Anti-Inflammatory, Anti-Tumor-Promoting, and Cytotoxic Activities of Constituents of Marigold (*Calendula officinalis*) Flowers

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Ten oleanane-type triterpene glycosides, **1−10**, including four new compounds, calendulaglycoside A 6′-*O*-methyl ester (**2**), calendulaglycoside A 6′-*O*-*n*-butyl ester (**3**), calendulaglycoside B 6′-*O*-*n*-butyl ester (**5**), and calendulaglycoside C 6′-*O*-*n*-butyl ester (**8**), along with five known flavonol glycosides, **11−15**, were isolated from the flowers of marigold (*Calendula officinalis*). Upon evaluation of compounds **1−9** for inhibitory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation (1 μg/ear) in mice, all of the compounds, except for **1**, exhibited marked anti-inflammatory activity, with ID_{50} values of 0.05−0.20 mg per ear. In addition, when **1−15** were evaluated against the Epstein−Barr virus early antigen (EBV-EA) activation induced by TPA, compounds **1−10** exhibited moderate inhibitory effects (IC_{50} values of 471−487 mol ratio/32 pmol TPA). Furthermore, upon evaluation of the cytotoxic activity against human cancer cell lines in vitro in the NCI Developmental Therapeutics Program, two triterpene glycosides, **9** and **10**, exhibited their most potent cytotoxic effects against colon cancer, leukemia, and melanoma cells.
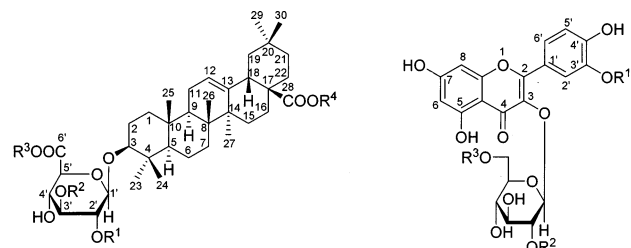
Calendula officinalis L. (Asteraceae) is commonly called (pot) marigold. The dried flower heads or the dried ligulate flowers ("Calendulae Flos") have been used for their anti-inflammatory, antipyretic, antitumor, and cicatrizing effects.[1] It has been reported that extracts of marigold show anti-HIV and hypoglycemic activities, gastric emptying inhibitory activity, and gastroprotective effects.[2,3] In addition, triterpene fatty acid esters isolated from this flower extract showed anti-inflammatory and antiedematous activities.[4−6]

In the course of our search for potential bioactive compounds from natural sources,[7,8] we have demonstrated that various mono-, di-, and trihydroxylated triterpenoids and *syn*-alkane-6,8-diols isolated from extracts of Asteraceae flowers, including the flowers of marigold (*Calendula officinalis* L.), exhibited marked anti-inflammatory and anti-tumor-promoting effects.[9−14] We now report the isolation and characterization of four new (**2**, **3**, **5**, and **8**) and six known oleanane-type triterpene glycosides (**1**, **4**, **6**, **7**, **9**, and **10**), along with five known flavonol glycosides (**11−15**), from an *n*-butanol (*n*-BuOH)-soluble fraction of the methanol (MeOH) extract of *C. officinalis* flowers. Inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice for nine triterpene glycosides, **1−9**, and on Epstein−Barr virus early antigen (EBV-EA) activation induced by TPA for all compounds **1−15** were evaluated as a preliminary screen for their potential cancer chemopreventive activities. In addition, the cytotoxic activity of two triterpene glycosides, **9** and **10**, against a 60-cell-line human cancer panel is reported.

Results and Discussion

Ten triterpene glycosides, calendulaglycoside A (**1**),[15,16] calendulaglycoside A 6′-*O*-methyl ester (**2**), calendulaglycoside A 6′-*O*-*n*-butyl ester (**3**), calendulaglycoside B (**4**),[15,16] calendulaglycoside B 6′-*O*-*n*-butyl ester (**5**), calendulaglycoside C (**6**),[15,16] calendulaglycoside C 6′-*O*-methyl ester (**7**),[17,18] calendulaglycoside C 6′-*O*-*n*-butyl ester (**8**),[17,18] calenduloside F 6′-*O*-*n*-butyl ester (**9**),[17] and calenduloside G 6′-*O*-methyl ester (**10**),[17,18] and five flavonol

Chart 1

	R[1]	R[2]	R[3]	R[4]
1	Glc	Gal	H	Glc
2	Glc	Gal	Me	Glc
3	Glc	Gal	*n*−Bu	Glc
4	Glc	Gal	H	H
5	Glc	Gal	*n*−Bu	H
6	H	Gal	H	Glc
7	H	Gal	Me	Glc
8	H	Gal	*n*−Bu	Glc
9	H	H	*n*−Bu	Glc
10	H	Gal	Me	H

	R[1]	R[2]	R[3]
11	Me	Rha	H
12	Me	Rha	Rha
13	Me	H	Rha
14	H	H	H
15	H	H	Rha

Gal = β-D-galactopyranosyl
Glc = β-D-glucopyranosyl
Rha = α-L-rhamnopyranosyl
n-Bu = *n*-butyl
Me = methyl

glycosides, isorhamnetin 3-*O*-neohesperidoside (**11**),[19] isorhamnetin 3-*O*-2[G]-rhamnosylrutinoside (**12**),[20] isorhamnetin 3-*O*-rutinoside (**13**),[21] quercetin 3-*O*-glucoside (**14**),[22] and quercetin 3-*O*-rutinoside (**15**), were isolated and characterized from an *n*-BuOH-soluble fraction of a MeOH extract of marigold flowers in this study. Among these, **2**, **3**, **5**, and **8** are new compounds, and characterization and spectroscopic analysis of these compounds were performed by data comparison with literature values.[15−18] The ^{13}C NMR data for these compounds are shown in Table 1. Identification of all other compounds, except for **15**, was performed by ^{1}H NMR and MS comparison with the corresponding compounds in the literature. Identification of **15** was undertaken by direct comparison with a reference compound.

Compound **2** gave a sodiated molecular ion at *m/z* 1155.5600 [M + Na][+], indicating a molecular weight of 1132, in agreement with the formula $C_{55}H_{88}O_{24}$ found by HRESIMS. The ^{1}H and ^{13}C NMR spectra of **2** were almost superimposable with those of calendulaglycoside A (**1**)[15,16] except that the former showed additional *O*-methyl signals [δ_H 3.72 (s); δ_C 52.2 (q)], suggesting that **2** is a methyl ester derivative of **1**. The *O*-methyl group was located at the C-6 carboxyl group of a glucuronyl moiety, since **2**

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Table 1. ^{13}C NMR Spectroscopic Data (δ values, 150 MHz, pyridine-d_5) of Compounds **2**, **3**, **5**, and **8**

carbon	2	3	5	8
oleanolic acid				
1	38.6	38.7	38.6	38.7
2	26.5	26.5	26.5	26.6
3	89.8	89.7	89.7	89.3
4	39.6	39.6	39.6	39.5
5	55.8	55.8	55.7	55.7
6	18.5	18.5	18.5	18.5
7	33.1	33.1	33.2	33.2
8	39.9	39.9	39.7	39.9
9	48.0	48.0	48.0	48.0
10	36.9	36.9	36.9	37.0
11	23.8	23.8	23.7	23.8
12	123.1	123.3	122.5	123.3
13	144.1	144.1	144.8	144.2
14	42.2	42.2	42.1	42.2
15	28.3	28.3	28.3	28.3
16	23.4	23.4	23.8	23.4
17	47.0	47.0	46.6	47.0
18	41.8	41.8	41.9	41.8
19	46.9	46.2	46.5	46.2
20	30.8	30.7	30.9	30.8
21	34.0	34.0	34.2	34.0
22	32.6	32.6	33.2	32.6
23	27.9	27.9	27.9	28.0
24	16.6	16.6	16.6	16.9
25	15.5	15.5	15.4	15.5
26	17.5	17.5	17.4	17.5
27	26.1	26.1	26.2	26.1
28	176.4	176.4	180.1	176.4
29	33.1	33.1	33.3	33.2
30	23.7	23.7	23.8	23.7

carbon	2	3	5	8
3-O-β-D-glucuronyl				
1'	105.2	105.2	105.3	106.8
2'	78.7	78.8	78.8	74.1
3'	87.6	87.5	87.5	87.4
4'	71.5	71.4	71.4	71.3
5'	76.5	76.5	76.5	76.7
6'	169.9	169.4	169.4	169.7
R^3 (Me)	52.2			
R^3 (n-Bu)		65.1	65.1	65.1
		30.8	30.8	30.9
		19.2	19.2	19.2
		13.7	13.7	13.7
R^1 (Glc)				
1''	103.9	103.9	103.9	
2''	76.3	76.3	76.3	
3''	77.8	77.8	77.7	
4''	72.6	72.6	72.5	
5''	78.6	78.6	78.5	
6''	63.4	63.4	63.4	
R^2 (Gal)				
1'''	105.2	105.3	105.1	106.5
2'''	72.9	72.9	72.9	73.1
3'''	75.3	75.3	75.3	75.1
4'''	70.1	70.1	70.1	70.1
5'''	77.4	77.3	77.3	77.3
6'''	61.9	61.9	61.9	62.0
R^4 (Glc)				
1''''	95.8	95.8		95.8
2''''	74.2	74.2		74.1
3''''	79.3	79.3		79.3
4''''	71.2	71.2		71.2
5''''	78.9	78.9		78.9
6''''	62.3	62.3		62.3

exhibited ^{13}C NMR signals at C-4', C-5', C-6', and for a *O*-methyl group, consistent with the corresponding signals of calendulaglycoside C 6'-*O*-methyl ester (**7**).[17,18] Hence, the structure of **2** was determined as calendulaglycoside A 6'-*O*-methyl ester {28-*O*-β-D-glucopyranosyloleanolic acid 3-*O*-β-D-glucopyranosyl(1→2)-[β-D-galactopyranosyl(1→3)]-β-D-glucuronopyranoside-6-*O*-methyl ester}.

Compound **3** was assigned the molecular formula $C_{58}H_{94}O_{24}$ (HRESIMS *m/z* 1197.6119 [M + Na]$^+$), corresponding to three CH_2 groups (42 mass units) more than **2**. The ^1H and ^{13}C NMR spectra of **3** were superimposable with those of **2** except for the resonances of an *O*-n-butyl group [δ_H 0.78 (t); δ_C 65.1 (t), 30.8 (t), 19.2 (t), and 13.7 (q)] in **3** instead of a *O*-methyl group in **2**, suggesting that the carboxyl group of a glucuronyl moiety of **3** is esterified with *n*-BuOH. This was supported by a comparison of the NMR signals for C-6' and the *O*-n-butyl moiety of **3** with the corresponding signals of calenduloside F 6'-*O*-n-butyl ester (**9**).[17] Accordingly, compound **3** was proposed as calendulaglycoside A 6-*O*-n-butyl ester {28-*O*-β-D-glucopyranosyloleanolic acid 3-*O*-β-D-glucopyranosyl(1→2)-[β-D-galactopyranosyl(1→3)]-β-D-glucuronopyranoside-6-*O*-n-butyl ester}.

Compound **5** exhibited a [M + Na]$^+$ peak at *m/z* 1035.5547 in the HRESIMS, corresponding to a molecular formula of $C_{52}H_{84}O_{19}$. The NMR spectra of **5** were very similar to those of calendulaglycoside B (**4**)[15,16] except that the former showed additional signals consistent with the presence of an *O*-n-butyl unit [δ_H 0.78 (t); δ_C 65.1 (t), 30.8 (t), 19.2 (t), and 13.7 (q)], suggesting that **5** is an *n*-butyl ester derivative of **4**. The ^{13}C NMR signals of the 3-*O*-glycosyl moiety of **5** were good in agreement with those of **3**, indicating that the glucuronyl moiety of **5** is esterified at C-6' with *n*-BuOH. Hence, the structure of **5** was assigned as calendulaglycoside B 6'-*O*-n-butyl ester {oleanolic acid 3-*O*-β-D-glucopyranosyl(1→2)-[β-D-galactopyranosyl(1→3)]-β-D-glucuronopyranoside-6-*O*-n-butyl ester}.

Compound **8** gave a [M + Na]$^+$ ion, 56 mass units more than calendulaglycoside C (**6**) (corresponding to four CH_2 groups), in the HRESIMS at *m/z* 1035.5574, consistent with a molecular formula of $C_{52}H_{84}O_{19}$. Its ^1H and ^{13}C NMR spectra were almost superimposable with those of **6**[15,16] except for the presence of signals for an additional *O*-n-butyl group [δ_H 0.78 (t); δ_C 65.1 (t), 30.9 (t), 19.2 (t), and 13.7 (q)]. Comparison of the NMR signals of the glucuronyl moiety of **8** with those of compounds **3** and **5** (Table 1) and **9**[17] permitted the assignment of the additional unit as an *n*-butyl ester at C-6' of the glucuronyl moiety of **8**. Hence, the structure of **8** was established as calendulaglycoside C 6'-*O*-n-butyl ester [28-*O*-β-D-glucopyranosyloleanolic acid 3-*O*-β-D-galactopyranosyl-(1→3)-β-D-glucuronopyranoside-6-*O*-n-butyl ester].

The inhibitory effects on TPA-induced inflammation in mice of the MeOH extract, ethyl acetate (EtOAc)-, *n*-BuOH-, and H_2O-soluble fractions (Table S1, Supporting Information), and nine triterpene glycosides, **1**−**9** (Table 2), were evaluated. The EtOAc-soluble fraction exhibited the most potent inhibition (84% inhibition at 1.0 mg/ear) among the extract and fractions tested (Table S1, Supporting Information). This fraction was considered to contain triterpene fatty acid esters, which were reported to have marked anti-inflammatory effects.[6,11] All of the triterpene glycosides tested showed potent inhibitory effects with ID$_{50}$ values of 0.05−0.32 mg/ear and were almost comparable with or more inhibitory than the positive control anti-inflammatory agent indomethacin (ID$_{50}$ = 0.3 mg/ear). Among them, six compounds, **2**−**5**, **8**, and **9**, exhibited fairly strong inhibitory effects (0.05−0.07 mg/ear), which were almost the same order of potency as that of a second anti-inflammatory agent, hydrocortisone (ID$_{50}$ = 0.03 mg/ear). These triterpene glycosides therefore contribute to the anti-inflammatory activity of the *n*-BuOH-soluble fraction of the MeOH extract of marigold flowers. The bisdesomosides calendulaglycosides A (**1**) and C (**6**), which esterified with MeOH (**2**, **7**) or with *n*-BuOH (**3**, **8**) at the 6'-carboxyl group of the glucuronic acid moiety, showed increased inhibitory effects, suggesting that esterification at this position might enhance the anti-inflammatory effects of the bisdesmosides. The inhibitory effect against TPA-induced inflammation has been demonstrated to closely parallel that of the

Table 2. Inhibitory Effects of Compounds **1**−**15** on TPA-Induced Inflammation in Mice and on Induction of the Epstein−Barr Virus Early Antigen

	inhibition of inflammation $ID^{50\,b}$ (mg/ear)	percentage of EBV-EA induction[a]				IC_{50} (mol/ratio/ 32 pmol TPA)
compound		concentration (mol ratio/TPA)				
		1000	500	100	10	
calendulagycoside A (**1**)	0.32	15.8 (70)	52.3 (>80)	75.3 (>80)	75.3 (>80)	484
calendulagycoside A 6′-*O*-*n*-methyl ester (**2**)	0.06	16.0 (70)	53.6 (>80)	77.8 (>80)	100 (>80)	487
calendulagycoside A 6′-*O*-*n*-butyl ester (**3**)	0.06	10.6 (70)	43.2 (>80)	71.6 (>80)	100 (>80)	480
calendulagycoside B (**4**)	0.05	5.1 (70)	40.3 (>80)	69.2 (>80)	100 (>80)	470
calendulagycoside B 6′-*O*-*n*-butyl ester (**5**)	0.06	11.0 (70)	49.3 (>80)	71.0 (>80)	100 (>80)	473
calendulagycoside C (**6**)	0.20	13.2 (70)	51.0 (>80)	73.1 (>80)	100 (>80)	475
calendulagycoside C 6′-*O*-*n*-methyl ester (**7**)	0.10	14.4 (70)	52.1 (>80)	74.3 (>80)	100 (>80)	480
calendulagycoside C 6′-*O*-*n*-butyl ester (**8**)	0.06	5.4 (70)	41.5 (>80)	70.1 (>80)	100 (>80)	471
calenduloside F 6′-*O*-*n* butyl ester (**9**)	0.07	10.1 (70)	48.1 (>80)	70.0 (>80)	100 (>80)	479
calenduloside G6'-*O*-methyl ester (**10**)		10.0 (70)	47.2 (>80)	71.2 (>80)	100 (>80)	479
isorhamnetin 3-*O*-neohesperidoside (**11**)		14.1 (70)	60.0 (>80)	81.1 (>80)	100 (>80)	574
isorhamnetin 3-*O*-2^G-rhamnosylrutinoside (**12**)		19.6 (70)	64.8 (>80)	86.0 (>80)	100 (>80)	592
isorhamnetin 3-*O*-rutinoside (**13**)		16.3 (70)	62.4 (>80)	84.3 (>80)	100 (>80)	585
quercetin 3-*O*-glucoside (**14**)		14.1 (70)	58.0 (>80)	78.5 (>80)	100 (>80)	570
quercetin 3-*O*-rutinoside (**15**)		16.2 (70)	60.1 (>80)	81.1 (>80)	100 (>80)	578
indomethacin	0.30					
hydrocortosone	0.03					
β-carotene		8.6 (70)	34.2 (>80)	82.1 (>80)	100 (>80)	397

[a] ID_{50}: 50% inhibitory does. [b] Values represent percentages relative to the positive control value. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are the viability percentages of Raji cells.

inhibition of tumor promotion in two-stage carcinogenesis initiated by 7,12-dimethylbenz[*a*]anthracene (DMBA) and TPA, a well-known promoter, in a mouse skin model;[7,26] thus, the triterpene glycosides, i.e., **1**−**9**, from marigold flowers might be expected to be potent anti-tumor promoters in the same animal model and deserve to be further investigated in this manner.

The inhibitory effect on EBV-EA activation induced by TPA was further examined as a preliminary evaluation of the potential antitumor-promoting activities of the MeOH extract, EtOAc-, *n*-BuOH- and H_2O-soluble fractions (Table S1, Supporting Information), and 10 triterpene glycosides, **1**−**10**, and five flavonol glycosides, **11**−**15**, isolated from the *n*-BuOH-soluble fraction (Table 2). The MeOH extract and the EtOAc-, *n*-BuOH-, and H_2O-soluble fractions showed inhibitory effects against EBV activation with 27−33% inhibition at 10 μg/mL (Table S1, Supporting Information). Ten triterpene glycosides, **1**−**10**, showed moderate inhibitory effects, with IC_{50} values of 471−487 mol ratio/32 pmol TPA, while preserving the high viability of Raji cells, which were somewhat less potent than the vitamin A precursor studied widely in cancer chemoprevention animal models, β-carotene (IC_{50} = 397 mol ratio/32 pmol TPA). On the other hand, the five flavonol glycosides, **11**−**15**, exhibited only weak inhibitory effects, with IC_{50} values of 570−592 mol ratio/32 pmol TPA.

The cytotoxic activities of calendulaglycoside B (**4**), calendula-glycoside B 6′-*O*-*n*-butyl ester (**5**), calenduloside F 6′-*O*-*n*-butyl ester (**9**), and calenduloside G 6′-*O*-methyl ester (**10**) were evaluated in the National Cancer Institute 60 human cancer cell line panel.[25,26] Among the four compounds tested, potent cytotoxicity was observed for compounds **9** and **10**, as shown in Table S2 (Supporting Information) as concentrations that yield 50% growth inhibition (GI_{50}). Compound **9** showed GI_{50} values of less than 10 μM against all of the cancer cells tested except for leukemia (CCRF-CEM: GI_{50} = 23.1 μM), renal (CAKI-1: 17.2 μM; UO-31: 12.7 μM), and breast (NCI/ADR-RES: >50 μM) cells. Compound **9** showed the most potent cytotoxicity against leukemia (MOLT-4 and RPMI-8226), colon (HCC-2998), and melanoma (LOX IMVI, SK-MEL-5, and UACC-62) cells, with GI_{50} values of 0.77−0.99 μM. Compound **10** exhibited GI_{50} values less than 20 μM against all of the cancer cells tested, except for ovarian (IGROV1: GI_{50} = 20.1 μM) and renal (UO-31: 33.3 μM) cells.

In conclusion, 10 triterpene glycosides, **1**−**10**, isolated from an *n*-BuOH-soluble fraction of a MeOH extract of marigold flowers, showed potent anti-inflammatory effects in the mouse ear edema

assay, and these compounds are worthy of further study as potential inhibitors of tumor promotion (potential cancer chemopreventive agents). In addition, two triterpene glycosides, **9** and **10**, exhibited their most potent cytotoxic activities against colon cancer, leukemia, and melanoma cells. These triterpene glycosides are expected to be responsible, in part, for the cytotoxicity of the extracts of marigold flowers.[27] The natural occurrence of triterpene glycosides possessing an *n*-butyl esterified glucuronic acid moiety in the sugar portion is extremely rare, and only **8** and two other related oleanolic acid glycosides isolated from the root bark of *Aralia armata* have been reported previously as natural products.[17] The four *n*-butyl esters of oleanolic acid glycosides, **3**, **5**, **8**, and **9**, isolated in this study, which exhibited potent biological activities in the assay systems used in this study, are considered to be genuine natural products and not artifacts formed during extraction by *n*-BuOH. This is because of a supplementary experiment in which compound **1** was treated with a slightly acidic (0.001% (w/v) H_2SO_4) *n*-BuOH solution (70 °C for 2 h), resulting in the detection of only unreacted **1** without the formation of esterified **3** in the reaction mixture.[28]

Experimental Section

General Experimental Procedures. Crystallizations were performed in MeOH, and melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1030 polarimeter in MeOH at 25 °C. IR spectra were recorded in KBr disks. NMR spectra were recorded with a JEOL LA-500 (¹H, 500 MHz; ¹³C, 125 MHz) spectrometer in pyridine-d_5 with tetramethylsilane as an internal standard. HRESIMS were recorded on an Agilent 1100 LC/MSD TOF (time-of-flight) system [ionization mode: positive; nebulizing gas (N_2) pressure: 35 psig; drying-gas (N_2): flow, 12 L/min, temp, 325 C; capillary voltage: 3000 V; fragmentor voltage: 225 V]. Silica gel (silica gel 60, 230−400 mesh, Merck) and Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) were used for open column chromatography. Reversed-phase prepara-tive HPLC was carried out on a 25 cm × 10 cm i.d. C_{18} silica column (Pegasil ODS II column; Senshu Scientific Co., Ltd., Tokyo, Japan) at 25 °C with MeOH−H_2O (4:1; 2 mL/min; HPLC system I) or MeOH−H_2O (9:11; 2 mL/min; HPLC system II) as mobile phase.

Plant Material. Dried marigold flowers (*Calendula officinalis* L.) cultivated in Egypt and harvested in April 2005 were purchased at Herbal K Corporation (Tokyo). The plant was authenticated by one (K.Y.) of the authors, and a voucher specimen (SM-0606) has been deposited in the College of Pharmacy, Nihon University.

Chemicals and Reagents. Chemicals were purchased as follows: TPA from ChemSyn Laboratories (Lenexa, KS), quercetin, quercetin

3-*O*-rutinoside, indomethacin, hydrocortisone, and β-carotene from Sigma Chemical Co. (St. Louis, MO), and the EBV cell culture reagents and *n*-butanoic acid from Nacalai Tesque, Inc. (Kyoto, Japan).

Animals. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the College of Pharmacy, Nihon University, Chiba, Japan. Specific pathogen-free female ICR mice were obtained from Japan SLC (Shizuoka, Japan). The animals were housed, five per polycarbonate cage, in an air-conditioned specific pathogen-free room at 24 ± 2 °C. Food and water were available ad libitum.

Extraction and Isolation. The dried flowers of *C. officinalis* (285 g) were extracted with MeOH (2 L) by soaking for one week each at room temperature three times. Evaporation of the combined solvent under reduced pressure provided an extract (152 g). The extract was suspended in water and partitioned successively with EtOAc and *n*-BuOH to yield EtOAc- (42.7 g), *n*-BuOH- (9.5 g), and H$_2$O- (90.0 g) soluble fractions separately. The *n*-BuOH-soluble fraction was subjected to chromatography on a Diaion HP-20 (120 g) column. Step gradient elution was conducted with H$_2$O–MeOH (1:0 → 0:1) to give fractions 1 (3.7 g), 2 (0.3 g), 3 (0.9 g), 4 (2.1 g), and 5 (2.4 g), listed in the increasing order of polarity. A portion (200 mg) of fraction 3 was further separated by HPLC system II to give five flavonol glycosides: **11** [15.0 mg, ca. 0.7% in the *n*-BuOH-soluble fraction as estimated from the weight of the isolated compound; retention time (t_R) 12.4 min], **12** (49.4 mg, 2.3%; t_R 10.5 min), **13** (38.5 mg, 1.8%; t_R 29.8 min), **14** (1.8 mg, 0.1%; t_R 18.0 min), and **15** (1.1 mg, 0.05%; t_R 16.7 min). In addition, a portion (384 mg) of fraction 5 was subjected to HPLC system I, giving 10 triterpene glycosides: **1** (30.4 mg, 0.3%; t_R 2.7 min), **2** (14.4 mg, 0.2%; t_R 5.1 min), **3** (45.0 mg, 0.5%; t_R 14.1 min), **4** (15.4 mg, 0.2%; t_R 12.3 min), **5** (6.4 mg, 0.07%; t_R 50.4 min), **6** (37.6 mg, 0.4%; t_R 3.9 min), **7** (13.0 mg, 0.1%; t_R 6.7 min), **8** (54.0 mg, 0.6%; t_R 17.8 min), **9** (7.4 mg, 0.08%; t_R 24.9 min), and **10** (2.4 mg, 0.03%; t_R 31.5 min).

Calendulaglycoside A 6′-*O*-methyl ester {**28-*O*-β-d-glucopyranosyloleanolic acid 3-*O*-β-d-glucopyranosyl(1→2)-[β-d-galactopyranosyl(1→3)]-β-d-glucuronopyranoside-6-*O*-methyl ester**} (2): fine needles, mp 230–234 °C; [α]$^{25}_D$ +4.6 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3424 (OH), 1739 (COOH) cm^{-1}; ^{1}H NMR (pyridine-*d$_5$*, 500 MHz) δ 0.79 (3H, s, H-25), 0.89 (3H, s, H-29), 0.92 (3H, s, H-30), 1.07 (3H, s, H-26), 1.08 (3H, s, H-24), 1.23 (3H, s, H-23), 1.25 (3H, s, H-27), 3.23 (2H, m, H-3α, H-18β), 3.72 (3H, s, OMe), 4.90 (1H, d, *J* = 7.9 Hz, H-1′), 5.28 (1H, d, *J* = 8.1 Hz, H-1″), 5.41 (1H, br s, H-12), 5.68 (1H, d, *J* = 7.6 Hz, H-1‴), 6.35 (1H, d, *J* = 7.8 Hz, H-1⁗); ^{13}C NMR, see Table 1; HRESIMS *m/z* 1155.5600 [M + Na]$^+$ (calcd for C$_{55}$H$_{88}$O$_{24}$-Na, 1155.5563).

Calendulaglycoside A 6′-*O*-*n*-butyl ester {**28-*O*-β-d-glucopyranosyloleanolic acid 3-*O*-β-d-glucopyranosyl(1→2)-[β-d-galactopyranosyl(1→3)]-β-d-glucuronopyranoside-6-*O*-*n*-butyl ester**} (3): fine needles, mp 220–225 °C; [α]$^{25}_D$ +8.0 (*c* 0.4, MeOH); IR (KBr) ν_{max} 3419 (OH), 1737 (COOH) cm^{-1}; ^{1}H NMR (pyridine-*d$_5$*, 500 MHz) δ 0.78 (3H, t, *J* = 7.6 Hz, *n*-butyl ester Me), 0.81 (3H, s, H-25), 0.89 (3H, s, H-29), 0.92 (3H, s, H-30), 1.06 (3H, s, H-26), 1.08 (3H, s, H-24), 1.23 (3H, s, H-23), 1.26 (3H, s, H-27), 3.21 (2H, m, H-3α, H-18β), 4.90 (1H, d, *J* = 7.3 Hz, H-1′), 5.28 (1H, d, *J* = 7.3 Hz, H-1″), 5.42 (1H, br s, H-12), 5.65 (1H, d, *J* = 7.6 Hz, H-1‴), 6.33 (1H, d, *J* = 8.3 Hz, H-1⁗); ^{13}C NMR, see Table 1; HRESIMS *m/z* 1197.6119 [M + Na]$^+$ (calcd for C$_{58}$H$_{94}$O$_{24}$Na, 1197.6119).

Calendulaglycoside B 6′-*O*-*n*-butyl ester {**oleanolic acid 3-*O*-β-d-glucopyranosyl(1→2)-[β-d-galactopyranosyl(1→3)]-β-d-glucuronopyranoside-6-*O*-*n*-butyl ester**} (5): fine needles, mp 193–198 °C; [α]$^{25}_D$ +10.5 (*c* 0.4, MeOH); IR (KBr) ν_{max} 3417 (OH), 1733 (COOH) cm^{-1}; ^{1}H NMR (pyridine-*d$_5$*, 500 MHz) δ 0.78 (3H, t, *J* = 7.6 Hz, *n*-butyl ester Me), 0.79 (3H, s, H-25), 0.96 (3H, s, H-29), 1.01 (3H, s, H-30), 0.98 (3H, s, H-26), 1.07 (3H, s, H-24), 1.25 (3H, s, H-23), 1.31 (3H, s, H-27), 3.27 (2H, m, H-3α, H-18β), 4.93 (1H, d, *J* = 6.6 Hz, H-1), 5.29 (1H, d, *J* = 7.6 Hz, H-1″), 5.46 (1H, br s, H-12), 5.67 (1H, d, *J* = 7.6 Hz, H-1‴); ^{13}C NMR, see Table 1; HRESIMS *m/z* 1035.5547 [M + Na]$^+$ (calcd for C$_{52}$H$_{84}$O$_{19}$Na, 1035.5504).

Calendulaglycoside C 6′-*O*-*n*-butyl ester [**28-*O*-β-d-glucopyranosyloleanolic acid 3-*O*-β-d-galactopyranosyl(1→3)-β-d-glucuronopyranoside-6-*O*-*n*-butyl ester**] (8): fine needles, mp 210–212 °C; [α]$^{25}_D$ +10.5 (*c* 0.4, MeOH); IR (KBr) ν_{max} 3424 (OH), 1735 (COOH) cm^{-1}; ^{1}H NMR (pyridine-*d$_5$*, 500 MHz) δ 0.78 (3H, t, *J* = 7.3 Hz, *n*-butyl ester Me), 0.83 (3H, s, H-25), 0.89 (3H, s, H-29), 0.92 (3H, s, H-30), 1.10 (3H, s, H-26), 0.96 (3H, s, H-24), 1.28 (6H, s,

H-23, H-27), 3.20 (1H, dd, *J* = 2.4, 13.2 Hz, H-18β), 3.33 (1H, dd, *J* = 4.4, 11.5 Hz, H-3α), 4.95 (1H, d, *J* = 7.8 Hz, H-1′), 5.29 (1H, d, *J* = 7.3 Hz, H-1″), 5.43 (1H, br s, H-12), 6.34 (1H, d, *J* = 7.8 Hz, H-1‴); ^{13}C NMR, see Table 1; HRESIMS *m/z* 1035.5574 [M + Na]$^+$ (calcd for C$_{52}$H$_{84}$O$_{19}$Na, 1035.5504).

Assay of TPA-Induced Inflammation Ear Edema in Mice. For the protocol for this in vivo assay, refer to a previous article.[29]

In Vitro EBV-EA Activation Experiment. For the protocol for this in vitro assay, refer to a previous article.[29]

In Vitro Cytotoxicity Assay. A panel of 60 human tumor cell lines derived from seven cancer types (lung, colon, melanoma, renal, ovarian, breast, and leukemia) was used to evaluate compounds at a minimum of five concentrations at 10-fold dilutions starting from a high of 10^{-4} M. A 48 h continuous drug exposure protocol was used, with a sulforhodamine B protein assay used to estimate cell viability or growth. Details of this assay procedure have been reported.[25]

Supporting Information Available: Tables of biological activities of *C. officinalis* extracts and of the cytotoxicity of compounds **4**, **5**, **9**, and **10** against a panel of cancer cell lines. This information is available free of charge via the Internet at http://pubs.acs.org.

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