

Medicinal Flowers. III.¹⁾ Marigold. (1): Hypoglycemic, Gastric Emptying Inhibitory, and Gastroprotective Principles and New Oleanane-Type Triterpene Oligoglycosides, Calendasaponins A, B, C, and D, from Egyptian *Calendula officinalis*

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The methanolic extract and its 1-butanol-soluble fraction from the flowers of *Calendula officinalis* were found to show a hypoglycemic effect, inhibitory activity of gastric emptying, and gastroprotective effect. From the 1-butanol-soluble fraction, four new triterpene oligoglycosides, calendasaponins A, B, C, and D, were isolated, together with eight known saponins, seven known flavonol glycosides, and a known sesquiterpene glucoside. Their structures were elucidated on the basis of chemical and physicochemical evidence. The principal saponin constituents, glycosides A, B, C, D, and F, exhibited potent inhibitory effects on an increase in serum glucose levels in glucose-loaded rats, gastric emptying in mice, and ethanol- and indomethacin-induced gastric lesions in rats. Some structure–activity relationships are discussed.

Key words calendasaponin; *Calendula officinalis*; marigold; hypoglycemic activity; gastroprotective activity; gastric emptying inhibitory activity

The Compositae plant, *Calendula (C.) officinalis* L., which is commonly called “Marigold”, is widely cultivated as an ornamental. The flowers of this plant are very important in European and western Asian folk medicines and are used to treat inflammatory conditions of internal organs, gastrointestinal ulcers, and dysmenorrhea and as a diuretic and diaphoretic in convulsions. This natural medicine is also known to be used externally for inflammation of the oral and pharyngeal mucosa, wounds, and burns. As chemical constituents of *C. officinalis*, some triterpenes, triterpene oligoglycosides, and flavonol glycosides have been obtained from the flowers.²⁾

In the course of our studies on the bioactive principles of medicinal flowers,^{1,3)} we found that the methanolic extract and its 1-butanol-soluble fraction from the dried flowers of Egyptian *C. officinalis* showed inhibitory effects on the increase of serum glucose levels in glucose-loaded mice, gastric emptying, and ethanol-induced gastric lesions. From the 1-butanol-soluble fraction, we have isolated four new triterpene oligoglycosides called calendasaponins A (1), B (2), C (3), and D (4), two new ionone glucosides named officinosides A and B, and two new sesquiterpene glycosides termed officinosides C and D. This paper deals with the structural elucidation of calendasaponins (1–4). In addition, we describe the inhibitory effects of the glycoside fraction and principal saponin constituents on the increase of serum glucose levels in glucose-loaded mice, gastric emptying, and ethanol- and indomethacin-induced gastric lesions.

The dried flowers of *C. officinalis* cultivated in Egypt were extracted with methanol under reflux. The methanol extract was partitioned into an ethyl acetate–water mixture to furnish the ethyl acetate-soluble fraction and water phase. The water phase was further extracted with 1-butanol to give a 1-butanol-soluble fraction (the glycoside fraction) and water-soluble fraction. The methanolic extract was found to exhibit inhibitory activity on the increase of serum glucose levels in glucose-loaded mice after a single oral administration of

1000 mg/kg. In the same bioassay, the 1-butanol-soluble fraction showed potent activity at a lower dose (500 mg/kg), as shown in Table 1. As shown in Table 2, the methanolic extract also inhibited gastric emptying in carboxymethyl cellulose sodium salt (CMC-Na) meal-loaded mice after an oral application (500, 1000 mg/kg). The 1-butanol-soluble fraction showed a potent inhibitory effect on gastric emptying at lower doses (250, 500 mg/kg). Furthermore, these extracts were found to show a potent gastroprotective effect in rats (Table 3). Namely, the methanolic extract inhibited gastric lesions induced by ethanol after an oral application (100, 200 mg/kg) in rats, while the 1-butanol-soluble fraction was found to completely inhibit ethanol-induced gastric lesions at a 100 mg/kg dose. On the other hand, the methanolic extract and 1-butanol-soluble fraction completely inhibited indomethacin-induced gastric lesions. The 1-butanol-soluble fraction was subjected to normal-phase silica gel column chromatography to provide six fractions (Fr. 1–6). Fractions 2–6 were separated by normal-phase and reversed-phase silica gel column chromatography and HPLC to give calendasaponins A (1, 0.0012%), B (2, 0.0024%), C (3, 0.0011%), and D (4, 0.0056%), officinosides A (0.0013%), B (0.0016%), C (0.0028%), and D (0.0028%), glycosides A (5,^{2f)} 0.090%), B (6,^{2f)} 0.080%), C (7,^{2f)} 0.085%), D (8,^{2f)} 0.060%), D₂ (9,^{2f)} 0.030%), F (10,^{2f)} 0.0091%), calenduloside D (11,⁴⁾ 0.0009%), arvensoside A (12,⁵⁾ 0.0014%), rutin (13,⁶⁾ 0.0025%), quercetin 3-*O*-neohesperidoside (14,⁷⁾ 0.0036%), quercetin 3-*O*-2^G-rhamnosylrutinoside (15,⁸⁾ 0.018%), isorhamnetin 3-*O*-rutinoside (17,⁷⁾ 0.060%), isorhamnetin 3-*O*-neohesperidoside (18,⁷⁾ 0.0099%), isorhamnetin 3-*O*-2^G-rhamnosylrutinoside (19,⁸⁾ 0.024%), and icaricide C₃ (20,¹⁰⁾ 0.0006%).

Structures of Calendasaponins A (1), B (2), C (3), and D (4) Calendasaponin A (1) was obtained as colorless fine crystals of mp 226.5–228.6 °C from MeOH–H₂O. The IR spectrum of 1 showed absorption bands at 1736 cm⁻¹ ascribable to a carbonyl function, and strong absorption bands at

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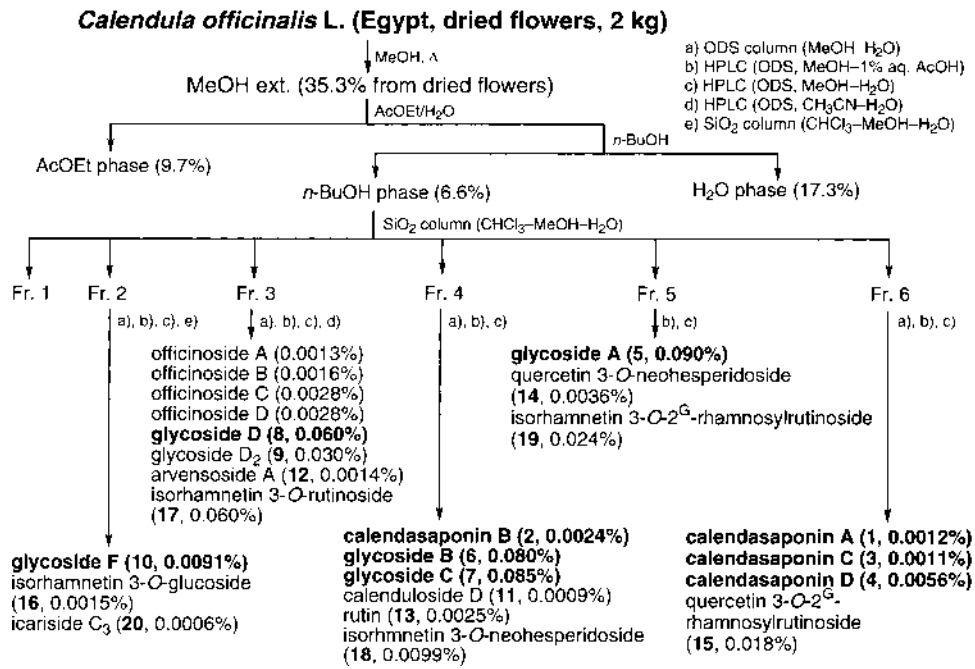


Chart 1

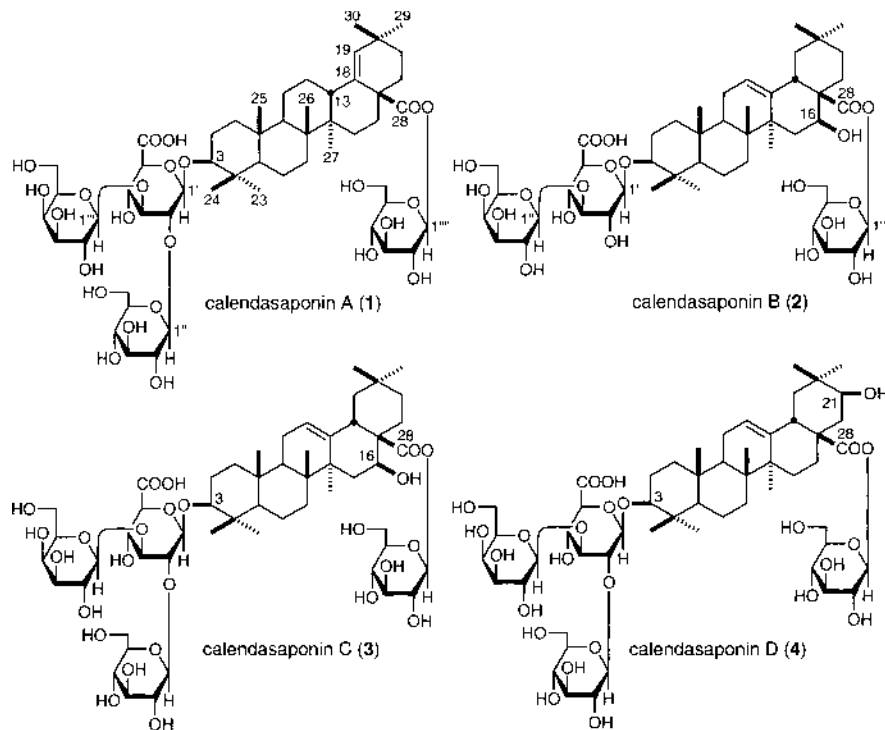


Chart 2

Table 1. Effects of MeOH Extract and *n*-BuOH Fraction from *C. officinalis* on Increase of Serum Glucose Levels in Glucose-Loaded Mice

Treatment	Dose (mg/kg, <i>p.o.</i>)	<i>n</i>	Serum glucose levels (mg/dl)		
			0.5h	1.0h	2.0h
Normal	—	7	109.2±6.1**	127.0±7.9**	136.8±11.0*
Control	—	7	200.9±19.3	175.9±19.3	181.4±13.6
MeOH ext.	1000	7	168.7±18.5	171.0±16.4	148.5±18.7
<i>n</i> -BuOH fr.	500	7	130.0±20.0**	138.9±10.6	126.4±10.8**

Values represent the means±S.E. Significantly different from the control group, **p*<0.05, ***p*<0.01.

3432, 1076, and 1048 cm^{-1} suggestive of an oligoglycosidic structure. In the negative- and positive-ion FAB-MS of **1**, quasimolecular ion peaks were observed at m/z 1117 ($M-H$)⁻ and m/z 1141 ($M+Na$)⁺, respectively, and the molecular formula $C_{54}H_{86}O_{24}$ was determined by high-resolution MS measurement of the quasimolecular ion peak ($M+Na$)⁺. Furthermore, fragment ion peaks at m/z 955 ($M-C_6H_{11}O_5$)⁻, m/z 793 ($M-C_{12}H_{21}O_{10}$)⁻, and m/z 631 ($M-C_{18}H_{31}O_{15}$)⁻,

Table 2. Effects of MeOH Extract and *n*-BuOH Fraction from *C. officinalis* on Gastric Emptying in 1.5% CMC-Na Meal-Loaded Mice

Treatment	Dose (mg/kg, <i>p.o.</i>)	<i>n</i>	GE (%) at 30 min
Control	—	7	86.6±2.4
MeOH ext.	250	7	85.0±1.3
	500	7	69.6±4.8**
	1000	7	58.6±4.8**
<i>n</i> -BuOH fr.	125	7	76.2±4.4
	250	7	57.6±1.4**
	500	7	49.1±3.7**

Values represent the means±S.E. Significantly different from the control group, ***p*<0.01.

which were derived by cleavage of the glycosidic linkages, were observed in the negative-ion FAB-MS of **1**. Acid hydrolysis of **1** with 5% aqueous sulfuric acid (H_2SO_4)–1,4-dioxane (1:1, v/v) liberated moronic acid (**21**)¹¹ as a saponogenol and *D*-glucuronic acid, *D*-glucose, and *D*-galactose, which were identified by GLC¹² of their trimethylsilyl thiazolidine derivatives. The ¹H-NMR (pyridine-*d*₅) and ¹³C-NMR (Table 4) spectra of **1**, which were assigned by various NMR analytical methods,¹³ showed the presence of a moronic acid moiety [δ 2.64 (br d, *J*=ca. 12 Hz, 13-H), 3.25 (dd-like, 3-H), 5.27 (br s, 19-H)], a β -*D*-glucopyranosiduronic acid moiety [δ 4.90 (d, *J*=6.4 Hz, 1'-H)], two β -*D*-glucopyranosyl moieties [δ 5.63 (d, *J*=8.0 Hz, 1''-H), 6.39 (d, *J*=7.9 Hz, 1'''-H)], and a β -*D*-galactopyranosyl moiety [δ 5.27 (d-like, 1''''-H)]. In the HMBC experiments of **1**, long-range correlations were observed between the anomeric proton (1'-H) of the glucopyranosiduronic acid moiety and the 3-carbon of the moronic acid moiety, between the anomeric proton (1''-H) of the glucopyranosyl moiety and the 2'-carbon of the glucopyranosiduronic acid moiety, between the anomeric proton (1'''-H) of the galactopyranosyl moiety and the 3'-carbon of the glucopyranosiduronic acid moiety, and

Table 3. Effects of MeOH Extract and *n*-BuOH Fraction from *C. officinalis* on Ethanol- or Indomethacin-Induced Gastric Lesions in Rats

Treatment	Dose (mg/kg, <i>p.o.</i>)	<i>n</i>	EtOH-induced gastric lesion (mm)	<i>n</i>	Indomethacin-induced gastric lesion (mm)
Control	—	6	134.4±24.3	6	28.2±5.5
MeOH ext.	100	6	46.0±11.9**	6	1.7±1.1**
	200	6	21.3±6.4**	6	3.6±1.3**
<i>n</i> -BuOH fr.	50	6	21.1±11.0**	6	6.5±3.2**
	100	6	2.5±2.5**	6	3.6±1.8**

Values represent the means±S.E. Significantly different from the control group, ***p*<0.01.

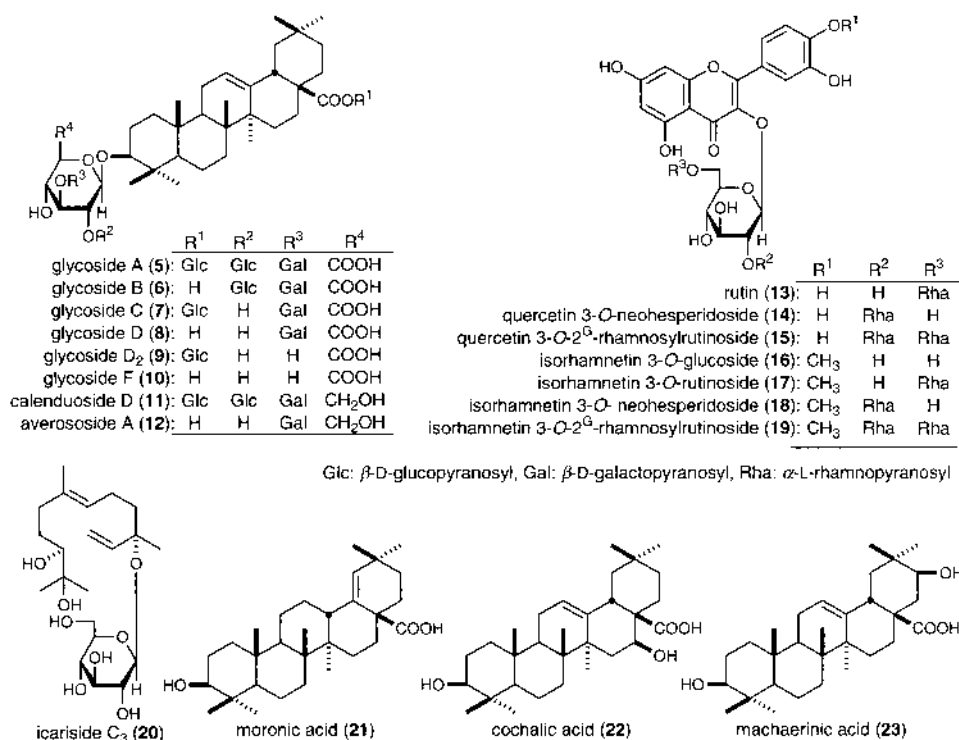


Chart 3

Table 4. ¹³C-NMR Data of Calendasaponins A (1), B (2), C (3), and D (4)

	1	2	3	4		1	2	3	4
C-1	39.1	38.7	38.7	38.6	C-1'	105.2	106.6	105.2	105.0
2	26.7	26.5	26.5	26.5	2'	79.2	74.3	78.9	78.7
3	89.6	89.3	89.6	89.5	3'	88.0	87.6	88.0	87.8
4	39.7	39.5	39.6	39.6	4'	71.9	71.6	71.8	71.6
5	56.0	55.8	55.8	55.7	5'	77.3	77.3	77.3	77.2
6	18.4	18.4	18.5	18.5	6'	171.7	172.6	171.7	171.4
7	35.0	33.1	33.2	33.2	C-1''	103.9	106.2	103.9	103.7
8	41.1	39.9	39.9	39.9	2''	76.4	73.0	76.3	76.2
9	51.4	47.2	47.2	48.0	3''	77.6	75.0	77.6	77.5
10	37.1	36.9	36.9	36.9	4''	72.7	70.2	72.7	72.5
11	21.2	23.8	23.8	23.8	5''	78.6	77.3	78.6	78.5
12	26.3	122.9	122.9	122.9	6''	63.5	62.2	63.5	63.4
13	41.4	143.1	143.1	143.1	C-1'''	105.2	96.1	105.2	105.0
14	43.0	44.5	44.6	42.2	2'''	73.0	74.1	73.0	72.8
15	29.8	38.1	38.3	28.4	3'''	75.3	79.3	75.4	75.2
16	34.0	64.8	64.8	36.7	4'''	70.2	71.2	70.2	70.0
17	48.8	51.4	51.4	41.5	5'''	77.3	78.4	77.3	77.2
18	138.1	44.1	44.0	48.8	6'''	62.1	62.3	62.0	61.9
19	132.8	46.5	46.5	47.1	C-1''''	96.0		96.1	95.7
20	32.3	30.6	30.6	38.2	2''''	74.3		74.2	74.0
21	33.5	33.8	33.8	72.2	3''''	79.2		79.4	79.1
22	33.9	26.4	26.4	24.9	4''''	71.4		71.3	71.1
23	27.9	28.1	28.0	27.9	5''''	79.0		78.5	78.7
24	16.6	16.9	16.7	16.6	6''''	62.5		62.4	62.1
25	16.7	15.6	15.6	15.5					
26	16.3	17.4	17.4	17.5					
27	15.3	26.9	26.9	25.9					
28	175.4	175.6	175.5	175.2					
29	29.3	23.9	23.9	29.6					
30	30.6	33.0	33.0	17.6					

125 MHz, pyridine-*d*₅.

between the anomeric proton (1'''-H) of the glucopyranosyl moiety and the 28-carbon of the moronic acid moiety. On the basis of this evidence, the structure of calendasaponin A was determined to be 28-*O*-β-D-glucopyranosyl moronic acid 3-*O*-β-D-glucopyranosyl(1→2)-[β-D-galactopyranosyl(1→3)]-β-D-glucopyranosiduronic acid (1).

Calendasaponin B (2) was also isolated as colorless fine crystals of mp 245.6–247.0 °C from MeOH–H₂O, and its IR spectrum showed absorption bands due to hydroxyl and carbonyl functions. The negative- and positive-ion FAB-MS of 2 showed quasimolecular ion peaks at *m/z* 971 (M–H)[–] and *m/z* 995 (M+Na)⁺, and the molecular formula C₄₈H₇₆O₂₀ was determined by high-resolution MS measurement. Upon acid hydrolysis with 5% aqueous H₂SO₄–1,4-dioxane, 2 liberated cochalic acid (22),¹⁴ together with D-glucuronic acid, D-galactose, and D-glucose.¹² The ¹H-NMR (pyridine-*d*₅) and ¹³C-NMR (Table 4) spectra¹³ of 2 showed the presence of a cochalic acid moiety [δ 3.27 (dd-like, 18-H), 3.35 (dd-like, 3-H), 4.55 (dd, *J*=5.5, 10.4 Hz, 16-H), 5.44 (br s, 12-H)], a β-D-glucopyranosiduronic acid moiety [δ 4.95 (d, *J*=7.6 Hz, 1'-H)], a β-D-galactopyranosyl moiety [δ 5.23 (d, *J*=7.8 Hz, 1''-H)], and a β-D-glucopyranosyl moiety [δ 6.19 (d, *J*=7.9 Hz, 1'''-H)]. In the HMBC experiment of 2, long-range correlations were observed between the 1'-proton and the 3-carbon, between the 1''-proton and the 3'-carbon, and between the 1'''-proton and the 28-carbon. Furthermore, in the pNOESY experiment, NOE correlations were observed between the 1'-H and the 3-H, between the 1''-H and the 3'-H, and between the 27-H₃ and the 16-H. Based on this evidence, calendasaponin B was determined to be 28-*O*-β-D-glucopyra-

nosyl cochalic acid 3-*O*-β-D-galactopyranosyl(1→3)-β-D-glucopyranosiduronic acid (2).

Calendasaponins C (3) and D (4) were isolated as colorless fine crystals of mp 228.5–230.2 °C and mp 226.9–229.0 °C, respectively. Calendasaponins C (3) and D (4) were found to have the same molecular formula, C₅₄H₈₆O₂₅, which was determined from the quasimolecular ion peaks in their negative-ion FAB-MS [*m/z* 1133 (M–H)[–]] and positive-ion FAB-MS [*m/z* 1157 (M+Na)⁺] and by high-resolution MS measurement. The acid hydrolysis with 5% aqueous H₂SO₄–1,4-dioxane of 3 liberated cochalic acid (22),¹⁴ D-glucuronic acid, D-glucose, and D-galactose.¹² On the other hand, 4 furnished machaerinic acid (23),¹⁵ D-glucuronic acid, D-glucose, and D-galactose upon acid hydrolysis.¹² The proton and carbon signals in ¹H-NMR (pyridine-*d*₅) and ¹³C-NMR (Table 4) spectra¹³ of 3 and 4 were superimposable on those of 1 except for a few signals due to the sapogenol moiety. The NMR data of 3 and 4 showed signals assignable to a cochalic acid moiety [δ 3.27 (dd-like, 3, 18-H), 4.54 (dd, *J*=3.9, 16.8 Hz, 16-H), 5.42 (br s, 12-H)] for 3, and a machaerinic acid moiety [δ 3.27 (dd-like, 3, 18-H), 3.89 (dd, *J*=4.6, 11.6 Hz, 21-H), 5.45 (br s, 12-H)] for 4. Furthermore, in the HMBC experiments of 3 and 4, long-range correlations were observed between the 1'-proton [3: δ 4.92 (d, *J*=7.9 Hz, 1'-H); 4: δ 4.92 (d, *J*=7.5 Hz, 1'-H)] of the glucopyranosiduronic acid moiety and the 3-carbon of the sapogenol moiety, between the 1''-proton [3: δ 5.65 (d, *J*=7.6 Hz, 1''-H); 4: δ 5.64 (d, *J*=7.8 Hz, 1''-H)] of the glucopyranosyl moiety and the 2'-carbon of the glucopyranosiduronic acid moiety, between the 1'''-proton [3: δ 5.27 (d, *J*=7.9 Hz, 1'''-H); 4: δ 5.26 (d,

Table 5. Effects of Glycosides A (5), B (6), C (7), D (8), and F (10) from *C. officinalis* on Increase of Serum Glucose Levels in Glucose-Loaded Rats

Treatment	Dose (mg/kg, <i>p.o.</i>)	<i>n</i>	Serum glucose levels (mg/dl)		
			0.5 h	1.0 h	2.0 h
Normal	—	5	94.6±1.9**	96.6±3.7**	100.4±4.3
Control	—	5	159.0±4.0	135.6±4.4	117.0±12.9
Glycoside A (5)	50	5	174.1±5.7	136.5±2.1	125.0±2.8
Glycoside B (6)	50	5	159.0±6.6	133.3±3.4	114.7±3.7
Glycoside C (7)	50	5	149.7±3.7	121.9±2.6	111.2±6.4
Glycoside D (8)	50	5	116.1±6.8**	131.3±9.2	119.8±5.6
Glycoside F (10)	50	5	106.8±10.2**	119.2±5.7	118.6±5.5
Normal	—	5	74.0±2.4**	94.3±3.3**	86.1±6.5*
Control	—	7	129.7±6.1	118.2±3.1	93.5±4.0
Tolbutamide	25	5	86.0±5.6**	78.9±3.4**	59.5±2.2**
	50	5	61.4±8.9**	67.9±7.2**	47.7±6.2**

Values represent the means±S.E. Significantly different from the control group, **p*<0.05, ***p*<0.01.

J=7.1 Hz, 1^{'''}-H)] of the galactopyranosyl moiety and the 3'-carbon of the glucopyranosiduronic acid moiety, and between the 1^{'''}-proton [3: δ 6.22 (d, *J*=7.9 Hz, 1^{'''}-H); 4: δ 6.28 (d, *J*=8.1 Hz, 1^{'''}-H)] of the glucopyranosyl moiety and the 28-carbon of the sapogenol moiety. Consequently, the structures of calendasaponins C and D were determined to be 28-*O*-β-D-glucopyranosyl cochalic acid 3-*O*-β-D-glucopyranosyl(1→2)-[β-D-galactopyranosyl(1→3)]-β-D-glucopyranosiduronic acid (3) and 28-*O*-β-D-glucopyranosyl machaerinic acid 3-*O*-β-D-glucopyranosyl(1→2)-[β-D-galactopyranosyl(1→3)]-β-D-glucopyranosiduronic acid (4), respectively.

Inhibitory Effects of Principle Saponins (5—8, 10) on the Elevation of Serum Glucose Levels in an Oral Glucose Tolerance Test Recently, we have characterized many saponins, such as elatosides, betavulgarosides, escins, and senegasaponins, which exhibit potent hypoglycemic effect in oral glucose tolerance tests in rats, from *Aralia elata*,¹⁶⁾ *Beta vulgaris*,¹⁷⁾ *Aesculus hippocastanum*,¹⁸⁾ and *Polygala senega* var. *latifolia*.¹⁹⁾ Furthermore, by examination of the structural requirement for hypoglycemic activity, the active saponins were found to be classified into the following three types of structure: 1) oleanen-28-oic acid 3-monodesmosides, 2) acylated polyhydroxyoleanene 3-monodesmosides, and 3) oleanene 3,28-acylated bisdesmosides.²⁰⁾ Since the methanolic extract and 1-butanol-soluble fraction from the flowers of *C. officinalis* showed an inhibitory effect on the increase in serum glucose levels in oral glucose-loaded mice (Table 1), we examined the hypoglycemic activity of the principle saponins, glycosides A (5), B (6), C (7), D (8), and F (10), from the 1-butanol-soluble fraction. As shown in Table 5, two oleanolic acid 3-monodesmosides (8, 10) showed potent hypoglycemic activity after a single oral administration of 50 mg/kg in rats, while two oleanolic acid 3,28-bisdesmosides (5, 7) showed no activity. Contrary to our expectation, an oleanolic acid 3-monodesmoside (6) having a 2'-*O*-β-D-glucopyranosyl moiety showed no activity (Table 5). In a previous paper, we reported as an exception that an oleanolic acid 3-monodesmoside having a 2'-*O*-β-D-glucopyranosyl moiety (28-deglucopyranosyl-chikusetsusaponin V) did not inhibit the increase in serum glucose levels in oral glucose-loaded rats at a dose of 50 mg/kg.²¹⁾ Taking this evidence into account, the 2'-*O*-β-D-glucopyranosyl moiety was assumed to markedly reduce the hypoglycemic activity of oleanolic acid 3-monodesmoside.

Table 6. Effects of Glycosides A (5), B (6), C (7), D (8), and F (10) from *C. officinalis* on Gastric Emptying in 1.5% CMC-Na-Loaded Mice

Treatment	Dose (mg/kg, <i>p.o.</i>)	<i>n</i>	GE (%) at 30 min
Control	—	7	76.3±3.2
Glycoside A (5)	50	7	60.4±7.6
Glycoside C (7)	50	7	72.6±2.6
Control	—	7	88.1±1.7
Glycoside B (6)	12.5	7	77.1±5.1
	25	7	84.8±2.6
	50	7	81.7±2.3
	100	7	64.5±2.1**
Glycoside D (8)	12.5	7	76.2±4.7
	25	7	53.5±4.9**
	50	7	50.9±5.4**
	100	7	45.4±3.7**
Control	—	10	89.9±1.0
Glycoside F (10)	5	8	87.2±1.3
	12.5	8	78.2±2.8*
	25	8	54.4±4.3**
	50	8	38.3±2.8**
Control	—	8	89.4±1.2
Atropine Sulfate monohydrate	5	8	78.7±1.0**
	12.5	8	73.1±3.0**
	20	8	67.7±1.2**

Values represent the means±S.E. Significantly different from the control group, **p*<0.05, ***p*<0.01.

Inhibitory Effects of Principle Saponins (5—8, 10) on Gastric Emptying in CMC-Na Meal-Loaded Mice By investigation of the modes of action for the hypoglycemic activity of saponins, active saponins were found to inhibit gastric emptying and glucose uptake in the small intestine.^{21,22)} In order to confirm the modes of action for the hypoglycemic activity, the effects of principle saponins on gastric emptying were examined. As shown in Table 6, two oleanolic acid 3-monodesmosides (8, 10) with potent hypoglycemic activity significantly inhibited gastric emptying at doses of 12.5, 25, 50, and 100 mg/kg, whereas two oleanolic acid 3,28-bisdesmosides (5, 7) did not exhibit the activity at a dose of 50 mg/kg. Oleanolic acid 3-monodesmoside (6) with a 2'-*O*-β-D-glucopyranosyl moiety did not show the activity at doses of 12.5, 25, and 50 mg/kg, but weak inhibitory activity was observed at a dose of 100 mg/kg. No hypoglycemic activity of 6 may be attributed to its weak inhibitory activity on gas-

Table 7. Effects of Glycosides A (5), B (6), C (7), D (8), and F (10) from *C. officinalis* on Ethanol- or Indomethacin-Induced Gastric Lesions in Rats

Treatment	Dose (mg/kg, p.o.)	n	EtOH-induced gastric lesion (mm)	n	Indomethacin-induced gastric lesion (mm)
Control	—	6	134.4±24.3	6	28.2±5.5
Glycoside A (5)	20	6	88.5±24.4	6	7.0±3.8**
Glycoside B (6)	20	6	25.8±9.6**	6	38.0±9.4
Glycoside C (7)	20	6	74.0±11.2*	6	2.4±1.0**
Glycoside D (8)	20	6	23.6±7.0**	6	8.8±4.1*
Glycoside F (10)	10	6	38.2±8.3**	6	15.4±1.5*
Control	—	7	123.8±21.3	6	22.8±3.3
Omeprazole	2.5	—	—	6	16.1±4.6
	5	—	—	6	4.0±1.6**
	10	6	86.5±11.3	6	1.8±1.2**
	20	6	65.7±9.0**	6	0.3±0.2**
	50	6	60.0±13.1**	—	—

Values represent the means±S.E. Significantly different from the control group, * $p<0.05$, ** $p<0.01$.

tric emptying.

Protective Effects of Principle Saponins (5–8, 10) on Ethanol- or Indomethacin-Induced Gastric Mucosal Lesions in Rats The flowers of *C. officinalis* have been said to be useful for treating gastrointestinal ulcers in traditional medicines, and also, we found that several saponins showed a potent gastroprotective effect²³; therefore we examined the gastroprotective effect of the methanolic extract, 1-butanol-soluble portion, and principle saponins (5–8, 10). By intragastric or subcutaneous administration of ethanol (1.5 mg/rat) or indomethacin (30 mg/kg), marked gross gastric mucosal lesions were induced in rats. These lesions were characterized by multiple hemorrhage red bands (patches) of different sizes along the long axis of the glandular stomach. As shown in Table 7, the oral application (20 mg/kg) of principle saponins (5–8, 10) exhibited protective effects against ethanol-induced gastric lesions in rats. Especially, oleanolic acid 3-monodesmoside (6, 8, 10) more strongly reduced the lengths than a reference drug, omeprazole.

On the other hand, two oleanolic acid 3,28-bisdesmosides (5, 7) and two oleanolic acid 3-monodesmosides (8, 10) showed inhibitory activity on indomethacin-induced gastric mucosal lesions in rats at a dose of 20 mg/kg. The activities of oleanolic acid 3,28-bisdesmosides (5, 7) and an oleanolic acid 3-monodesmoside (8) were much more potent than those of two oleanolic acid 3-monodesmosides (6, 10).

Experimental

The instruments used to obtain physical data and the experimental conditions for chromatography were the same as described in our previous paper.¹⁾

Isolation of Calendasaponins A (1), B (2), C (3), and D (4), Officinoids A, B, C, and D, and Sixteen Known Compounds (5–20) from the Dried Flowers of *C. officinalis* L. The dried flowers of *C. officinalis* (2 kg, cultivated in Egypt) were cut and extracted three times with MeOH under reflux. Evaporation of the solvent under reduced pressure provided the MeOH extract (705 g, 35.3%). The MeOH extract (670 g) was partitioned in an AcOEt–H₂O (1:1, v/v) mixture. The aqueous layer was extracted with *n*-BuOH and removal of the solvent *in vacuo* from the AcOEt-soluble portion, *n*-BuOH-soluble portion, and aqueous phase yielded 194 g (9.7%), 132 g (6.6%), and 345 g (17.3%) of the residue, respectively. The *n*-BuOH extract (121 g) was subjected to normal-phase silica gel column chromatography [3 kg, CHCl₃–MeOH–H₂O (10:3:0.5→7:3:0.5, v/v)→MeOH] to give six fractions [Fr. 1 (2.5 g), 2 (6.0 g), 3 (17.3 g), 4 (38.0 g), 5 (39.0 g), 6 (17.5 g)]. Reversed-phase silica gel column chromatography [Chromatorex ODS DM1020T (Fuji Silysia Co., Ltd., 150 g), MeOH–H₂O (40:60→60:40→80:20→90:10, v/v)→MeOH] of fraction 2 (5.0 g) gave four fractions [Fr. 2-1 (3.0 g), 2-2 (603 mg), 2-3 (488 mg), 2-4 (563 mg)]. Fraction 2-

(603 mg) was purified by HPLC [YMC-Pack ODS-A (250×20 mm i.d., YMC Co., Ltd.), MeOH–H₂O (50:50, v/v)] and normal-phase silica gel column chromatography [BW-200 (Fuji Silysia Co., Ltd., 100 mg), CHCl₃–MeOH–H₂O (15:3:1, lower layer→10:3:1, lower layer, v/v)→MeOH] to give icaricide C₃ (20, 12 mg, 0.0006%) and isorhamnetin 3-*O*-glucoside (16, 29 mg, 0.0015%). Fraction 2-4 (538 mg) was purified by HPLC [MeOH–1% aq. AcOH (70:30, v/v)] to give glycoside F (10, 181 mg, 0.0091%). Fraction 3 (17.0 g) was separated by reversed-phase silica gel column chromatography [500 g, MeOH (40:60→50:50→60:40→70:30→80:20, v/v)→MeOH] to give six fractions [Fr. 3-1 (4.2 g), 3-2 (1.4 g), 3-3 (2.3 g), 3-4 (740 mg), 3-5 (1.6 g), 3-6 (3.5 g)]. Fraction 3-2 (1.4 g) was purified by repeated HPLC [1) MeOH–H₂O (40:60, v/v); 2) CH₃CN–H₂O (15:85, v/v)] to give officinoids A (25 mg, 0.0013%) and B (32 mg, 0.0016%). Fraction 3-3 (1.0 g) was purified by HPLC [MeOH–H₂O (50:50, v/v)] to give isorhamnetin 3-*O*-rutinoside (17, 1.2 g, 0.060%). Fraction 3-4 (740 mg) was purified by HPLC [MeOH–1% aq. AcOH (60:40, v/v)] to give officinoids C (56 mg, 0.0028%) and D (56 mg, 0.0028%). Fraction 3-5 (1.0 g) was purified by repeated HPLC [1) MeOH–1% aq. AcOH (75:25, v/v); 2) MeOH–1% aq. AcOH (70:30, v/v)] to give glycoside D₂ (9, 591 mg, 0.030%) and arvensoside A (12, 28 mg, 0.0014%). Fraction 3-6 (2.0 g) was purified by HPLC [MeOH–1% aq. AcOH (85:15, v/v)] to give glycoside D (8, 1.2 g, 0.060%). Fraction 4 (35 g) was separated by reversed-phase silica gel column chromatography [1.0 kg, MeOH–H₂O (40:60→50:50→60:40→70:30→80:20, v/v)→MeOH] to give five fractions [Fr. 4-1 (5.4 g), 4-2 (6.0 g), 4-3 (480 mg), 4-4 (13.5 g), 4-5 (6.8 g)]. Fraction 4-2 (1 g) was purified by repeated HPLC [1) MeOH–H₂O (50:50, v/v); 2) MeOH–H₂O (45:55, v/v)] to give rutin (13, 50 mg, 0.0025%) and isorhamnetin 3-*O*-neohesperidoside (18, 197 mg, 0.0099%). Fraction 4-3 (480 mg) was purified by HPLC [MeOH–1% aq. AcOH (70:30, v/v)] to give calendasaponin B (2, 48 mg, 0.0024%). Fraction 4-4 (2 g) was purified by repeated HPLC [1) MeOH–1% aq. AcOH (75:25, v/v); 2) MeOH–1% aq. AcOH (70:30, v/v)] to give calendulose D (11, 18 mg, 0.0009%) and glycoside C (7, 1.7 g, 0.085%). Fraction 4-5 (6.8 g) was purified by reversed-phase silica gel column chromatography [200 g, MeOH–H₂O (80:20, v/v)→MeOH] and HPLC [MeOH–1% aq. AcOH (80:20, v/v)] to give glycoside B (6, 1.6 g, 0.080%). Fraction 5 (2.0 g) was purified by repeated HPLC [1) MeOH–H₂O (75:25, v/v); 2) MeOH–H₂O (45:55, v/v)] to give glycoside A (5, 1.8 g, 0.090%), quercetin 3-*O*-neohesperidoside (14, 72 mg, 0.0036%), and isorhamnetin 3-*O*-2^G-rhamnosylrutinoside (19, 487 mg, 0.024%). Fraction 6 (16 g) was separated by reversed-phase silica gel column chromatography [500 g, MeOH–H₂O (20:80→50:50→70:30, v/v)→MeOH] to give five fractions [Fr. 6-1 (10.9 g), 6-2 (831 mg), 6-3 (369 mg), 6-4 (3.5 g), 6-5 (509 mg)]. Fraction 6-2 (831 mg) was purified by HPLC [MeOH–H₂O (40:60, v/v)] to give quercetin 3-*O*-2^G-rhamnosylrutinoside (15, 356 mg, 0.018%). Fraction 6-3 (320 mg) was purified by HPLC [MeOH–1% aq. AcOH (60:40, v/v)] to give calendasaponins C (3, 21 mg, 0.0011%) and D (4, 111 mg, 0.0056%). Fraction 6-4 (400 mg) was purified by HPLC [MeOH–1% aq. AcOH (75:25, v/v)] to give calendasaponin A (1, 23 mg, 0.0012%). The known compounds (5–20) were identified by comparison of their physical data ([α]_D, IR, ¹H-, ¹³C-NMR) with reported values.^{2/4–10)}

Calendasaponin A (1): Colorless fine crystals from MeOH–H₂O, mp 226.5–228.6 °C, [α]_D²⁷ +8.6° ($c=0.1$, MeOH). IR (KBr): 3432, 1736, 1076,

1048 cm^{-1} . High-resolution positive-ion FAB-MS: Calcd for $\text{C}_{54}\text{H}_{86}\text{O}_{24}\text{Na}$ ($\text{M}+\text{Na}^+$): 1141.5407. Found: 1141.5403. $^1\text{H-NMR}$ (500 MHz, pyridine- d_5) δ : 0.71, 0.92, 1.01, 1.03, 1.05, 1.08, 1.22 (3H each, all s, 25, 27, 30, 24, 29, 26, 23- H_3), 2.64 (1H, br d, $J=ca.$ 12 Hz, 13-H), 3.25 (1H, dd-like, 3-H), 4.90 (1H, d, $J=6.4$ Hz, 1'-H), 5.27 (1H, d-like, 1''-H), 5.27 (1H, br s, 19-H), 5.63 (1H, d, $J=8.0$ Hz, 1''-H), 6.39 (1H, d, $J=7.9$ Hz, 1'''-H). $^{13}\text{C-NMR}$ (125 MHz, pyridine- d_5) δ_c : given in Table 4. Negative-ion FAB-MS: m/z 1117 ($\text{M}-\text{H}^-$), 955 ($\text{M}-\text{C}_6\text{H}_{11}\text{O}_3^-$), 793 ($\text{M}-\text{C}_{12}\text{H}_{21}\text{O}_{10}^-$), 631 ($\text{M}-\text{C}_{18}\text{H}_{31}\text{O}_{15}^-$). Positive-ion FAB-MS: m/z 1141 ($\text{M}+\text{Na}^+$).

Calendasaponin B (2): Colorless fine crystals from $\text{MeOH}-\text{H}_2\text{O}$, mp 245.6—247.0 $^\circ\text{C}$, $[\alpha]_D^{27} +6.4^\circ$ ($c=0.1$, MeOH). IR (KBr): 3432, 1736, 1075, 1032 cm^{-1} . High-resolution positive-ion FAB-MS: Calcd for $\text{C}_{48}\text{H}_{76}\text{O}_{20}\text{Na}$ ($\text{M}+\text{Na}^+$): 995.4828. Found: 995.4825. $^1\text{H-NMR}$ (500 MHz, pyridine- d_5) δ : 0.84, 0.97, 1.07, 1.28, 1.37 (3H each, all s, 25, 24, 26, 23, 27- H_3), 0.91 (6H s, 29, 30- H_3), 3.27 (1H, dd-like, 18-H), 3.35 (1H, dd-like, 3-H), 4.55 (1H, dd, $J=5.5$, 10.4 Hz, 16-H), 4.95 (1H, d, $J=7.6$ Hz, 1'-H), 5.23 (1H, d, $J=7.8$ Hz, 1''-H), 5.44 (1H, br s, 12-H), 6.19 (1H, d, $J=7.9$ Hz, 1'''-H). $^{13}\text{C-NMR}$ (125 MHz, pyridine- d_5) δ_c : given in Table 4. Negative-ion FAB-MS: m/z 971 ($\text{M}-\text{H}^-$), 809 ($\text{M}-\text{C}_6\text{H}_{11}\text{O}_3^-$). Positive-ion FAB-MS: m/z 995 ($\text{M}+\text{Na}^+$).

Calendasaponin C (3): Colorless fine crystals from $\text{MeOH}-\text{H}_2\text{O}$, mp 228.5—230.2 $^\circ\text{C}$, $[\alpha]_D^{26} +8.1^\circ$ ($c=1.0$, MeOH). IR (KBr): 3410, 1736, 1076, 1042 cm^{-1} . High-resolution positive-ion FAB-MS: Calcd for $\text{C}_{54}\text{H}_{86}\text{O}_{25}\text{Na}$ ($\text{M}+\text{Na}^+$): 1157.5356. Found: 1157.5338. $^1\text{H-NMR}$ (500 MHz, pyridine- d_5) δ : 0.82, 1.06, 1.08, 1.24, 1.35 (3H each, all s, 25, 26, 24, 23, 27- H_3), 0.91 (6H s, 29, 30- H_3), 3.27 (2H, dd-like, 3, 18-H), 4.54 (1H, dd, $J=3.9$, 11.9 Hz, 16-H), 4.92 (1H, d, $J=7.9$ Hz, 1'-H), 5.27 (1H, d, $J=7.9$ Hz, 1''-H), 5.42 (1H, br s, 12-H), 5.65 (1H, d, $J=7.6$ Hz, 1''-H), 6.22 (1H, d, $J=7.9$ Hz, 1'''-H). $^{13}\text{C-NMR}$ (125 MHz, pyridine- d_5) δ_c : given in Table 4. Negative-ion FAB-MS: m/z 1133 ($\text{M}-\text{H}^-$), 971 ($\text{M}-\text{C}_6\text{H}_{11}\text{O}_3^-$), 809 ($\text{M}-\text{C}_{12}\text{H}_{21}\text{O}_{10}^-$). Positive-ion FAB-MS: m/z 1157 ($\text{M}+\text{Na}^+$).

Calendasaponin D (4): Colorless fine crystals from $\text{MeOH}-\text{H}_2\text{O}$, mp 226.9—229.0 $^\circ\text{C}$, $[\alpha]_D^{24} +33.0^\circ$ ($c=1.1$, MeOH). IR (KBr): 3453, 1736, 1074, 1042 cm^{-1} . High-resolution positive-ion FAB-MS: Calcd for $\text{C}_{54}\text{H}_{86}\text{O}_{25}\text{Na}$ ($\text{M}+\text{Na}^+$): 1157.5356. Found: 1157.5364. $^1\text{H-NMR}$ (500 MHz, pyridine- d_5) δ : 0.81, 1.06, 1.07, 1.15, 1.23, 1.24, 1.28 (3H each, all s, 25, 26, 24, 29, 30, 23, 27- H_3), 3.27 (2H, dd-like, 3, 18-H), 3.89 (1H, dd, $J=4.6$, 11.6 Hz, 21-H), 4.92 (1H, d, $J=7.5$ Hz, 1'-H), 5.26 (1H, d, $J=7.1$ Hz, 1''-H), 5.45 (1H, br s, 12-H), 5.64 (1H, d, $J=7.8$ Hz, 1''-H), 6.28 (1H, d, $J=8.1$ Hz, 1'''-H). $^{13}\text{C-NMR}$ (125 MHz, pyridine- d_5) δ_c : given in Table 4. Negative-ion FAB-MS: m/z 1133 ($\text{M}-\text{H}^-$), 971 ($\text{M}-\text{C}_6\text{H}_{11}\text{O}_3^-$), 809 ($\text{M}-\text{C}_{12}\text{H}_{21}\text{O}_{10}^-$). Positive-ion FAB-MS: m/z 1157 ($\text{M}+\text{Na}^+$).

Acid Hydrolysis of Calendasaponins (1—4) A solution of 1—4 (5 mg each) in 5% aq. H_2SO_4 -1,4-dioxane (2 ml, 1:1, v/v) was heated under reflux for 1 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH^- form) and the residue was removed by filtration. After removal of the solvent from the filtrate *in vacuo*, the residue was transferred to a Sep-Pak C18 cartridge with H_2O and MeOH . The MeOH eluate from 1—4 was concentrated and the residue was purified by normal-phase silica gel column chromatography [1.0 g, *n*-hexane-AcOEt (1:1, v/v) \rightarrow MeOH] to give saponenols [moronic acid (21,¹¹) 1.8 mg, 88% from 1], cochalic acid (22,¹⁴) 2.3 mg, 93% from 2; 2.0 mg, 96.1% from 3], machaerinic acid (23,¹⁵) 1.9 mg, 90% from 4]. The H_2O eluate was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (4 mg) in pyridine (0.5 ml) at 60 $^\circ\text{C}$ for 1 h. After reaction, the solution was treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (0.2 ml) at 60 $^\circ\text{C}$ for 1 h. The supernatant was then subjected to GLC analysis to identify the derivatives of D-glucuronic acid (i), D-glucose (ii), and D-galactose (iii); GLC conditions: column: Supelco STBTM-1, 30 m \times 0.25 mm (i.d.) capillary column, column temperature: 230 $^\circ\text{C}$, He flow rate: 15 ml/min, t_R : i: 24.4 min, ii: 24.2 min, iii 25.5 min.

Serum Glucose in Glucose-Loaded Mice or Rats Mice weighing 28—32 g or rats weighing 130—170 g were fasted for 20—24 h, and the test compounds suspended in 5% acacia solution were given orally. Thirty minutes later, 10% D-glucose solution was administered orally (*p.o.*) at 10 ml/kg for mice or 5 ml/kg for rats. Water was given orally instead of glucose solution in the normal group. Blood samples were collected at 0.5, 1, and 2 h after glucose loading. The blood was centrifuged to obtain serum, and serum glucose levels were determined enzymatically by the glucose-oxidase method (Glucose CII-test Wako, Wako Pure Chemical Industries).

Gastric Emptying in Mice A solution of 1.5% CMC-Na containing 0.05% phenol red as a marker was given intragastrically (0.5 ml/mouse) to conscious mice. Thirty minutes later, mice were sacrificed by cervical dislocation. The abdominal cavity was opened, and the gastroesophageal junction

and pylorus were clamped, then the stomach was removed, weighed and placed in 14 ml of 0.1 M NaOH and homogenized. The suspension was allowed to settle for 1 h at room temperature and 5 ml of the supernatant were added to 0.5 ml of 20% trichloroacetic acid (w/v) and then centrifuged at 3000 rpm for 20 min. The supernatant was mixed with 4 ml of 0.5 M NaOH, and the amount of phenol red was determined from the absorbance at 560 nm. Phenol red recovered from animals sacrificed immediately after the administration of the CMC-Na solution was used as a standard (0% emptying). Gastric emptying (%) in the 30 min period was calculated according to the following equation:

$$\text{gastric emptying (\%)} = (1 - \text{amount of test sample/amount of standard}) \times 100$$

Ethanol-Induced Gastric Lesions in Rats Acute gastric lesions were induced by intragastric (*i.g.*) application of ethanol. Briefly, 99.5% ethanol (1.5 ml) was administered *i.g.* to 24-h fasted rats by means of a metal oro-gastric tube. One hour later, the animals were sacrificed by cervical dislocation under ether anesthesia, then the stomach was removed and inflated by the injection of 10 ml of 1.5% formalin to fix the inner and outer layers of the gastric walls. Subsequently, the stomach was incised along the greater curvature and the lengths of the necrotizing lesions were examined at 10 \times magnification by 2 or 3 observers unaware of the treatment. The samples were given orally 1 h prior to the application of ethanol.

Indomethacin-Induced Gastric Lesions in Rats Acute gastric lesions were induced by the subcutaneous (*s.c.*) application of indomethacin. Briefly, indomethacin (30 mg/kg) was subcutaneously injected into the back of 24-h fasted rats. After 4 h, the animals were sacrificed. The stomachs were dissected out and treated with 1.5% formalin, and were then opened along the greater curvature. The numbers of necrotizing lesions were counted and their lengths were measured at 10 \times magnification by 2 or 3 observers unaware of the treatment. The samples were given orally 1 h prior to the application of indomethacin.

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References and Notes

- 1) Part II: Yoshikawa M., Morikawa T., Toguchida I., Harima S., Matsuda H., *Chem. Pharm. Bull.*, **48**, 651—656 (2000).
- 2) a) Kasprzyk Z., Wojciechowski Z., *Phytochemistry*, **6**, 69—75 (1967); b) Kasprzyk Z., Pyrek J., *ibid.*, **7**, 1631—1639 (1968); c) Kasprzyk Z., Wilkomirski B., *ibid.*, **12**, 2299—2300 (1973); d) Gracza L., *Planta Med.*, **53**, 227 (1987); e) Willuhn G., Westhanus R. G., *ibid.*, **53**, 304 (1987); f) Vidal-Ollivier E., Balansard G., *J. Nat. Prod.*, **52**, 1156—1159 (1989); g) Verljen J., Liptak A., Wagner H., *Phytochemistry*, **28**, 2379—2383 (1989); h) Della Loggia R., Tubaro A., Sosa S., Becker H., Saar S., Isaac O., *ibid.*, **60**, 516—520 (1994).
- 3) Yoshikawa M., Morikawa T., Murakami T., Toguchida I., Harima S., Matsuda H., *Chem. Pharm. Bull.*, **47**, 340—345 (1999).
- 4) Vidal-Ollivier E., Babadjamian A., Faure R., Chemli R., Boukef D., Balansard G., Vincent E. J., *Spectrosc. Lett.*, **22**, 579—584 (1989).
- 5) Pizza C., Zhong-Liang Z., Tommasi De N., *J. Nat. Prod.*, **50**, 927—931 (1987).
- 6) Wenkert E., Gottlieb H. E., *Phytochemistry*, **16**, 1811—1816 (1977).
- 7) Markham K. R., Ternai B., Stanley R., Geiger H., Mabry T. J., *Tetraedron*, **34**, 1389—1397 (1978).
- 8) Vildal-Ollivier E., Elias R., Faure R., Bobadjamian A., Crespin F., Balansard G., Boudon G., *Planta Med.*, **55**, 73—74 (1989).
- 9) Lei Z. H., Yahara S., Tai B. S., Tian R. H., Takiguchi Y., Nohara T., *Nat. Med.*, **49**, 475—477 (1989).
- 10) Miyase T., Ueno A., Takizawa N., Kobayashi H., Karasawa H., *Chem. Pharm. Bull.*, **35**, 1109—1117 (1987).
- 11) Majumder P. L., Maiti R. N., Panda S. K., Mal D., *J. Org. Chem.*, **44**, 2811—2812 (1979).
- 12) Hara S., Okabe H., Mihashi K., *Chem. Pharm. Bull.*, **34**, 1843—1845 (1986).
- 13) The ^1H - and ^{13}C -NMR spectra of 1—4 were assigned on the basis of homo- and hetero-correlation spectroscopy (^1H - ^1H , ^1H - ^{13}C COSY), homo- and hetero-nuclear Hartmann-Hahn spectroscopy (^1H - ^1H , ^1H - ^{13}C HOHAHA), heteronuclear multiple bond correlation (HMBC), phase-sensitive nuclear Overhauser effect spectroscopy (pNOESY) experiments.
- 14) Segal R., Reicher F., Youssefeyeh R. D., *J. Chem. Soc. Chem. Comm.*,

- 1974, 481—482.
- 15) Delgado M. C. C., Silva M. S. D., Fo R. B., *Phytochemistry*, **23**, 2289—2292 (1984).
- 16) a) Yoshikawa M., Yoshizumi S., Ueno T., Matsuda H., Murakami T., Yamahara J., Murakami N., *Chem. Pharm. Bull.*, **43**, 1878—1882 (1995); b) Yoshikawa M., Murakami T., Harada E., Murakami N., Yamahara J., Matsuda H., *ibid.*, **44**, 1923—1927 (1996).
- 17) a) Yoshikawa M., Murakami T., Kadoya M., Matsuda H., Muraoka O., Yamahara J., Murakami N., *Chem. Pharm. Bull.*, **44**, 1212—1217 (1996); b) Yoshikawa M., Murakami T., Kadoya M., Yamahara J., Matsuda H., *ibid.*, **46**, 1758—1763 (1998); c) Murakami T., Matsuda H., Inadzuki M., Hirano K., Yoshikawa M., *ibid.*, **47**, 1717—1724 (1999).
- 18) a) Yoshikawa M., Murakami T., Matsuda H., Yamahara J., Murakami N., Kitagawa I., *Chem. Pharm. Bull.*, **44**, 1454—1464 (1996); b) Yoshikawa M., Murakami T., Yamahara J., Matsuda H., *ibid.*, **46**, 1764—1769 (1998).
- 19) a) Yoshikawa M., Murakami T., Ueno T., Kadoya M., Matsuda H., Yamahara J., Murakami N., *Chem. Pharm. Bull.*, **43**, 2115—2122 (1995); b) Yoshikawa M., Murakami T., Matsuda H., Ueno T., Kadoya M., Yamahara J., Murakami N., *ibid.*, **44**, 1305—1313 (1996).
- 20) a) Yoshikawa M., Murakami T., Matsuda H., “Towards Natural Medicine Research in the 21st Century,” ed. by Ageta H., Aimi N., Ebizuka Y., Fujita T., Honda G., Elsevier, Amsterdam, 1998, pp. 137—149; b) Yoshikawa M., Murakami T., Li Y., Shimada H., Yamahara J., Matsuda H., “Advances in Plant Glycosides, Chemistry and Biology,” ed. by Yang C. R., Tanaka O., Elsevier, Amsterdam, 1999, pp. 19—35; c) Yoshikawa M., Matsuda H., “Saponins in Food, Foodstuffs and Medicinal Plants,” ed. by Oleszek W., Marston A., Kluwer Academic Publishers, Dordrecht, Boston, London, 2000, pp. 189—203.
- 21) Matsuda H., Li Y., Murakami T., Matsumura N., Yamahara J., Yoshikawa M., *Chem. Pharm. Bull.*, **46**, 1399—1403 (1998).
- 22) a) Matsuda H., Murakami T., Li Y., Yamahara J., Yoshikawa M., *Bioorg. Med. Chem.*, **6**, 1019—1023 (1998); b) Matsuda H., Li Y., Yamahara J., Yoshikawa M., *J. Pharmacol. Exper. Ther.*, **289**, 729—734 (1999); c) Matsuda H., Li Y., Murakami T., Yamahara J., Yoshikawa M., *Bioorg. Med. Chem.*, **7**, 323—327 (1999); d) Matsuda H., Li Y., Murakami T., Yamahara J., Yoshikawa M., *Eur. J. Pharmacol.*, **368**, 237—243 (1999); e) Matsuda H., Li Y., Yoshikawa M., *Life Sci.*, **66**, PL41—46 (2000); f) *Idem, ibid.*, **67**, 2921—2927 (2000).
- 23) a) Matsuda H., Li Y., Murakami T., Yamahara J., Yoshikawa M., *Life Sci.*, **63**, PL245—250 (1998); b) Matsuda H., Li Y., Yoshikawa M., *Eur. J. Pharmacol.*, **373**, 63—70 (1999); c) *Idem, Life Sci.*, **65**, PL27—32 (1999); d) Murakami T., Nakamura J., Matsuda H., Yoshikawa M., *Chem. Pharm. Bull.*, **47**, 1759—1764 (1999); e) Yoshikawa M., Murakami T., Oominami H., Matsuda H., *ibid.*, **48**, 1045—1050 (2000).