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Anti-Tobacco Mosaic Virus (TMV) Triterpenoid Saponins from the Leaves of *Ilex oblonga*

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Ten triterpene saponins have been isolated from a MeOH extract of the leaves of *Ilex oblonga*. In their structures, six new triterpenoid saponins were named as oblonganosides H−M (**2**, **4**, **5**, and **8**−**10**). All structures were elucidated on the basis of spectroscopic analysis. Among the triterpenoid saponins, three compounds (**7**, **8**, and **10**) showed obvious activities in inhibiting multiplication of the tobacco mosaic virus (TMV).

KEYWORDS: **Aquifoliaceae; *Ilex oblonga*; triterpenoid saponin; TMV**

INTRODUCTION

In a continuation of our study on the constituents of the medicinal plants of the Aquifoliaceae family (*1*−*4*), we investigated the water-soluble fraction of *Ilex oblonga*. This plant is a well-known endemic herb used to treat gumboil, eczema, rheumatism, scald, and bruise in the Guangxi province of China (*5*). Recently, the occurrence of flavonoids (*6*), xanthines (*7*), aldehydes (*8*), hemiterpene glycosides (*9*, *10*), triterpenes and alkanes (*11*), anthocyanins (*12*), pentyl esters, hexyl esters, and other lipophilic compounds (*13*) has been reported in *Ilex* species. Several biological activities were related to the compounds isolated from them, including hypocholesterolemic (*14*) and antioxidant (*15*, *16*) activities. In this paper, we describe the isolation, biological activity to inhibit multiplication of the tobacco mosaic virus (TMV), and structure elucidation of six new triterpenoid saponins designated oblonganoside H (**2**), oblonganoside I (**4**), oblonganoside J (**5**), oblonganoside K (**8**), oblonganoside L (**9**), and oblonganoside M (**10**) (**Figure 1**) along with four known triterpenoid saponins: pomolic acid-28-*O*-β-D-glucopyranosyl ester (**1**) (*17*), rotundic acid-28-*O*-β-D-glucopyranosyl ester (**3**) (*18*), nigaichigoside F1 (**6**) (*19*), and siaresinolic acid-28-*O*-β-D-glucopyranosyl ester (**7**) (*20*) from the leaves of *I. oblonga*.

MATERIALS AND METHODS

General Experimental Procedures. IR spectra were recorded with a Perkin-Elmer 1750 FTIR spectrometer, and the films of the all samples were measured on KBr disks. Optical rotations were measured with a Jasco DIP-180 digital polarimeter spectrophotometer. The ^{1}H, ^{13}C, DEPT, $^{1}H-^{1}H$ COSY, NOESY, HMQC, and HMBC NMR spectra were performed using a Bruker AM-400 and a DRX-500 spectrometer. FAB mass spectra were recorded on a JEOL JMS-HX 110 instrument. Chromatographic stationary phases used were RP-18 (40−60 μm, Merck), silica gel (160−200 mesh), Sephadex LH-20 (25−100 μm, Pharmacia Fine Chemical Co. Ltd.), and MCI-gel CHP20P (75−150 μm, Mitsubishi Chemical Industries, Ltd.). HPLC was a P-230-UV-230 (Dalian Elite Analytical Instruments Co., Ltd.) and HPLC column (YMC-Pack ODS-A, S-5 μm, 250 × 10 mm). The following solvent systems were used: (a) $CHCl_3$/MeOH/H_2O (80:20:3), $CHCl_3$/MeOH/H_2O (70:30:5), and MeOH/H_2O (0−100%) for the glycosides; and (b) $CHCl_3$/MeOH/H_2O (7:3:1) lower-layer 9 mL + 1 mL HOAc for the sugars. Compounds on TLC were detected by spraying with 5% H_2SO_4 followed by heating. Sugars were detected by spraying with aniline−phthalate reagent.

Plant Material. The leaves of *I. oblonga* C.J. Tseng were collected at the Plant Garden of the Guangxi Institute of Botany, Chinese Academy of Sciences, in July 1999. A voucher specimen (no. 13523) is deposited in the Herbarium of the Guangxi Institute of Botany. The plant was identified by Prof. C. H. Li.

Extraction and Isolation. The leaves of *I. oblonga* (690 g) were extracted (2 × 4 L) with MeOH at room temperature (7 days × 2). The extract was evaporated in vacuo to yield a residue, which was dissolved in water and filtered. The water-soluble fraction was passed through a Diaion column and eluted with water and methanol. Evaporation of the methanol eluate yielded 37 g of a brown fraction (A). Fraction A was subjected to dry column chromatography (DCC) on silica gel (1.0 kg), eluted with $CHCl_3$/MeOH/H_2O (10:2:0.2) to afford 13 fractions. Each fraction was purified by Sephadex LH-20, RP-8 gel column chromatography (solvent: MeOH/H_2O, 10−70%), then purified by a silica gel column with $CHCl_3$/MeOH/H_2O (100:10:1−70:30:5), and finally repeatedly purified by RP-HPLC with MeOH/H_2O (60%-80%) as solvent to yield **1** (44 mg), **2** (21 mg), **3** (13 mg), **4** (16 mg), **5** (24 mg), **6** (17 mg), **7** (65 mg), **8** (15 mg), **9** (35 mg), and **10** (17 mg).

Compound 1: amorphous powder, $C_{36}H_{58}O_9$; $[\alpha]_D^{21}$ +7.3 (c 0.17, MeOH); FABMS, *m/z* 633 $[M - H]^-$, 471 $[M - H - 162]^-$; IR, ν_{max}-

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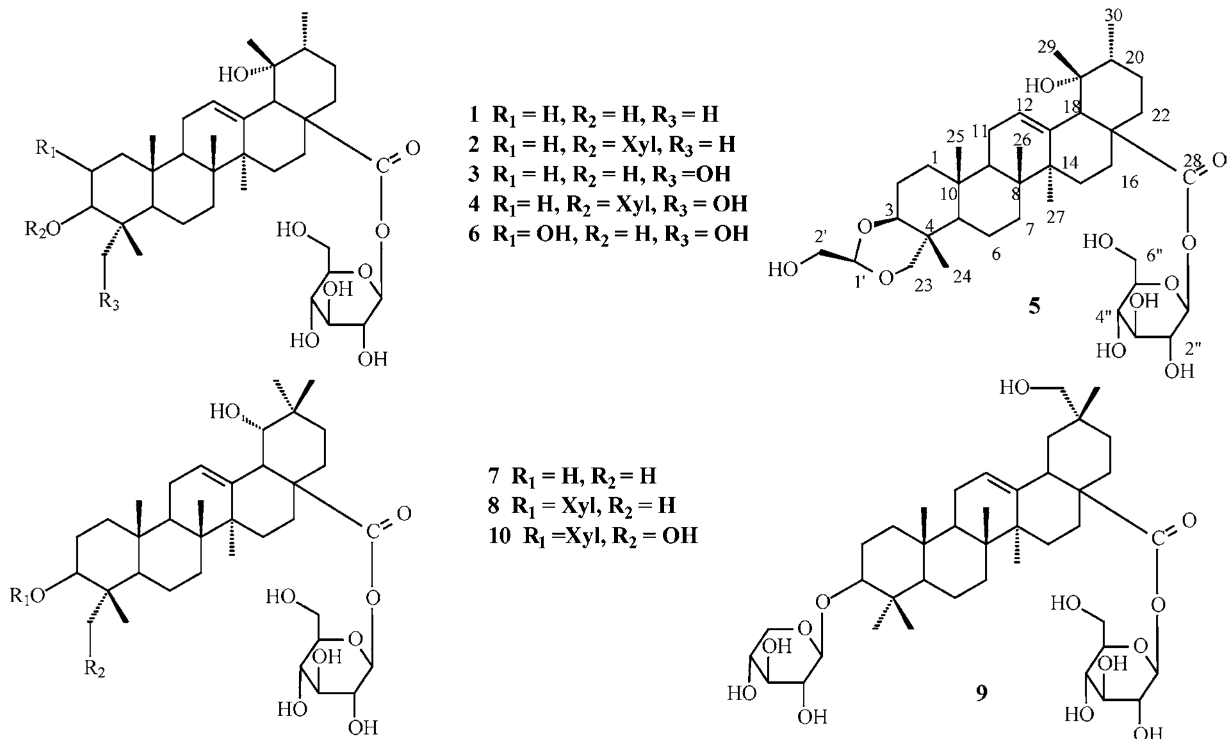

Figure 1. Triterpene saponins **1**–**10**.

(film)/cm^{-1} 3427, 2940, 1728, 1639, 1455, 1378, 1072, 1021; ^{1}H and ^{13}C NMR (see **Table 1**).

Compound 2: amorphous powder, $C_{41}H_{66}O_{13}$; $[\alpha]_D^{21}$ +71 (*c* 0.10, MeOH); FABMS, *m/z* 765 [M − H]$^-$, 603 [M − H − 162]$^-$, 471 [M − H − 162 − 132]$^-$; HRFAB-MS, *m/z* 765.44249 [M − H]$^-$; IR, ν_{max}(film)/cm^{-1} 3440, 2920, 1729, 1640, 1445, 1388, 1086, 1103; ^{1}H and ^{13}C NMR (see **Table 1**).

Compound 3: amorphous powder, $C_{36}H_{58}O_{10}$; $[\alpha]_D^{21}$ −23.3 (*c* 0.24, MeOH); FABMS, *m/z* 649[M − H]$^-$, 487 [M − H − 162]$^-$; IR, ν_{max}(film)/cm^{-1} 3337, 2936, 1730, 1636, 1451, 1388, 1092, 1001; ^{1}H and ^{13}C NMR (see **Table 1**).

Compound 4: amorphous powder, $C_{41}H_{66}O_{14}$; $[\alpha]_D^{21}$ −3.8 (*c* 0.30, MeOH); FABMS, *m/z* 781 [M − H]$^-$, 619 [M − H − 162]$^-$, 487 [M − H − 162 − 132]$^-$; HRFAB-MS, *m/z* 781.43738 [M − H]$^-$; IR, ν_{max}(film)/cm^{-1} 3430, 2923, 1729, 1640, 1449, 1398, 1046, 1003; ^{1}H and ^{13}C NMR (see **Table 1**).

Compound 5: amorphous powder, $C_{38}H_{60}O_{11}$; $[\alpha]_D^{21}$ −72.3 (*c* 0.37, MeOH); FABMS, *m/z* 691 [M − H]$^-$, 529 [M − H − 162]$^-$; HRFAB-MS, *m/z* 691.40572 [M − H]$^-$; IR, ν_{max}(film)/cm^{-1} 3432, 2934, 1729, 1639, 1465, 1348, 1032, 1001; ^{1}H and ^{13}C NMR (see **Table 1**).

Compound 6: amorphous powder, $C_{36}H_{58}O_{11}$; $[\alpha]_D^{21}$ +21 (*c* 0.11, MeOH); FABMS, *m/z* 665 [M − H]$^-$, 503 [M − H − 162]$^-$; IR, ν_{max}(film)/cm^{-1} 3341, 2930, 1729, 1631, 1475, 1418, 1026, 963; ^{1}H and ^{13}C NMR (see **Table 1**).

Compound 7: amorphous powder, $C_{36}H_{58}O_{9}$; $[\alpha]_D^{21}$ +63.5 (*c* 0.39, MeOH); FABMS, *m/z* 633[M − H]$^-$, 471 [M − H − 162]$^-$; IR, ν_{max}(film)/cm^{-1} 3357, 2916, 1730, 1641, 1471, 1418, 1292, 1101; ^{1}H and ^{13}C NMR (see **Table 1**).

Compound 8: amorphous powder, $C_{41}H_{66}O_{13}$; $[\alpha]_D^{21}$ +25.3 (*c* 0.27, MeOH); FABMS, *m/z* 765 [M − H]$^-$, 603 [M − H − 162]$^-$, 471 [M − H − 162 − 132]$^-$; HRFAB-MS, *m/z* 765.44248 [M − H]$^-$; IR, ν_{max}(film)/cm^{-1} 3336, 2903, 1730, 1642, 1469, 1328, 1016, 937; ^{1}H and ^{13}C NMR (see **Table 1**).

Compound 9: amorphous powder, $C_{41}H_{66}O_{13}$; $[\alpha]_D^{21}$ +37.2 (*c* 0.14, MeOH); FABMS, *m/z* 765 [M − H]$^-$, 603 [M − H − 162]$^-$, 471 [M − H − 162 − 132]$^-$; HRFAB-MS, *m/z* 765.44250 [M − H]$^-$; IR, ν_{max}(film)/cm^{-1} 3437, 2933, 1731, 1636, 1419, 1338, 1146, 903; ^{1}H and ^{13}C NMR (see **Table 1**).

Compound 10: amorphous powder, $C_{41}H_{66}O_{14}$; $[\alpha]_D^{21}$ −2.9 (*c* 0.43, MeOH); FABMS, *m/z* 781 [M − H]$^-$, 619 [M − H − 162]$^-$, 487 [M

Table 1. ^{13}C NMR Spectral Data (in Parts per Million, *J* in Hertz, in CD_3OD) for **1**–**10**

no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	39.8	39.8	39.6	39.8	40.0	47.1	39.8	39.6	39.8	38.9
2	27.9	27.1	27.2	27.4	27.2	69.7	27.9	27.2	27.1	25.9
3	79.8	90.8	74.3	74.0	86.8	78.7	79.8	90.7	90.7	83.2
4	39.8	40.2	41.2	43.3	41.4	41.3	40.3	40.2	40.2	40.4
5	56.7	57.0	49.0	49.0	52.7	55.0	56.9	57.1	57.1	49.0
6	19.6	19.4	19.3	19.1	18.9	19.2	19.7	19.5	19.3	19.3
7	34.1	34.1	33.7	33.5	33.6	33.5	34.0	33.9	34.0	32.9
8	41.2	41.2	42.7	42.6	42.9	42.8	40.9	40.9	40.6	42.2
9	49.0	49.0	48.6	48.9	48.6	49.0	48.6	48.6	49.0	49.0
10	38.1	37.8	38.3	38.4	38.3	39.0	38.3	38.0	38.0	37.7
11	24.7	24.7	24.7	24.5	24.5	24.8	24.8	24.8	24.6	24.3
12	129.7	129.7	129.7	129.6	129.4	129.5	125.0	125.0	123.9	124.5
13	139.6	139.6	139.6	139.6	139.6	139.7	144.3	144.3	144.8	143.9
14	42.6	42.6	42.9	42.8	42.6	42.9	44.6	42.6	42.9	44.6
15	30.8	29.7	29.6	29.7	29.6	29.6	29.6	29.4	28.9	29.1
16	26.5	26.5	26.6	26.7	26.5	26.4	28.5	28.4	24.0	28.0
17	48.8	48.7	48.8	48.8	49.4	49.8	47.2	47.1	49.0	46.7
18	54.9	54.9	55.0	54.9	54.9	55.0	45.1	45.0	41.9	44.6
19	73.8	73.6	73.7	73.8	73.6	73.8	82.5	82.4	41.4	82.0
20	42.9	42.6	43.3	43.4	42.9	44.1	35.9	35.9	36.8	35.5
21	27.2	27.3	27.5	27.5	27.2	27.2	29.6	29.5	29.3	29.1
22	38.3	37.3	37.9	37.8	37.9	38.3	33.3	33.3	32.5	32.9
23	28.7	28.5	67.8	67.7	79.1	66.6	28.7	28.6	28.9	64.5
24	16.6	17.0	12.7	12.8	13.8	13.8	17.0	16.9	17.0	12.9
25	15.9	16.0	16.6	16.6	17.0	17.4	16.3	15.9	16.0	15.9
26	17.8	17.6	17.7	17.8	17.5	17.7	17.8	17.7	17.8	17.4
27	24.6	24.6	24.7	24.6	24.7	24.7	26.2	25.1	26.3	24.6
28	178.6	178.5	178.6	178.6	178.5	178.6	178.6	178.5	178.0	178.1
29	27.0	27.0	27.1	27.3	27.1	27.1	28.6	28.4	74.4	28.1
30	16.0	16.6	16.3	16.7	16.6	16.6	25.0	25.0	19.5	24.8
1′		107.5		107.4	103.1			107.4	107.4	106.0
2′		75.4		75.3	64.6			75.3	75.4	75.0
3′		78.0		78.2				78.0	78.0	77.6
4′		71.2		71.3				71.0	71.2	70.7
5′		66.7		66.7				66.7	66.7	66.3
1″	95.8	95.7	95.8	95.7	95.7	95.8	95.9	95.8	95.7	95.3
2″	73.8	73.8	73.8	73.7	73.8	73.9	74.0	73.9	73.9	73.5
3″	78.3	78.3	78.3	78.2	78.1	78.3	78.4	78.3	78.3	77.9
4″	71.1	71.2	71.2	71.2	71.1	71.2	71.2	71.0	71.3	70.9
5″	78.6	78.6	78.6	78.4	78.5	78.6	78.7	78.7	78.7	78.1
6″	62.4	62.4	62.5	62.6	62.4	62.5	62.5	62.3	62.5	62.0

Table 2. ^{1}H NMR Spectral Data (in Parts per Million, *J* in Hertz, in CD_3OD) for **1**–**10**

no.	1	2	3	4	5	6	7	8	9	10
1	1.64, m 0.99, m	1.62, m 0.98, m	1.72, m 1.15, m	1.73, m 1.11, m	1.68, m 1.10, m	2.25, m 2.02, m	1.62, m 1.00, m	1.60, m 0.99, m	1.58, m 0.94, m	1.60, m 0.98, m
2	1.87, m 1.73, m	1.86, m 1.68, m	1.86, m 1.69, m	1.85, m 1.70, m	1.87, m 1.71, m	3.42, m	1.77, m 1.70, m	1.81, m 1.68, m	1.79, m 1.66, m	1.80, m 1.67, m
3	3.17, overlap	3.17, overlap	3.60, dd 11.1, 4.5	3.61, overlap	3.33, overlap	3.67, overlap	3.11, br d, 11.3	3.12, overlap	3.09, overlap	3.13, overlap
5	0.78, m	0.77, m	0.73, m	0.74, m	0.85, br d 11.6	0.80, m	0.78, br d, 11.2	0.79, br d, 11.0	0.75, m	0.80, br d, 10.1
6	1.53, m 1.38, m	1.52, m 1.38, m	1.51, m 1.39, m	1.50, m 1.38, m	1.51, m 1.44, m	1.52, m 1.40, m	1.50, m 1.38, m	1.55, m 1.42, m	1.53, m 1.38, m	1.52, m 1.40, m
7	1.52, m 1.30, m	1.52, m 1.30, m	1.53, m 1.28, m	1.53, m 1.28, m	1.53, m 1.29, m	1.57, m 1.32, m	1.49, m 1.30, m	1.47, m 1.29, m	1.46, m 1.29, m	1.47, m 1.28, m
9	1.67, m	1.67, m	1.73, m	1.74, m	1.74, m	1.76, m	1.72, m	1.72, m	1.56, m	1.72, m
11	1.96, m	1.97, m	1.97, m	1.96, m	1.97, m	1.99, m	1.93, m	1.93, m	1.87, m	1.91, m
12	5.29, br s	5.29, br s	5.29, br s	5.28, br s	5.29, br s	5.30, br s	5.32, br s	5.31, br s	5.26, br s	5.30, br s
15	1.79, m 1.27, m	1.80, m 1.28, m	1.84, m 1.30, m	1.85, m 1.27, m	1.80, m 1.00, m	1.83, m 1.09, m	1.92, m 1.17, m	1.94, m 1.20, m	1.90, m 1.20, m	1.89, m 1.19 m
16	2.61, m 1.59, m	2.60, dt, 13.3 4.3, 1.60, m	2.60, m 1.63, m	2.60, m 1.58, m	2.60, dt, 13.3 4.3, 1.60, m	2.60, mm 1.62, m	2.33, m 1.69, m	2.31, t, 13.0 1.69, m	2.02, dt, 13.6, 3.0 1.70, m	2.03, m 1.69, m
18	2.50, s	2.50, s	2.50, s	2.50, s	2.50, s	2.51, s	3.04, overlap	3.04, overlap	2.85, m	3.04, overalp
19							3.26, overlap	3.26, overlap	1.80, m 1.08, m	3.26, overlap
20	1.34, m	1.34, m	1.33,m	1.43, m	1.34, m	1.35, m				
21	2.23, m 1.87, m	2.22, m 1.84, m	2.23, m 1.85, m	2.21, m 1.86, m	1.69, m 1.53, m	2.24, m 1.85, m	1.23, m 0.93, m	1.24, m 0.93, m	1.27, m 0.93, m	1.24, m 0.93, m
22	1.78, m 1.62, m	1.78, m 1.62, m	1.79, m 1.62, m	1.78, m 1.62, m	1.76, m 1.59, m	1.78, m 1.60, m	1.78, m 1.67, m	1.78, m 1.67, m	1.72, m 1.63, m	1.78, m 1.67, m
23	0.96, s	1.04, s	3.52, d, 11.1 3.28, d, 11.1	3.55, d, 11.0 3.30, d, 11.0	3.79, d, 10.7 3.28, d, 10.7		0.96, s	1.04, s	1.01, s	3.52, d, 11.3 3.27, d, 11.3
24	0.77, s	0.84, s	0.70, s	0.70, s	1.04, s	0.69, s	0.77, s	0.84, s	0.81, s	0.78, s
25	0.94, s	0.95, s	0.97, s	0.94, s	1.00, s	1.03, s	0.94, s	0.93, s	0.92, s	0.93, s
26	0.78, s	0.77, s	0.74, s	0.75, s	0.76, s	0.77, s	0.73, s	0.73, s	0.77, s	0.76, s
27	1.31, s	1.32, s	1.32, s	1.30, s	1.33, s	1.33, s	1.28, s	1.28, s	1.14, s	1.15, s
29	1.19, s	1.19, s	1.19, s	1.19, s	1.19, s	1.19, s	0.93, s	0.93, s	3.30, d, 10.7 3.16, d, 10.7	0.93
30	0.92, d, 6.8	0.92, d, 6.8	0.92, d, 6.8	0.94, d, 6.8	0.92, d, 6.7	0.92, d, 6.5	0.93, s	0.93, s	0.89, s	0.93
1′		4.26, d, 7.6		4.27, d, 7.4	4.65, t, 5.6			4.26, d, 7.6	4.23, d, 7.8	4.24, d, 7.6
2′		3.18, m		3.15, m	3.57, br d, 5.6 3.50, br d, 5.6			3.17, m	3.15, m	3.16, m
3′		3.26, m		3.28, m				3.16, m	3.09, m	3.26, m
4′		3.45, m		3.47, m				3.45, m	3.43, m	3.52, m
5′		3.81, br d, 11.1 3.17, overlap		3.84 d, 11.2 3.18, overlap				3.81, d, 10.3 3.17, overlap	3.79, d, 11.6 3.15, overlap	3.80, d, 11.4 3.17, overlap
1″	5.31, d, 7.7	5.31, d, 7.9	5.31, d, 7.8	5.34, d, 7.9	5.31, d, 7.7	5.31, d, 8.0	5.36, d, 7.7	5.36, d, 8.0	5.35, d, 8.0	5.37, d, 8.0
2″	3.30, m	3.30, m	3.30, m	3.28, m	3.30, m	3.30, m	3.30, m	3.30, m	3.29, m	3.30, m
3″	3.39, m	3.38, m	3.39, m	3.38, m	3.39, m	3.39, m	3.390, m	3.39, m	3.37, m	3.40, m
4″	3.24, m	3.24, m	3.24, m	3.24, m	3.25, m	3.24, m	3.24, m	3.25, m	3.22, m	3.24, m
5″	3.32, m	3.35, m	3.33, m	3.33, m	3.34, m	3.33, m	3.34, m	3.33, m	3.31, m	3.34, m
6″	3.79, br d, 11.5 3.67, dd, 11.5, 4.2	3.79, br d, 12.3 3.67, dd, 12.3, 4.0	3.79, br d, 12.0 3.67, dd, 12.0, 4.7	3.80, br d, 11.0 3.67, dd, 11.0, 4.9	3.79, br d, 11.0 3.67, dd, 11.0, 3.9	3.79, br d, 10.0 3.67, br d, 10.0	3.81 br d, 11.6 3.67, br d, 11.6	3.80, br d, 11.0 3.67, br d, 11.0	3.80, br d, 11.0 3.65, br d, 11.6, 4.1	3.83, br d, 11.3 3.67, dd, 11.3, 3.2

Table 3. TMV Multiplication Inhibition of Compounds **1**–**10**[a]

	–[b]	+[b]	1	2	3	4	5	6	7	8	9	10
TR[c]	0	0.3262	0.2381	0.2042	0.1908	0.2228	0.2345	0.2450	0.0506	0.0466	0.1732	0.0701
%[e]	100	0	27.0	37.4	41.5	31.7	28.1	24.9	84.5	85.7	46.9	78.5

[a] The concentration of each compound is 0.2 mg/mL. [b] "+" is positive control, and "–" is negative control. [c] Concentration of TMV in every treatment (ng). [d] Inhibition rate.

− H − 162 − 132]$^-$; HRFAB-MS, *m/z* 781.43740 [M − H]$^-$; IR, ν_{max}(film)/cm^{-1} 3350, 2913, 1729, 1636, 1469, 1398, 1266, 1120; ^{1}H and ^{13}C NMR (see **Table 1**).

Acid Hydrolysis. A solution of each compound was heated at 100 °C in 2 M aqueous CF_3COOH (5 mL) refluxed on a water bath for 3 h. After this period, the reaction mixture was diluted with H_2O (15 mL) and extracted with CH_2Cl_2 (3 × 5 mL). The combined CH_2Cl_2 extract was washed with H_2O and then evaporated to dryness in vacuo. After evaporation to dryness of the aqueous layer with MeOH to wash until neutral, the sugars were analyzed by silica gel HPTLC by comparison with standard sugars [solvent system $CHCl_3$/MeOH/H_2O (7:3:1) lower-layer 9 mL + 1 mL of HOAc for sugars] on silica gel HPTLC.

Alkaline Hydrolysis. The sample was refluxed with 5% KOH (10 mL) for 1 h. The reaction mixture was adjusted to pH 6 with dilute HCl and then extracted with H_2O-saturated *n*-BuOH (3 × 10 mL). The combined *n*-BuOH extracts were washed by H_2O. Evaporation of the *n*-BuOH gave the progenin. Acid hydrolysis of progenin in 2 N CF_3COOH for 2 h at 120 °C furnished glucose (HPTLC with authentic sample).

Screening Material Preparations. *Nicotiana tabacum* cv. K_{326} was cultivated in a glasshouse without pests. Different compounds were weighed precisely with an electrobalance, dissolved with a small quantity of DMSO, and diluted with water to provide solutions having a concentration of 0.2 mg/mL; all solutions were in Petri dishes.

Leaf-Disk Method. Leaves of *N. tabacum* cv. K_{326} were mechanically inoculated with TMV at 10 μg/mL. Leaf disks of 10-mm diameter were punched and floated on the solutions of different compound solutions 7 h after inoculation and incubated at 25 ± 1 °C for 48 h. Disks were treated with solvent only as the positive control, whereas disks of healthy leaves were used as the negative control; 48 h later, leaf disks were ground in coating buffer, and virus concentrations in them were measured by indirect ELISA. There were six repetitions in every treatment. A series of TMV solutions at known concentrations

was incorporated into every microliter plate to provide an internal calibration curve. Linearity of absorbance with TMV concentrations was obtained within a range of about 2 orders of magnitude (0.04888–12.5 ng of TMV).

The inhibition of virus replication was calculated as [1 − (virus concentration of compound-treated leaf disk)/(virus concentration of leaf disk of the positive control] × 100, where virus concentration was calculated by the TMV standard curve with OD_{405} value of indirect ELISA.

Indirect ELISA Procedure. One hundred microliters of diluted antigen was added to each well of a micro-ELISA plate and incubated overnight at 4 °C. After incubation, the antigen solution was discarded and the plates were washed three times in phosphate-buffered saline (PBS), pH 7.2, containing 0.001% Tween 20 (PBS-T). Rabbit anti-TMV serum (1:2000) was added to the antigen-coated wells. The plates were incubated for 1 h at 37 °C and then washed three times in PBS-T. Goat anti-rabbit alkaline phosphatase (Sigma) conjugate(1:30000) in PBS-T was then added and a further incubation for 1 h carried out at 37 °C. The plates were again washed three times, 100 μL of PNP solution per well was added, and after 10 min, the reaction was stopped by adding 100 μL of 3 M sodium hydroxide. The intensity of color development was determined by measuring absorbance using a micro-ELISA reader equipped with a 405 nm filter.

RESULTS AND DISCUSSION

The leaves of *I. oblonga* were extracted with MeOH under reflux. The MeOH extract was subjected to Diaion HP-20 column chromatography to give H_2O, MeOH, and acetone-eluted fractions. The MeOH-eluted fraction was subjected to ordinary and reversed phase silica gel column chromatography and finally HPLC to furnish triterpenoid saponins **1**−**10**.

Compound **2** was obtained as an amorphous powder and exhibited a positive specific rotation, $[\alpha]_D^{21}$ + 71.0 (*c* 0.10, MeOH), with IR ν_{max} (film)/cm^{-1} of 3440, 2920, 1729, 1640, 1445, 1388, 1086, and 1103 assignable to ester carbonyl and double bond. The negative FABMS of **2** showed a quasimolecular ion peak at *m/z* 765 [M − H]$^-$, indicating a molecular weight of 766, and HRFABMS analysis revealed the molecular formula of **2** to be $C_{41}H_{66}O_{13}$. The other significant ion peaks were at *m/z* 603 [(M − H) − 162]$^-$ and 471 [M − H − 162 − 132]$^-$, corresponding to the loss of one hexosyl and one pentosyl units. Acid hydrolysis afforded a mixture of sugars, which were identified as D-glucose and D-xylose by detected HPTLC. Alkaline hydrolysis of **2** performed with 1 M KOH yielded glucose and a prosapogenin, which furnished xylose and a genin by subsequent acid hydrolysis. These chemical reactive results manifested that **2** should be a triterpene-bidesmosidic saponin in which xylose was bound to the aglycone by a glycosidic linkage at C-3, whereas the remaining sugar should be bound to the genin by a glycosidic ester linkage at C-28; these results were also confirmed by the carbon signals of **2** at δc 90.8 (downfield shift of C-3 of the aglycone) and δc 178.5 (upfield shift of C-28 of the aglycone). In comparison with the NMR data of pomolic acid (*21*) and the aglycone of **2**, they were the same triterpene. In the HMBC spectrum, the correlation cross-peaks were observed between H-1′ and C-3 and between H-1″ and C-28. Thus, the structure of **2** was determined to be 3-*O*-*β*-D-xylopyranosylpomolic acid-28-*O*-*β*-D-glucopyranosyl ester and named oblonganoside H.

The negative FAB-MS of **4** gave a quasimolecular ion peak at *m/z* 781 [M − H]$^-$ and main fragment peaks at *m/z* 619 [M − H − 162]$^-$ and 487 [M − H − 162 − 132]$^-$. The molecular formula $C_{416}H_{66}O_{14}$ was as shown by HRFABMS. The ^{1}H NMR spectrum of **4** gave five singlet methyl signals and one doublet methyl signal [δ 1.30, 1.19, 0.94, 0.75, 0.70, and 0.94 (3H, d, *J* = 6.8 Hz)] and two anomeric proton signals at δ 5.34 (1H, d, *J* = 7.9 Hz) and 4.27 (1H, d, *J* = 7.4 Hz). The ^{13}C NMR spectrum of **4** presented a characteristic carbon signal at δ 12.8 C-24 (by shielding of the hydroxyl of C-23) and at δ 67.7 C-23 (bearing oxygen). Comparison with the NMR data of the genin (**4**) and pomolic acid revealed that characteristic signals of pomolic acid presented in compound **4** such as C-19 (quarternary carbon δ 73.8) and C-12(13) at δ 139.6 and 129.6. In the HMBC experiment, the key correlations were observed between H-23 and C-3, C-4, C-5, between H-1′ and C-3, and between H-1″ and C-28. Therefore, the positions of the C-23 and the sugar linkages of **4** were elucidated, and the structure was assigned to be 3-*O*-*β*-D-xylopyranosyl-3*β*,19α,23-trihydroxyurs-12(13)-dien-28-oic acid-28-*O*-*β*-D-glucopyranosyl ester and named oblonganoside I.

Compound **5** was obtained as an amorphous powder and exhibited a negative specific rotation $[\alpha]_D^{21}$ −72.3 (*c* 0.37, MeOH), with IR ν_{max} (film)/cm^{-1} of 3432, 2934, 1729, 1639, 1465, 1348, 1032, and 1001 assignable to ester carbonyl and double bond. The negative FABMS of **5** showed a quasimolecular ion peak at *m/z* 691 [M − H]$^-$, and HRFABMS analysis revealed the molecular formula of **5** to be $C_{38}H_{60}O_{11}$. The other significant ion peak was at *m/z* 529 [M − H − 162]$^-$, corresponding to the loss of one hexosyl unit. The ^{13}C NMR spectrum and DEPT of **5** exhibited 6 methyl, 12 methylene, 12 methine, and 8 quarternary carbon signals, including 1 ester carbonyl at δ_C 178.5, 2 sp^2 carbon signals at δ_C 139.6 and 129.4, and two anomeric carbon signals at δ_C 103.1 and 95.7. The ^{1}H NMR spectrum of **5** presented five singlet methyl signals and one doublet methyl signal [δ_H 1.33, 1.19, 1.04, 1.00, 0.76, and (3H, d, *J* = 6.7 Hz)], an olefin proton signal at δ_H 5.29 (br s), and two anomeric proton signals at δ_H 5.31 (1H, d, *J* = 7.7 Hz) and 4.65 (1H, d, *J* = 5.6 Hz). In comparison with the NMR data of pomolic acid and the aglycone of **5**, the position C-23 was changed. The HBMC experiment confirmed the position of C-23. The correlations were observed between H-23 and C-4, C-3, and C-5. Thus, the aglycone was 3*β*,19α23-trihydroxyurs-12-dien-28-oic acid. Alkaline hydrolysis of **5** performed with 1 M KOH yielded a prosaopgenin and D-glucose by detected HPTLC. The data of the anomeric carbon signal at δ_C 95.7 and the anomeric proton signal at δ_H 5.31 (d, *J* = 7.7 Hz) suggestinged that glucose was in the *β* form and was bound to the aglycone by a glycosidic linkage at C-28. The carbon signal at δ_C 103.1, a methine signal, should be an acetal carbon signal. The connection of the ethylidene group was confirmed by the HMBC and the ^{13}C NMR spectra (see **Table 1**). The HMBC spectrum exhibited cross-peaks between H-1′ of the ethylidene group and C-23 and C-3 of the genin and between H-2′ and C-1′. The NOESY spectrum presented cross-peaks between H-1′ and H-3, H-23, and H-2′. These observations indicated that the ethylidene group was attached to C-3 and C-23. The structure of **5** was therefore defined as 3,23-*O*-hydroxyethylidene-3*β*,-19α,23-trihydroxyurs-12-dien-28-oic acid 28-*O*-*β*-D-glucopyranosyl ester, named oblonganoside J.

Compounds **8**−**10** were all triterpenes of oleanane types because of their double bond signals of ^{13}C NMR data about δ 143 and 124, suggesting the oleanane type (see **Table 1**). They also had the same sugars and linkages in NMR spectra. **8** was obtained as a white powder and exhibited a quasimolecular ion peak at *m/z* 765 [M − H]$^-$ and main fragment ions at *m/z* 603 [M − H − 162]$^-$ and 471 [M − H − 162 − 132]$^-$ in negative FABMS. HRFABMS analysis revealed the molecular formula of **8** to be $C_{41}H_{66}O_{13}$. The ^{1}H NMR spectrum of **8** gave seven singlet methyl signals at δ 1.28, 1.04, 0.93 × 3, 0.84, and 0.73 and two anomeric proton signals at δ 5.36 (1H, d, *J* = 8.0 Hz)

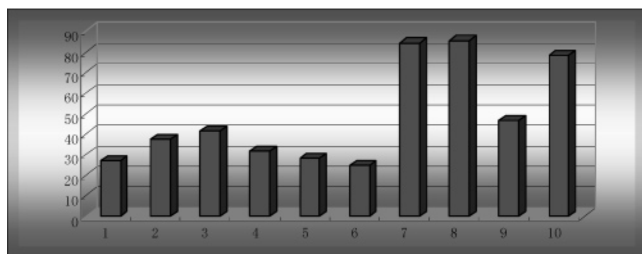

Figure 2. Inhibitory activity of compounds **1**–**10** from the leaves of *I. oblonga* against TMV replication at 0.2 mg/mL.

and 4.26 (1H, d, $J = 7.6$ Hz). In the HMBC experiment of **8**, long-range correlations presented between the following proton and carbon pairs: between H-19 and C-13, C-17, C-18, C-20, C-29, and C-30; between H-1′ and C-3; and between H-1″ and C-28. These cross-peaks disclosed the positions of hydroxyl in C-19 and two sugar linkages. The aglycone of **8** was identified as siaresinolic acid by comparison with the NMR data of a published paper (*21*). Therefore, **8** was determined to be 3-*O*-β-D-xylopyranosylsiaresinolic acid-28-*O*-β-D-glucopyranosyl ester and named oblonganoside K.

Compounds **9** and **8** were isomers. They had the same oleanane type, sugars, and their linkages. The main differences were hydroxyl positions of the aglycones. In the ^{1}H NMR spectrum of **9**, six singlet signals were observed at δ 1.14, 1.01, 0.92, 0.89, 0.81, and 0.77. The number of methyls implied that a methyl was oxidated. The HMBC experiment showed cross-peaks between H-29 (δ 3.30) and C-20 (δ 36.8) and C-30 (δ 19.5) and between H-30 (δ 0.89) and C-29 (δ 74.4). Consequentyl, the structure of **9** was assigned as 3-*O*-β-D-xylopyranosyl-3β,29-dihydroxyoleanolic acid-28-*O*-β-D-glucopyranosyl ester and named oblonganoside L.

Although compound **10** was a derivative of **8**, **10** exhibited a quasimolecular ion peak at *m/z* 781 $[M - H]^-$ and main fragment peaks at *m/z* 619 $[M - H - 162]^-$ and 487 $[M - H - 162 - 132]^-$. In the ^{1}H NMR spectrum of **10**, six singlet signals were observed at δ 1.15, 0.93 $\times$ 3, 0.78, and 0.76. The number of methyls implied that a methyl should be oxidated, and the ^{13}C NMR data gave the methyl signal at δ 12.9, for which the methyl could be C-24. This assignment was confirmed by the HMBC experiment. Correlations were observed between H-23 (δ 3.52) and C-4 (δ 40.4), C-3 (δ 83.2), and C-5 (δ 49.0). Therefore, the structure of **10** was assigned as 3-*O*-β-D-xylopyranosyl-3β,19α,23-trihydroxyoleanolic acid-28-*O*-β-D-glucopyranosyl ester and named oblonganoside M.

The inhibitory activities of compounds **1**−**10** against TMV were evaluated in vitro. Of the 10 compounds tested at a concentration of 0.2 mg/mL, compounds **7**, **8**, and **10** showed higher inhibitory activity against TMV replication than other compounds, with 84.5, 85.7, and 78.5% inhibitions, respectively (see **Figure 2**). The results of inhibitory effect of compounds **7**, **8**, and **10** at several concentrations are shown in **Table 4**. The EC_{50} values of compounds **7**, **8**, and **10** were determined to be 0.08, 0.076, and 0.085 mg/mL respectively. On the basis of the above results, it should concluded that the three triterpenoid saponins, namely, siaresinolic acid-28-*O*-β-D-glucopyranosyl ester (**7**), oblonganoside K (**8**), and oblonganoside M (**10**), were main active components of leaf extracts of *I. oblonga* against TMV replication. Further investigations are in progress to study the mechanism of active components in inhibiting viral replication.

Table 4. Inhibitory Activity of Compounds **7**, **8**, and **10** against TMV Replication

compound	final concentration (mg/mL)	inhibition rate(%)	EC_{50} (mg/mL)
siaresinolic acid 28-*O*-β-D-glucopyranosyl ester (**7**)	0.4	89.2	0.080
	0.2	84.5	
	0.1	65.7	
	0.05	25.3	
	0.025	7.6	
oblonganoside K (**8**)	0.4	88.5	0.076
	0.2	85.7	
	0.1	63.9	
	0.05	31.4	
	0.025	13.1	
oblonganoside M (**10**)	0.4	85.3	0.085
	0.2	78.5	
	0.1	61.5	
	0.05	32.7	
	0.025	5.2	

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