacteria (*Escherichia coli*), and protozoa (*Leishmania donovani*) may have significantly different susceptibilities to a compound. For example, *Candida* TOP1 was more susceptible to aminocatechol A-3253 than was human TOP1, while the opposite result was observed for CPT.[14] Das et al. found that luteolin had IC_{50} values of 0.66 μM and 2.5 μM against rat liver TOP1 and *L. donovani* TOP1, respectively, when the enzyme and luteolin were preincubated together prior to the addition of DNA substrate.[14] However, when the TOP1 enzyme and luteolin were simultaneously added to DNA and incubated, the IC_{50} were 5 μM and 14 μM, respectively.

In our experiment, luteolin showed an IC_{50} of 182.3 μM against human TOP1. Also, it has been found that the simultaneous addition of enzyme, testing agent, and DNA in the reaction mixture dramatically reduces inhibitory potency when compared to reactions

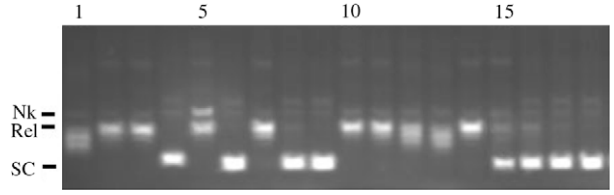


Figure 2. Electrophoresis gel assay showing the activities of aesculioside IV-23D1 (**4**) on human TOP1. Lane 1, relaxed pHOT-1 DNA; Lane 2 and 3, relaxed pHOT-1 DNA + TOP1 (10×); Lane 4, relaxed pHOT-1 DNA + TOP1 (10×) + ethidum bromide at 4.4 μg/ml; Lane 5, relaxed pHOT-1 DNA + TOP1 (10×) + camptothecin (100 μM); Lane 6, supercoiled pHOT-1 DNA; Lane 7, supercoiled pHOT-1 DNA + TOP1 (10×); Lane 8, supercoiled pHOT-1 DNA + TOP1 (2×) + luteolin (312 μM); Lane 9, supercoiled pHOT-1 DNA + **4** (312 μM); Lane 10-13, relaxed pHOT-1 DNA + TOP1 (10×) + **4** at 30, 63, 125, and 312 μM, respectively; Lane 14, supercoiled pHOT-1 DNA + TOP1 (2×); Lane 15–18, supercoiled pHOT-1 DNA + TOP1 (2×) + **4** at 30, 63, 125, and 312 μM, respectively.

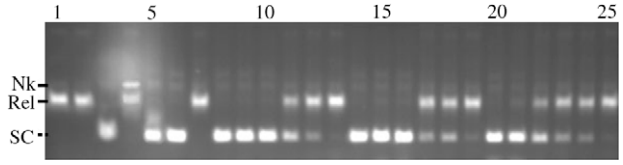


Figure 3. Electrophoresis gel showing inhibition of human TOP1 relaxation activity in a concentration series of aesculiosides **4**, **24**, and **35**. Lane 1 and 2, relaxed pHOT-1 DNA + TOP1 (10×); Lane 3, relaxed pHOT-1 DNA + TOP1 (10×) + ethidum bromide at 4.4 μg/ml; Lane 4, relaxed pHOT-1 DNA + TOP1 (10×) + camptothecin (100 μM); Lane 5, supercoiled pHOT-1DNA + TOP1 (2×) + luteolin(312 μM); Lane 6, supercoiled pHOT-1 DNA; Lane 7, supercoiled pHOT-1 DNA + TOP1 (2×); Lane 8, supercoiled pHOT-1 DNA + **35** (125 μM); Lane 9–13, supercoiled pHOT-1 DNA + TOP1 (2×) + **24** at 125, 63, 30, 20, and 10 μM, respectively; Lane 14, supercoiled pHOT-1 DNA + **4** (125 μM); Lane 15–19, supercoiled pHOT-1DNA + TOP1 (2×) + **4** at 125, 63, 30, 20, and 10 μM, respectively; Lane 20, supercoiled pHOT-1 DNA + **35** (312 μM); Lane 21–25, supercoiled pHOT-1DNA + TOP1 (2×) + **35** at 312, 125, 63, 45, and 30 μM, respectively.

in which enzyme is preincubated with the testing agent prior to the addition of DNA.[15–17] This is true for the compounds with two or three modes of poisoning, inhibition, and intercalation action. Testing against *L. donovani* DNA TOP1, Das et al. found that the IC_{50} values of luteolin, quercetin, and baicalein under simultaneous addition were 4.6, 2.1, and five times higher, respectively than those under enzyme preincubation.[15] Similarly, amarogentin demonstrated 28% inhibition at 30 μM under simultaneous addition as compared to 80% inhibition at the same concentration under preincubation with *L. donovani* TOP1.[16] In addition, treatment of DNA with luteolin before the addition of rat liver TOP1 decreased the inhibitory effect at higher concentrations.[17] In this study, the preincubation of DNA and testing compounds prior to the addition of human TOP1 showed a similar result. Luteolin exhibited intercalation at higher concentrations (1 mM) than lower concentrations at which the compound showed a potent inhibitory effect. Our DNA-compound preincubation may reduce the inhibitory effect in comparison to enzyme-compound preincubation because most of the testing compounds demonstrated intercalative activity. Therefore, IC_{50} values measuring the Topo I inhibitory potency in the present study could be higher than those from other studies.

The compounds **1**, **3**, **4**, **22**, and **24** with GI_{50} (equivalent to IC_{50}) values ranging of 0.649–8.18, 0.866–6.14, 0.175–6.42, 3.60–9.05, and 3.55–>25 μM, respectively, against 60 tumor lines of nine different human cancers in previous experiments[7b] demonstrated TOP1 inhibition activity (IC_{50}: 53.0, 32.7, 27.1, 110.5, and 23.8 μM, respectively). Compounds **20** and **23** showed somewhat cytotoxicity also had weak TOP1 inhibition (IC_{50}: >100 μM). TOP1 enzyme could be one of the targets of these cytotoxic triterpenoid glycosides.

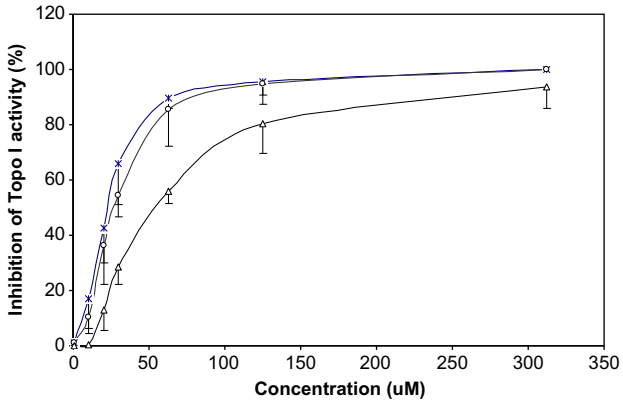


Figure 4. Inhibition of aesculiosides **4** (○), **24** (*), and **35** (△) on human TOP1 relaxation activity.

It seems commonly accepted that sugar moiety at C-3 is required for oleananes to have bioactivity and type and number of sugar moieties at this position affect the cytotoxicity.[7c] However, our data showed that most sapogenins without any sugar moieties at C-3 demonstrated more potent cytotoxicity than their glycosides with sugar moieties at C-3 (e.g., **25** vs **23** and **24**, **2** vs **1**, **9** vs **8**, and **11** vs **10**) while some had no significant change of cytotoxicity after hydrolysis (**5** vs **4** and **7** vs **6**).

It was found that some oleananes with 5-carbon acyl groups at both C-21 and C-22 showed potent cytotoxicity and the saponins without any 5-carbon acyl group at C-21 or C-22 showed no or weak cytotoxicity.[7b,c] As a result, some authors believed that angeloyl or tigeloyl at both C-21 and C-22 are required to have activity[7c] and 2-methylbutanoyl moiety increase cytotoxicity than 2-methylpropanoyl moiety at C-22.[3b] In fact, however, some compounds with acyl groups at both C-21 and C-22 may have weak cytotoxicity only.[3e] In our assays (Table 1), compounds with angeloyl groups at both C-21 and C-22 (**1–5**) or with an angeloyl group at C-21 and a 2-methylbutanoyl group at C-22 (**6–11**) showed potent or moderate activity, but most compounds with angeloyl at C-21 and either a 2-methylpropanoyl, acyl, or hydroxyl group at C-22 (**12**, **14–16**, **18**, **19**, **21**, and **22**) also showed moderate cytotoxicity. Some compounds with a hydroxyl group at C-21 and an angeloyl group at C-22 (**26** and **27**) had no activity. Further, compounds with tigeloyl groups at both C-21 and C-22 (**34** and **35**) and those with a tigeloyl group at C-21 and an angeloyl or other moieties at C-22 showed only weak or no cytotoxicity. Our data suggest that it is required at least one angeloyl moiety at either C-21 or C-22 for oleananes to have cytotoxicity. In some other cases, saponins without any 5-carbon acyl group at C-21, C-22, C-15, or C-16 but with saccharide chain or carboxyl at C-28 showed significant or moderate activity.[18]

It was believed that the occurrence of acetyl or hydroxyl group at any position of C-21 or C-22 reduces the activity.[19] Compounds with an angeloyl group at C-21 and an acetyl group at C-22 (**13** and **17**) had weaker activity than those with angeloyl groups at both C-21 and C-22 (**1**) or an angeloyl group at C-21 and a 2-methylbotanoyl group at C-22 (**6**). But it is not always true. The compounds **4**, **8**, **18**, and **22** all with an angeloyl group at C-21 but with an angeloyl (**4**), 2-methylbutanoyl (**8**), acetyl (**18**), and hydroxyl group (**22**) at C-22, respectively, did not show any significant difference in cytotoxicity.

Some authors speculated that saccharides at C-28 was crucial for oleananes to have activity against J774 and HEK-293 cells,[9] but this seems not true for some similar compounds against HL-60 cells, U937, HL-60 and 3LL cells, and HCT 116 and HT-29 cells.[3c] Hydroxyl group is a necessary group at C-17 for some oleananes to have activity against A-2780 cells,[3a] but some compounds without hydroxyl at C-17 still have activity against KB cells or J774 and HEK-293 cells.[9] All active compounds in our cytotoxicity assays have hydroxyl group rather than saccharides at C-17. Further, the sugar moiety at C-3 either decreased or no impact on the cytotoxicity of oleananes and all six sapogenins (**2**, **5**, **7**, **9**, **11**, and **25**) showed enhanced cytotoxicity in comparison with their glycosides (**1**, **4**, **6**, **8**, **10**, and **23**).

Structure–activity relationships of oleananes against TOP1 demonstrated different patterns from their cytotoxicities. Among the tested oleananes, those bearing 5-carbon acyl groups (angeloyl or tigeloyl) at both C-21 and C-22 atoms (**1**, **3**, **4**, **6**, **8**, **10**, **34**, **35**, and **36**) demonstrated TOP1 inhibition with IC_{50} values between 27.1 and 53.7 μM, with complete enzyme inhibition between 63 and 125 μM. Most oleananes with a 5-carbon acyl group at C-21 and a hydroxyl or methylpropanoyl group at C-22 (**12**, **19**, **20**, **22**, **24**, and **41**) demonstrated weak TOP1 inhibition (IC_{50}:109.3–160.0 μM) and completely inhibited the enzyme at 450 μM. Oleananes with an acyl group at C-21 but an acetyl group at C-22 (**13–18** and **37–40**) had no or negligible TOP1 inhibitory activity. Oleananes without any 5-carbon acyl group at either C-21 or C-22 (**28–33** and **42–50**) did not show any activity against TOP1. Further, compounds with an angeloyl group at C-22 only (**26** and **27**) had no TOP1 activity. Therefore, we conclude that a 5-carbon acyl group at C-21 is required for oleananes to bind the free DNA TOP1 enzyme. Additionally, a 5-carbon acyl group at C-22 enhances activity (e.g., IC_{50} values: below 55 μM of **1**, **3**, **4**, **6**, **8**, **10**, and **34–36** with acyl groups at both C-21 and C-22 vs. most above 100 μM of **12**, **19**, **20**, **22**, **23**, and **41** with an acyl group only at C-21). The existence of olefinic double bonds in an acyl moiety may be an important factor that enhances TOP1 inhibitory potency. The type and length of an acyl moiety also affects the activity. An acetyl moiety at C-22 may make a triterpenoid glycoside with a 5-carbon acyl group at C-21 decrease or lose its TOP1 inhibitory potency (e.g., compounds **13–18** and **37–40**). Compounds bearing a methylbutanoyl group (**6**, **8**, and **10**) are approximately three to five times more potent than those with a methylpropanyl group (**12** and **41**).

The existence of olefinic bonds in an acyl moiety is necessary for active quinones.[20] It seems that a conjugated keto group is probably a common feature for the TOP1 inhibitors identified to date, for examples, flavones such as velutin, 7,3′,5′-tri-*O*-methyltricetin, genkwanin, lethedocin, luteolin, orientin, and baicalein;[12,15,17,21] flavonols such as kaempferol, quercetin, fisetin, myricetin, morin, hyperoside, and trifolin;[22] triterpenoids including fomitellic acids;[11] iridoids such as amarogentin;[17] quinones such as β-lapachon and acylshikonins.[20] However, further investigations are needed to determine the pharmocophore of the active compounds inhibiting DNA TOP1.

Saccharide chains of the screened oleananes are all attached at C-3. The sugar moiety is necessary for the TOP1 inhibition of these compounds. The triterpenoid sapogenins (**2**, **5**, **7**, **9**, **11**, and **25**) of active triterpenoid glycosides (**1**, **4**, **6**, **8**, **10**, and **23**), the result of hydrolysis, exhibited low or no TOP1 activity. For example, the sapogenin (**5**) of the active compound **4** did not show any TOP1 inhibition until 450 μM. Additionally, the type of saccharide chains attached to the skeleton and the number of glycosyl residues per chain affected TOP1 inhibitory activity. Compounds **20** and **21** have similar structures except for the difference in attached sugar type at C-3, but the IC_{50} of aesculioside **20** was 109.3 μM while **21** did not show activity at 312 μM. Structures of aesculiosides **23**, **24**, and **25** are similar except the moiety attached at C-3. Compound **24** with a bisaccharide residue (AF) at C-3 showed highest TOP1 activity (IC_{50}: 23.8 μM), **23** with a trisaccharide residue (AFU) at C-3 showed moderate activity (IC_{50} >100 μM), and **25** without any sugar moiety had no any activity.

The above structure–activity relationship investigations showed that triterpenoid glycosides exhibiting TOP1 activity share the common feature of a conjugated keto group (e.g., 5-carbon acyl groups). Fomitellic acids bearing a conjugated keto group at C-3 showed TOP1 activity with an IC_{50} of 60 μM, while triterpenoids lacking a keto group did not show any activity.[13] However, some compounds bearing a conjugated keto group may not demonstrate TOP1 inhibition because sugar moieties impact activity. Without such a conjugated keto group, ginsenosides do not significantly inhibit TOP1. In this case, the impact of the sugar moiety on TOP1 inhibition seems minimal. For example, both F_2 (**70**) bearing one glucopyranosyl at C-3 and Rg_3 (**71** or **72**) bearing two glucopyranosyl moieties are inactive against TOP1 (Table S3).

Acknowledgments

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Supplementary data

Supplementary data (Tables S1–S3) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.063.

References and notes

1. Francis, G.; Kerem, Z.; Makkar, H. P. S.; Becker, K. *Br. J. Nutr.* **2002**, *88*, 587.
2. Vincken, J.; Heng, L.; de Groot, A.; Gruppen, H. *Phytochemistry* **2007**, *68*, 275.
3. (a) Liang, B.; Tian, J. K.; Xu, L. Z.; Yang, S. L. *Chem. Pharm. Bull.* **2006**, *54*, 1380; (b) Fu, G. M.; Liu, Y.; Yu, S. S.; Huang, X. Z.; Hu, Y. C.; Chen, X. G.; Zhang, F. R. *J. Nat. Prod.* **2006**, *69*, 1680; (c) Diome, C.; Mitaine-Offer, A. C.; Miyamoto, T.; Delaude, C.; Mirjolet, J. F.; Duchamp, O.; Lacaille-Dubois, M. A. *J. Nat. Prod.* **2007**, *70*, 1680; (d) Mujoo, K.; Haridas, V.; Hoffmann, J. J.; Wächter, G. A.; Hutter, L. K.; Lu, Y. L.; Blake, M. E.; Jayatilake, G. S.; Bailey, D.; Mills, G. B.; Gutterman, J. U. *Cancer Res.* **2001**, *61*, 5486; (e) Seo, Y.; Berger, J. M.; Hoch, J.; Neddermann, K. M.; Bursuker, I.; Mamber, S. W.; Kingston, D. G. I. *J. Nat. Prod.* **2002**, *65*, 65; (f) Zhang, Z. Z.; Li, S. Y.; Zhang, S. M.; Gorenstein, D. *Phytochemistry* **2006**, *67*, 784; (g) Yuan, W.; Li, S. Y.; Ownby, S.; Zhang, Z. Z.; Wang, P.; Zhang, W. L.; Beasley, R. S. *Planta Med.* **2007**, *73*, 1304.
4. Lacaille-Dubois, M. *Stud. Nat. Prod. Chem.* **2005**, *32*, 209.
5. Butler, M. S. *Nat. Prod. Rep.* **2005**, *22*, 162.
6. Hostettman, K.; Marston, A. *Chemistry and Pharmacology of Natural Products: Saponins*; Cambridge University Press: Cambridge, 1995.
7. (a) Bailly, C. *Curr. Med. Chem.* **2000**, *7*, 39; (b) Zhang, Z. Z.; Li, S. Y. *Phytochemistry* **2007**, *68*, 2075; (c) Chan, P. K. *Biochem. Pharmacol.* **2007**, *73*, 341.
8. Haridas, V.; Higuchi, M.; Jayatilake, J. U. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 5821.
9. De Tommasi, N.; Autore, G.; Bellino, A.; Pinto, A.; Pizza, C.; Sorrentino, R.; Venturella, P. *J. Nat. Prod.* **2000**, *63*, 308.
10. (a) Zhang, Z. Z.; Li, S. Y.; Zhang, S. M. *Planta Med.* **2005**, *71*, 355; (b) Zhang, Z. Z.; Li, S. Y.; Ownby, S.; Wang, P.; Yuan, W.; Zhang, W. L.; Beasley, R. S. *Phytochemistry* **2008**, *69*, 2070; (c) Zhang, Z. Z.; Li, S. Y.; Zhang, S. M.; Liang, C.; Gorenstein, D.; Beasley, R. S. *Planta Med.* **2004**, *70*, 1216.
11. Mizushina, Y.; Iida, A.; Ohta, K.; Sugawara, F.; Sakaguchi, K. *Biochem. J.* **2000**, *350*, 757.
12. Webb, M. R.; Ebeler, S. E. *Anal. Biochem.* **2003**, *321*, 22.
13. Liu, L. W.; Ye, G. C. *Chin. J. Cancer Prev. Treat.* **2004**, *11*, 957.
14. Fostel, J.; Montgomery, D. *Antimicrob. Agents Chemother.* **1995**, *39*, 586.
15. Das, B. B.; Sen, N.; Roy, A.; Dasgupta, S. B.; Ganguly, A.; Mohhanta, B. C.; Dinda, B.; Majumder, H. K. *Nucleic Acids Res.* **2006**, *34*, 1121.
16. Ray, S.; Majumder, H. K.; Chakravarty, A. K.; Mukhopadhyay, S.; Gil, R. R.; Cordell, G. A. *J. Nat. Prod.* **1996**, *59*, 27.
17. Chowdhury, A. R.; Sharma, S.; Mandal, S.; Goswami, A.; Mukhopadhyay, S.; Majumder, H. K. *Biochem. J.* **2002**, *366*, 653.
18. Zhang, L. T.; Zhang, Y. W.; Takaishi, Y.; Duan, H. Q. *Chin. Chem. Lett.* **2008**, *19*, 190.
19. Tian, J. K.; Xu, L. Z.; Zou, Z. M.; Yang, L. S. *Chem. Pharm. Bull.* **2006**, *54*, 567.
20. Ahn, B. Z.; Baik, K. U.; Kweon, G. R.; Lim, K.; Hwang, B. D. *J. Med. Chem.* **1995**, *38*, 1044.
21. Zahir, A.; Jossang, A.; Bodo, B. *J. Nat. Prod.* **1996**, *59*, 701.
22. Webb, M. R.; Ebeler, S. E. *Biochem. J.* **2004**, *384*, 527.