# Six New Triterpenoid Saponins from the Leaves of *Ilex oblonga* and Their Inhibitory Activities against TMV Replication

Zu-Jian Wu,[a] Ming-An Ouyang,*[,a,b] Cong-Zhou Wang,[b] and Zheng-Kun Zhang[a]

[a] *Key Laboratory of Bio-pesticide and Chemistry-Biology, Ministry of Education, Fujian Agriculture and Forestry University; Fuzhou, Fujian, 350002, P. R. China: and* [b] *Department of Bio-engineering & Technology, Huaqiao University; Quanzhou, Fujian, 362011, P. R. China.* Received November 3, 2006; accepted December 21, 2006

**Six new triterpenoid saponins of the ursane types were isolated from the MeOH extract of the leaves of *Ilex oblonga*. They were oblonganoside A (1), oblonganoside B (2), oblonganoside C (6), oblonganoside D (8), oblonganoside E (9), and oblonganoside F (10), together with three known triterpenoid saponins. The structures of these compounds were elucidated on the basis of spectroscopic analysis, and compound 1 showed appreciable inhibitory activity against TMV replication with $EC_{50}$ value 0.074 mM.**

**Key words** Aquifoliaceae; *Ilex oblonga*; triterpenoid saponin; Tobacco mosaic virus (TMV)

*Ilex* (Aquifoliaceae) species are distributed widely in the People's Republic of China, and some are used extensively in folk medicine. For example, *I. rotunda* is an antipyretic and antidote and used for the treatment of the common cold, tonsillitis, and stomach and intestinal ulcers. *I. pubescens* is used for the treatment of coronary disease, myocardial infarction, dysentery, and erysipelas. *I. cornuta* and *I. latifolia* are used for the treatment of headache, toothache, bloodshot eyes, and tinnitus. Many reports indicate that saponins are the major constituents of *Ilex* species.[1—5] 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] were reported. Several biological activities were related to the compounds isolated from *Ilex* species, including hypocholesterolemic[14] and antioxidant[15,16] activities.

In a continuation of our study on the constituents of the medicinal plants of the Aquifoliaceae family.[17,18] We investigated the water-soulble fraction of *Ilex oblonga*. This plant is a well-known endemic herb to treat gumboil, eczema, rheumatism, scald and bruise in Guangxi province of China.[19] No previous phytochemical investigation on *I. oblonga* has been reported. In this paper, we describe the isolation and structure elucidation of ten triterpenoid saponins designated as oblonganoside A (**1**), oblonganoside B (**2**), oblonganoside C (**6**), oblonganoside D (**8**), oblonganoside E (**9**), oblonganoside F (**10**) along with four known triterpenoid saponins: 3$\beta$,23-dihydroxyurs-12,19(20)-dien-28-oic acid 28-$\beta$-D-glucopyranosyl ester (**3**),[20] 3$\beta$,23-dihydroxyursa-12,18(19)-dien-28-oic acid 28-$\beta$-D-glucopyranosyl ester (**4**),[20] 3-*O*-$\beta$-D-xylopyranosyl-3$\beta$-hydroxyurs-12,18(19)-dien-28-oic acid 28-$\beta$-D-glucopyranosyl ester (**5**).[21] 3$\beta$,23-Dihydroxyurs-12,19(29)-dien-28-oic acid 28-$\beta$-D-glucopyranosyl ester (**7**)[22] from the leaves of *I. oblonga*.

## 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 saponons **1**—**10**.

Compound **1** was obtained as a white powder and exhib-

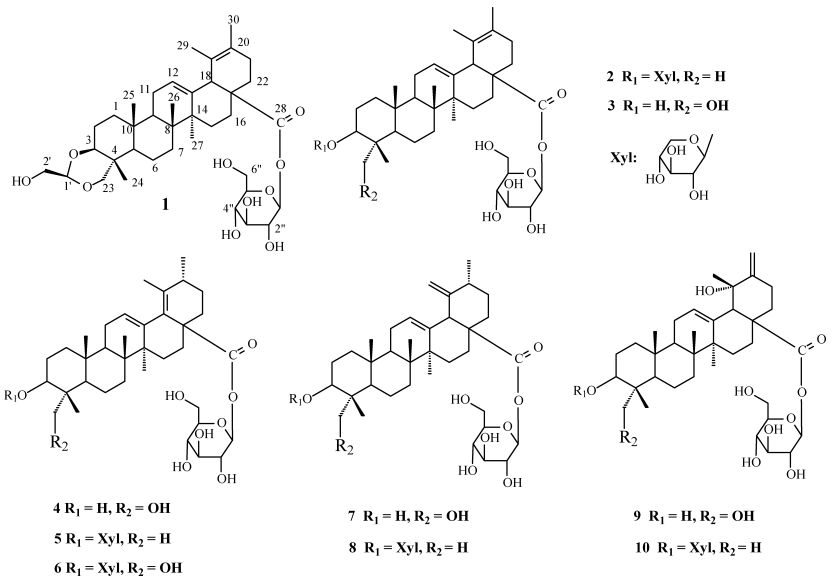


Fig. 1. Structures of Compounds **1**—**10**

* To whom correspondence should be addressed. e-mail: maouyang@yahoo.com

ited a negative specific rotation −29° ($c$=0.24, MeOH). IR $\nu_{max}$ (film)/cm$^{-1}$: 3410, 2925, 1729, 1640, 1440, 1210, 1082, 995 were assignable to ester carbonyl and double bond. The negative FAB-MS of **1** showed a quasimolecular ion peak at $m/z$ 673 [M−H]$^-$, indicating a molecular weight of 674, and HR-FAB-MS analysis revealed the molecular formula of **1** to be $C_{38}H_{58}O_{10}$. The other significant ion peak was at $m/z$ 511 [(M−H)−162]$^-$, corresponded to the loss of one hexosyl unit. The $^{13}$C-NMR spectrum and DEPT of **1** displayed six methyl, twelve methylene, eleven methine, and nine quarternary carbon signals, including one ester carbonyl at $\delta_C$ 177.8, four $sp^2$ carbon signals at $\delta_C$ 138.5, 129.3, 128.5, and 125.0, one anomeric carbon signal at $\delta_C$ 95.7, and one methine carbon signal at $\delta_C$ 103.1. The $^1$H-NMR spectrum of **1** exhibited six singlet methyl signals, an olefin proton signal at $\delta_H$ 5.47 (br s), one anomeric proton signal at $\delta_H$ 5.38 (d, $J$=7.7 Hz), and one methine proton signal at $\delta_H$ 4.64 (br s). Alkaline hydrolysis of **1** performed with 3% KOH yielded a prosaopgenin and glucose by confirmed HPTLC. The data of anomeric carbon signal at $\delta_C$ 95.7 and anomeric proton signal at $\delta_H$ 5.38 (d, $J$=7.7 Hz) indicated glucose was $\beta$ form and was bound to the aglycone by a glycosidic linkage at C-28. And all data of NMR spectra in **1** should be to imply an ursane type of the aglycone. Comparison with the NMR data of the aglycone and ursolic acid, triterpene of ursolic acid commonly was seven methyl groups and the methyl signal of C-19, C-20 were all a doublet peak in $^1$H-NMR spectrum. But compound **1** was six singlet methyl signal, these data indicated the position of C-20 was changed to make a double bond, or bearing oxygen. In comparing the NMR data of ursolic acid and the aglycone of **1**, the position C-19, C-20, and C-23 were changed. The C-23 signal was at $\delta$ 79.1, and the methyl of C-24 was at $\delta$ 13.9, whose the carbon signal was upfield due to this carbon by shielded of the methyl of C-25 and hydroxyl methylene of C-23 (see Figs. 2a, b). The HBMC experiments confirmed the positions of double bonds and the C-23. The correlations were observed between H-30 and C-20, C-19, C-21, between H-29 and C-19, C-20, C-18, between H-23 and C-4, C-3, C-5 (see Fig. 2). Thus, the aglycone was 3$\beta$,23-dihydroxyurs-12,19(20)-dien-28-oic acid. The carbon signal at $\delta_C$ 103.1, a methine signal, should be an acetal carbon. The connection of the ethylidene group was confirmed by the HMBC and the $^{13}$C-NMR spectra. The HMBC spectrum showed cross-peaks between H-1′ of the ethylidene group and C-23, C-3 of the genin, and between H-2′ and C-1′ (Fig. 2). NOESY spectrum observed cross-peaks between H-1′ and H-3, H-23, H-2′. These observations indicated that the ethylidene group was attached to C-3 and C-23. However, the configuration of the ethylidene group was not determined. The structure of **1** was therefore defined as 3,23-*O*-hydroxyethylidene-3$\beta$,23-dihydroxyurs-12,19(20)-dien-28-oic acid 28-$\beta$-D-glucopyranosyl ester, and named as oblonganoside A.

The negative FAB-MS of **2** gave a quasimolecular ion peak at $m/z$ 747 [M−H]$^-$, and the main fragment peak at $m/z$ 585 [M−H−162]$^-$, 453 [M−H−162−132]$^-$. Acid hydrolysis of **1** afforded a mixture of sugars, which were identified as glucose and xylose by detected HPTLC. Alkaline hydrolysis of **1** performed with 3% KOH yielded glucose and a prosapogenin, which furnished xylose and a genin by acid hydrolysis subsequently. These chemical reactive results manifested that **1** was a triterpene-bidesmosidic saponin in which xylose should link the aglycone as ether bond, and while the remaining sugar should be bound to the genin by a glycosidic ester. Compound **2** was a derivative of **1**. They were main different from sugar moiety and methyl signals. In NMR spectra, the methyl signals were seven and all singlet peak, the presence signals indicated C-23 of genin didn't be oxidated and C-19, C-20 positions were formed a double bond. The HBMC experiments confirmed the positions of double bonds and the sugars. The correlations were observed between H-30 and C-20, C-19, C-21, between H-29 and C-19, C-20, C-18, between H-1 [$\delta_H$ 4.25 (d, $J$=7.7 Hz), Xyl] and C-3 ($\delta_C$ 90.6), between H-1 [$\delta_H$ 5.39 (d, $J$=7.8 Hz), Glc] and C-28 ($\delta_C$ 177.8). Thus, compound **2** was determined to be 3-*O*-$\beta$-D-xylopyranosyl-3$\beta$-hydroxyurs-12,19(20)-dien-28-oic acid 28-$\beta$-D-glucopyranosyl ester, and named oblonganoside B.

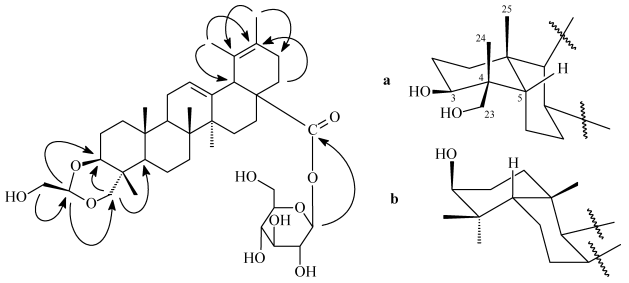


Fig. 2. The Key HMBC Correlations of Compound **1**

Compound **5** has the same molecular weight as compounds **2** and **8**. The negative FAB-MS of **5**, **2**, **8** all showed a quasimolecular ion peak at $m/z$ 747 [M−H]$^-$, HR-FAB-MS analysis revealed the molecular formula to be $C_{41}H_{64}O_{12}$. The other significant ion peaks were at $m/z$ 585 [M−H−162]$^-$, 453 [M−H−162−132]$^-$, corresponded to the loss of one hexosyl and pentosyl units. The NMR data exhibited the same sugar moiety and the linkages of **2**, **5**, **8**. The $^1$H-NMR spectrum of **5** gave seven methyl signals at $\delta$ 1.66, 1.09, 1.04, 1.00, 0.98, 0.92, and 0.82 including a doublet peak at $\delta$ 1.09 (3H, d, $J$=7.2 Hz). The $^{13}$C-NMR spectrum showed two double bonds at $\delta$ 139.6, 137.5, 134.0, and 127.7. In the HMBC experiment of **5**, long-range correlations were observed between the following proton and carbon pairs: between H-29 and C-18 (134.0), C-19 (137.5), C-20 (35.6); between H-30 and C-19 (137.5), C-20 (35.6); between H-1′ and C-3; between H-1″ and C-28. Thus the positions of the double bond and $\beta$-glucopyranosyl and $\beta$-xylcopyranosyl moieties in **5** were clarified. On the basis of those findings, the structure of compound **5** was elucidated as 3-*O*-$\beta$-D-xylopyranosyl-3$\beta$-hydroxyurs-12,18(19)-dien-28-oic acid 28-$\beta$-D-glucopyranosyl ester.[21]

The same reasoning, the $^1$H-NMR spectrum of **8** gave a terminal double bond signals [$\delta$ 4.99 (1H, d, $J$=2.0 Hz), 4.95 (1H, d, $J$=2.0 Hz)] and six methyl signals at $\delta$ 1.15, 1.12, 1.05, 0.98, 0.85, and 0.83 including a doublet peak at $\delta$ 1.12 (3H, d, $J$=6.5 Hz). The HMBC spectrum confirmed the terminal double bond position at C-19 (29). Thus oblonganoside D was determined as shown.

Comparison with the NMR data of **5** and **6** showed the same sugar moiety and linkages, the main difference were their methyl signals. In $^1$H-NMR spectrum of **6**, six methyl

Table 1. $^{13}$C-NMR (125 MHz, $CD_3OD$) Data of Compounds **1**—**10**

| No. | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 40.4 | 40.2 | 40.3 | 40.1 | 40.2 | 40.0 | 39.7 | 39.9 | 39.7 | 39.9 |
| 2 | 24.2 | 27.2 | 27.9 | 27.5 | 27.5 | 26.6 | 26.4 | 26.4 | 26.2 | 26.3 |
| 3 | 86.6 | 90.6 | 74.2 | 74.0 | 90.7 | 83.3 | 73.6 | 90.4 | 74.1 | 90.7 |
| 4 | 37.9 | 40.3 | 43.7 | 43.3 | 40.3 | 43.9 | 43.4 | 40.6 | 43.3 | 40.2 |
| 5 | 52.8 | 57.2 | 49.3 | 49.0 | 57.2 | 49.0 | 49.1 | 57.1 | 49.0 | 57.1 |
| 6 | 18.7 | 19.3 | 19.4 | 19.1 | 19.3 | 18.7 | 19.2 | 19.3 | 19.2 | 19.3 |
| 7 | 34.4 | 35.0 | 34.9 | 35.3 | 35.8 | 35.8 | 33.8 | 34.2 | 35.6 | 34.2 |
| 8 | 40.7 | 40.7 | 40.9 | 40.3 | 40.3 | 40.2 | 40.6 | 40.2 | 40.6 | 40.6 |
| 9 | 49.0 | 49.3 | 49.3 | 49.2 | 49.0 | 49.0 | 49.1 | 49.0 | 49.0 | 49.0 |
| 10 | 38.3 | 37.0 | 38.2 | 37.8 | 37.8 | 37.5 | 38.1 | 38.0 | 38.0 | 38.0 |
| 11 | 24.4 | 24.5 | 24.9 | 24.2 | 24.2 | 24.2 | 24.7 | 24.6 | 24.6 | 24.6 |
| 12 | 128.5 | 128.7 | 129.1 | 127.6 | 127.7 | 127.7 | 129.5 | 129.5 | 129.6 | 129.9 |
| 13 | 138.5 | 138.6 | 139.0 | 139.6 | 139.6 | 139.6 | 138.4 | 138.2 | 137.9 | 137.6 |
| 14 | 44.5 | 44.6 | 45.0 | 45.8 | 45.7 | 45.7 | 43.7 | 43.6 | 43.8 | 43.6 |
| 15 | 24.3 | 24.3 | 24.6 | 31.6 | 31.6 | 31.5 | 27.7 | 27.2 | 27.5 | 27.1 |
| 16 | 29.2 | 29.3 | 29.6 | 29.7 | 29.7 | 29.7 | 29.9 | 29.8 | 29.6 | 29.6 |
| 17 | 49.0 | 48.4 | 49.0 | 50.8 | 50.8 | 50.8 | 50.7 | 50.7 | 50.1 | 49.9 |
| 18 | 51.2 | 51.3 | 51.6 | 134.0 | 134.0 | 134.0 | 53.0 | 52.9 | 49.0 | 49.0 |
| 19 | 129.3 | 129.4 | 129.8 | 137.5 | 137.5 | 137.5 | 154.5 | 154.4 | 84.4 | 82.4 |
| 20 | 125.0 | 125.0 | 125.3 | 35.6 | 35.6 | 35.6 | 38.6 | 38.5 | 153.8 | 149.5 |
| 21 | 29.2 | 29.3 | 29.6 | 27.5 | 27.2 | 27.4 | 31.7 | 31.6 | 33.7 | 31.7 |
| 22 | 33.5 | 33.6 | 33.9 | 35.8 | 35.8 | 35.3 | 38.0 | 37.9 | 32.1 | 31.7 |
| 23 | 79.1 | 28.6 | 67.7 | 67.7 | 28.6 | 64.7 | 67.2 | 28.5 | 67.7 | 28.5 |
| 24 | 13.9 | 17.2 | 13.3 | 12.8 | 16.7 | 13.5 | 13.0 | 17.1 | 12.7 | 17.0 |
| 25 | 17.6 | 17.5 | 17.3 | 16.9 | 17.1 | 17.2 | 16.7 | 16.3 | 16.5 | 16.2 |
| 26 | 18.2 | 18.4 | 18.8 | 18.4 | 18.7 | 18.8 | 17.8 | 17.7 | 17.7 | 17.6 |
| 27 | 22.4 | 22.4 | 22.8 | 22.4 | 22.3 | 22.4 | 26.7 | 26.5 | 26.4 | 26.3 |
| 28 | 177.8 | 177.8 | 178.3 | 176.6 | 176.6 | 176.6 | 177.3 | 177.2 | 177.5 | 177.2 |
| 29 | 17.4 | 17.5 | 17.8 | 19.8 | 19.8 | 19.8 | 110.9 | 110.8 | 23.0 | 23.5 |
| 30 | 20.4 | 20.5 | 20.8 | 19.0 | 19.3 | 19.0 | 19.9 | 19.8 | 113.6 | 117.5 |
| 1′ | 103.1 | 107.4 | | | 107.4 | 106.6 | | 107.5 | | 107.5 |
| 2′ | 64.6 | 75.5 | | | 75.4 | 75.5 | | 75.4 | | 75.5 |
| 3′ | | 78.0 | | | 78.0 | 78.1 | | 78.0 | | 78.0 |
| 4′ | | 71.3 | | | 71.3 | 71.2 | | 71.1 | | 71.1 |
| 5′ | | 66.7 | | | 66.7 | 66.8 | | 66.8 | | 66.7 |
| 1″ | 95.7 | 95.8 | 96.1 | 95.7 | 95.8 | 95.7 | 95.9 | 95.8 | 96.0 | 95.8 |
| 2″ | 73.8 | 74.0 | 74.3 | 74.1 | 74.0 | 74.0 | 73.9 | 73.8 | 73.9 | 73.9 |
| 3″ | 78.2 | 78.3 | 78.6 | 78.3 | 78.6 | 78.3 | 78.3 | 78.2 | 78.2 | 78.2 |
| 4″ | 71.1 | 71.3 | 71.5 | 71.2 | 71.3 | 71.1 | 71.2 | 71.2 | 71.3 | 71.2 |
| 5″ | 78.7 | 78.7 | 79.1 | 78.6 | 78.7 | 78.6 | 78.9 | 78.7 | 78.7 | 78.7 |
| 6″ | 62.4 | 62.6 | 62.8 | 62.6 | 62.6 | 62.5 | 62.4 | 62.3 | 62.6 | 62.4 |

signals were observed including a doublet peak at $\delta$ 1.09 (3H, d, $J$=7.0 Hz), these signals indicated one methyl group should be oxidated. The $^{13}$C-NMR spectrum gave the signal of methyl at $\delta$ 13.5 (C-24), which the methyl signal was downfield 3—4 ppm, suggesting that C-23 of methyl was oxidated (see Fig. 1). The HBMC experiments confirmed the positions of the methylene and the sugars. The correlations were observed between H-23 and C-4, C-3, C-5, C-24, between H-1 [$\delta_H$ 4.33 (d, $J$=7.6 Hz), Xyl] and C-3 ($\delta_C$ 83.3), between H-1 [$\delta_H$ 5.38 (d, $J$=7.8 Hz), Glc] and C-28 ($\delta_C$ 176.6). Therefore, the structure of **6** was determined as shown and named as oblonganoside C.

The negative FAB-MS of **9** gave a quasimolecular ion peak at $m/z$ 647 $[M-H]^-$, and main fragment peak at $m/z$ 485 $[M-H-162]^-$. The molecular formula $C_{36}H_{56}O_{10}$ was as shown by HR-FAB-MS. The $^1$H-NMR spectrum of **9** displayed five singlet methyl signals ($\delta$ 1.40, 1.13, 1.00, 0.83, 0.71), a terminal double bond proton signals [$\delta$ 5.29 (1H, d, $J$=1.9 Hz) and 5.10 (1H, d, $J$=1.9 Hz)], and an anomeric proton signal at $\delta$ 5.37 (1H, d, $J$=8.3 Hz). The $^{13}$C-NMR spectrum of **9** showed a characteristic carbon signal at $\delta$ 12.7 (C-24) and 67.7 (C-23). Comparing with the NMR data of the genin in **9** and pomolic acid, the characteristic signals of pomolic acid were presence in compound **9** such as C-19 (quarternary carbon $\delta$ 84.4), C-12(13) at $\delta$ 137.9 and 129.6. The main difference was their methyls. In the HMBC experiment, the correlations were observed between H-23 and C-3, C-4, C-5, between H-30 and C-20, C-19, between H-1″ and C-28. Thus, the positions of the C-23, double bond C-20(30), and the sugar moiety of **9** were elucidated. Consequently, the structure was assigned to be 3$\beta$,19$\alpha$,23-trihydroxyurs-12,20(30)-dien-28-oic acid 28-$\beta$-D-glucopyranosyl ester, and named oblonganoside E.

Compound **10** was a derivative of **9**, and revealed a quasimolecular ion peak at $m/z$ 763 $[M-H]^-$, main fragment ion peaks at $m/z$ 601 $[M-H-162]^-$, 469 $[M-H-162-132]^-$ in the negative FAB-MS. Most NMR spectroscopic data of the aglycone moiety in **10** resemble those of **9**, except for sugar moiety and methyl signals. But, the NMR data of sugar moiety in **10** and **2**, **5**, **6**, **8** had the same sugars and linkages. The $^1$H-NMR spectrum of **10** presented six singlet methyl signals ($\delta$ 1.41, 1.12, 1.04, 0.97, 0.84, 0.82), a terminal olefin proton signals [$\delta$ 5.35 (1H, d, $J$=1.4 Hz) and 5.28 (1H, d, $J$=1.4 Hz)], and two anomeric proton signal at $\delta$ 5.37 (1H, d,

Table 2. $^1$H-NMR (500 MHz, $CD_3OD$) Data of Compounds **1**—**10**

| No. | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.78, m | 1.68, m | 1.70, m | 1.73, m | 1.64, m | 1.75, m | 1.67, m | 1.66, m | 1.67, m | 1.67, m |
| | 1.12, m | 0.99, m | 1.00, m | 1.01, m | 1.01, m | 1.02, m | 1.05, m | 1.04, m | 1.04, m | 1.02, m |
| 2 | 1.97, m | 1.96, m | 1.94, m | 1.95, m | 1.97, m | 1.92, m | 1.77, m | 1.80, m | 1.77, m | 1.81, m |
| | 1.93, m | 1.93, m | 1.78, m | 1.80, m | 1.82, m | 1.88, m | 1.73, m | 1.73, m | 1.73, m | 1.65, m |
| 3 | 3.37, overlap | 3.13, overlap | 3.61, overlap | 3.61, overlap | 3.14, overlap | 3.61, overlap | 3.61, br d, 11.3 | 3.13, overlap | 3.60, overlap | 3.13, overlap |
| 5 | 0.83, br d, 12.2 | 0.76, br d, 12.2 | 1.13, br d, 12.2 | 1.16, br d, 13.0 | 0.89, br d, 12.3 | 1.25, br d, 12.0 | 1.18, br d, 11.8 | 0.81, br d, 12.1 | 1.17, br d, 12.1 | 0.79, br d, 12.0 |
| 6 | 1.42, m | 1.52, m | 1.39, m | 1.74, m | 1.53, m | 1.45, m | 1.40, m | 1.52, m | 1.40, m | 1.53, m |
| | 1.17, m | 1.36, m | 1.36, m | 1.65, m | 1.38, m | 1.30, m | 1.38, m | 1.40, m | 1.36, m | 1.41, m |
| 7 | 1.53, m | 1.50, m | 1.58, m | 1.63, m | 1.55, m | 1.67, m | 1.59, m | 1.47, m | 1.59, m | 1.49, m |
| | 1.35, m | 1.39, m | 1.33, m | 1.43, m | 1.40, m | 1.45, m | 1.30, m | 1.32, m | 1.30, m | 1.33, m |
| 9 | 1.53, m | 1.43, m | 1.48, m | 1.47, m | 1.43, m | 1.42, m | 1.44, m | 1.46, m | 1.74, m | 1.71, m |
| 11 | 1.95, m | 1.93, m | 1.93, m | 1.95, m | 1.94, m | 1.95, m | 1.99, m | 1.98, m | 1.99, m | 1.98, m |
| 12 | 5.47, br s | 5.4, t, 3.5 | 5.47, br s | 5.38, br s | 5.39, br s | 5.38, br s | 5.32, br s | 5.30, br s | 5.34, br s | 5.36, br s |
| 15 | 1.56, m | 1.53, m | 1.51, m | 1.95, m | 1.93, m | 1.93, m | 1.88, m | 1.88, m | 1.93, m | 1.94, m |
| | 1.21, m | 1.26, m | 1.22, m | 1.14, m | 1.19, m | 1.14, m | 1.07, m | 1.14, m | 1.20, m | 1.20, m |
| 16 | 1.61, m | 1.79, m | 1.69, m | 1.90, m | 1.83, m | 1.65, m | 1.63, m | 1.63, m | 1.71, m | 1.71. m |
| | 1.09, m | 1.65, m | 1.60, m | 1.78, m | 1.58, m | 1.63, m | 1.59, m | 1.59, m | 1.65, m | 1.65, m |
| 18 | 3.18, s | 3.19, s | 3.19, s | | | | 3.44, s | 3.44, s | 3.86, s | 3.72, s |
| 20 | | | | 1.43, m | 1.47, m | 1.45, m | 1.98, m | 1.98, m | | |
| 21 | 2.25, m | 2.22, m | 2.23, m | 2.21, m | 2.24, m | 2.24, m | 1.63, m | 1.84, m | 2.07, m | 2.06, m |
| | 1.87, m | 1.84, m | 1.85, m | 1.86, m | 1.82, m | 1.82, m | 1.32, m | 1.68, m | 1.93, m | 1.93, m |
| 22 | 1.82, m | 1.82, m | 1.81, m | 2.24, br d, 13.1 | 2.24, br d, 13.0 | 2.25, br d, 13.0 | 1.88, m | 1.88, m | 1.56, m | 1.63, m |
| | 1.66, m | 1.65, m | 1.67, m | 2.17, m | 2.17, m | 2.16, m | 1.77, m | 1.77, m | 1.03, m | 1.56, m |
| 23 | 3.79, d, 10 | 1.04, s | 3.51, d, 11.1 | 3.52, d, 11.0 | 1.04, s | 3.61, d, 11.6 | 3.51, d, 10.8 | 0.85, s | 3.52, d, 10.4 | 0.84, s |
| | 3.27, d, 10 | | 3.28, d, 11.1 | 3.30, d, 11.0 | | 3.27, d, 11.6 | 3.27, d, 10.8 | | 3.26, d, 10.4 | |
| 24 | 1.05, s | 0.84, s | 0.68, s | 0.70, s | 0.82, s | 0.70, s | 0.70, s | 1.05, s | 0.71, s | 1.04, s |
| 25 | 1.03, s | 0.98, s | 1.00, s | 1.02, s | 1.00, s | 1.03, s | 1.00, s | 0.98, s | 1.00, s | 0.97, s |
| 26 | 0.87, s | 0.87, s | 0.87, s | 0.92, s | 0.92, s | 0.92, s | 0.84, s | 0.83, s | 0.83, s | 0.82, s |
| 27 | 1.00, s | 0.98, s | 0.99, s | 1.00, s | 0.98, s | 1.00, s | 1.16, s | 1.15, s | 1.13, s | 1.12, s |
| 29 | 1.56, s | 1.56, s | 1.56, s | 1.74, s | 1.66, s | 1.74, s | 5.00, d, 2 | 4.99, d, 2 | 1.40, s | 1.41, s |
| | | | | | | | 4.96, d, 2 | 4.95, d, 2 | | |
| 30 | 1.63, s | 1.63, s | 1.63, s | 1.09, d, 7.0 | 1.09, d, 7.2 | 1.09, d, 7.0 | 1.13, d, 6.3 | 1.12, d, 6.5 | 5.29, d, 1.9 | 5.35, d, 1.4 |
| | | | | | | | | | 5.10, d, 1.9 | 5.28, d, 1.4 |
| 1′ | 4.64, br s | 4.25, d, 7.7 | | | 4.26, d, 7.2 | 4.33, d, 7.6 | | 4.26, d, 7.6 | | 4.25, d, 7.6 |
| 2′ | 3.78, br d, 7.2 | 3.19, m | | | 3.17, m | 3.14, m | | 3.17, m | | 3.19, m |
| | 3.27, br d, 7.2 | | | | | | | | | |
| 3′ | | 3.26, m | | | 3.28, m | 3.27, m | | 3.26, m | | 3.26, m |
| 4′ | | 3.50, m | | | 3.48, m | 3.49, m | | 3.50, m | | 3.50, m |
| 5′ | | 3.81, br d, 11.6 | | | 3.82, d, 11.5 | 3.82, d, 11.1 | | 3.81, d, 11.4 | | 3.81, d, 11.3 |
| | | 3.17, overlap | | | 3.17, overlap | 3.19, overlap | | 3.17, overlap | | 3.17, overlap |
| 1″ | 5.38, d, 7.7 | 5.39, d, 7.8 | 5.38, d, 7.6 | 5.38, d, 7.7 | 5.38, d, 7.7 | 5.38, d, 7.8 | 5.39, d, 7.9 | 5.38, d, 8.0 | 5.37, d, 8.3 | 5.37, d, 8.3 |
| 2″ | 3.31, m | 3.30, m | 3.30, m | 3.28, m | 3.30, m | 3.30, m | 3.30, m | 3.30, m | 3.30, m | 3.30, m |
| 3″ | 3.39, m | 3.40, m | 3.39, m | 3.38, m | 3.38, m | 3.40, m | 3.40, m | 3.41, m | 3.39, m | 3.41, m |
| 4″ | 3.25, m | 3.24, m | 3.25, m | 3.24, m | 3.24, m | 3.24, m | 3.25, m | 3.24, m | 3.25, m | 3.24, m |
| 5″ | 3.33, m | 3.34, m | 3.34, m | 3.33, m | 3.33, m | 3.33, m | 3.34, m | 3.34, m | 3.34, m | 3.34, m |
| 6″ | 3.80, br d, 2.0 | 3.80, br d, 11.6 | 3.80, br d, 11.6 | 3.80, br d, 12.0 | 3.79, br d, 11.5 | 3.80, br d, 12.1 | 3.80 br d, 11.6 | 3.80, br d, 11.3 | 3.80, br d, 11.0 | 3.80, br d, 9.8 |
| | 3.67, dd, 11.6, 3.2 | 3.66, dd, 11.6, 4.0 | 3.67, dd, 11.8, 4.3 | 3.67, dd, 12.0, 3.9 | 3.67, dd, 11.5, 3.9 | 3.67, br d, 12.1 | 3.66, br d, 11.3 | 3.67, dd, 11.3, 3.2 | 3.67, br d, 11.6 | 3.67, br d, 11.0 |

Table 3. The TMV Multiplication Inhibition of Compounds **1**—**10**

| No. | − | + | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TR[a)] | 0 | 1.0456 | 0.2060 | 0.4559 | 0.6159 | 0.5510 | 0.7006 | 0.6420 | 0.7361 | 0.6514 | 0.8208 | 0.6305 |
| %[b)] | 100 | 0 | 80.3 | 56.4 | 41.1 | 47.3 | 33.0 | 38.6 | 29.6 | 37.7 | 21.5 | 39.7 |

*a*) Concentration of TMV in every treatment (ng). *b*) Inhibition rate. "+" is positive control and "−" is negative control. The concentration of each compound is 0.2 mg/ml. The TMV standard curve was $y=0.3276\ln(x)+0.6379$, in which the correlation coefficient was 0.9795, and the "$x$" was the value of $OD_{405}$. According the formula we obtained the inhibition rate of every compound.

Table 4. Inhibitory Activity of Compound **1** against TMV Replication

| Compound | Final concentration (mg/ml) | Inhibition rate (%) | $EC_{50}$ (mg/ml) |
|---|---|---|---|
| Oblonganoside A (**1**) | 0.4 | 87.3 | 0.074 |
| | 0.2 | 80.7 | |
| | 0.1 | 61.9 | |
| | 0.05 | 30.1 | |
| | 0.025 | 10.1 | |

$J$=8.3 Hz), 4.25 (1H, d, $J$=7.6 Hz). So, the aglycone of **10** was 3$\beta$,19$\alpha$-dihydroxyurs-12,20(30)-dien-28-oic acid. Based on the above evidences, the structure of **10** is elucidated as 3-*O*-$\beta$-D-xylopyranosyl-3$\beta$,19$\alpha$-dihydroxyurs-12,20(30)-dien-28-oic acid 28-$\beta$-D-glucopyranosyl ester, named oblonganoside F.

The inhibitory activities of compounds **1**—**10** against Tobacco mosaic virus (TMV) were evaluated *in vitro*.[24,25] Of the ten compounds tested, at a concentration of 0.2 mg/ml, compound **1** showed higher inhibitory activity against TMV replication with 80.3% than other compounds. The results of inhibitory effect of compound **1** at several concentrations are shown in Table 4. The straight line concentration-inhibition experiments showed a max inhibition to be close to 90% at 0.4 mg/ml and this concentration may be not potency and efficacy. Otherwise, the $EC_{50}$ value of compounds **1** was as high as 0.074 mg/ml, which one was an important value to potency of anti-TMV. On the basis of the above results, it may be concluded that the triterpenoid saponin, namely, oblonganoside A (**1**) should be a major active component of leave extracts of *Ilex oblonga* against TMV replication. Further investigations are in progress to study the action mechanism of active components on inhibiting viral replication.

## Experimental

**General Experimental Procedures** IR spectra were recorded with a Perkin-Elmer 1750 FT-IR spectrometer and the films of the all samples were measured on KBr disks. Optical rotations were measured with a Jasco DIP-180 digital polarimeter spectro-photometer. 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 phase used RP-18 (40—60 $\mu$m, Merck), silica gel (160—200 mesh), Sephadex LH-20 (25—100 $\mu$m, Pharmacia Fine Chemical Co. Ltd.), MCI-gel CHP20P (75—150 $\mu$m, 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 $\mu$m, 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 sugars. Compounds on TLC were detected by spraying with 5% $H_2SO_4$ followed heating. Sugars were detected by spraying with aniline-phthalate reagent.

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

**Extraction and Isolation** The leaves of *Ilex oblonga* (690 g) were extracted (2×4 l) with MeOH at room temperature (7 d×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). The 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 thirteen 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** (24 mg), **2** (14 mg), **3** (11 mg), **4** (26 mg), **5** (84 mg), **6** (22 mg), **7** (15 mg), and **8** (35 mg), **9** (45 mg), and **10** (25 mg).

Compound (**1**): Colorless amorphous powder, $C_{38}H_{58}O_{10}$; FAB-MS $m/z$ 673 $[M-H]^-$; 511 $[M-H-162]^-$; HR-FAB-MS $m/z$ 673.39513; $[\alpha]_D^{21}$ −29° ($c$=0.24, MeOH); IR (KBr) $\nu_{max}$/cm$^{-1}$: 3410, 2925, 1729, 1640, 1440, 1210, 1082, 995; $^1$H- and $^{13}$C-NMR (see Table 1).

Compound (**2**): Colorless amorphous powder, $C_{41}H_{64}O_{12}$; $[\alpha]_D^{21}$ −11° ($c$=0.12, MeOH); FAB-MS $m/z$ 747 $[M-H]^-$, 585 $[M-H-162]^-$, 453 $[M-H-162-132]^-$; HR-FAB-MS $m/z$ 747.43192 $[M-H]^-$; IR (KBr) $\nu_{max}$/cm$^{-1}$: 3380, 2927, 1726, 1644, 1442, 1248, 1062, 913; $^1$H- and $^{13}$C-NMR (see Table 1).

Compound (**3**): Amorphous powder, $C_{36}H_{56}O_9$; $[\alpha]_D^{21}$ −39° ($c$=0.26, MeOH); FAB-MS $m/z$ 631 $[M-H]^-$, 469 $[M-H-162]^-$; IR (KBr) $\nu_{max}$/cm$^{-1}$: 3320, 2921, 1728, 1642, 1430, 1278, 1032, 943; $^1$H- and $^{13}$C-NMR (see Table 1).

Compound (**4**): Amorphous powder, $C_{36}H_{56}O_9$; $[\alpha]_D^{21}$ +69° ($c$=0.16, MeOH); FAB-MS $m/z$ 631 $[M-H]^-$, 469 $[M-H-162]^-$; IR (KBr) $\nu_{max}$/cm$^{-1}$: 3360, 2925, 1730, 1653, 1622, 1446, 1386, 1366, 1258, 1072, 993; $^1$H- and $^{13}$C-NMR (see Table 1).

Compound (**5**): Colorless amorphous powder, $C_{41}H_{64}O_{12}$; $[\alpha]_D^{21}$ +41° ($c$=0.19, MeOH); FAB-MS $m/z$ 747 $[M-H]^-$, 585 $[M-H-162]^-$, 453 $[M-H-162-132]^-$; HR-FAB-MS $m/z$ 747.43194 $[M-H]^-$; IR (KBr) $\nu_{max}$/cm$^{-1}$: 3380, 2927, 1727, 1655, 1624, 1442, 1388, 1362, 1250, 996; $^1$H- and $^{13}$C-NMR (see Table 1).

Compound (**6**): Colorless amorphous powder, $C_{41}H_{64}O_{13}$; $[\alpha]_D^{21}$ +22° ($c$=0.07, MeOH); FAB-MS $m/z$ 763 $[M-H]^-$, 601 $[M-H-162]^-$, 469 $[M-H-162-132]^-$; HR-FAB-MS $m/z$ 763.42684 $[M-H]^-$; IR (KBr) $\nu_{max}$/cm$^{-1}$: 3389, 2929, 1728, 1627, 1439, 1368, 1342, 1260, 1002; $^1$H- and $^{13}$C-NMR (see Table 1).

Compound (**7**): Amorphous powder, $C_{36}H_{56}O_9$; $[\alpha]_D^{21}$ +11° ($c$=0.13, MeOH); FAB-MS $m/z$ 631 $[M-H]^-$, 469 $[M-H-162]^-$; IR (KBr) $\nu_{max}$/cm$^{-1}$: 3420, 2931, 1726, 1642, 1440, 1208, 1140, 1072, 843; $^1$H- and $^{13}$C-NMR (see Table 1).

Compound (**8**): Colorless amorphous powder, $C_{41}H_{64}O_{12}$; $[\alpha]_D^{21}$ −11° ($c$=0.12, MeOH); FAB-MS $m/z$ 747 $[M-H]^-$, 585 $[M-H-162]^-$, 453 $[M-H-162-132]^-$; HR-FAB-MS $m/z$ 747.43189 $[M-H]^-$; IR (KBr) $\nu_{max}$/cm$^{-1}$: 3420, 2937, 1730, 1644, 1442, 1208, 1138, 1062, 1002; $^1$H- and $^{13}$C-NMR (see Table 1).

Compound (**9**): Amorphous powder, $C_{36}H_{56}O_{10}$; $[\alpha]_D^{21}$ +39° ($c$=0.20, MeOH); FAB-MS $m/z$ 647 $[M-H]^-$, 485 $[M-H-162]^-$; HR-FAB-MS $m/z$ 647.37949 $[M-H]^-$; IR (KBr) $\nu_{max}$/cm$^{-1}$: 3440, 2940, 1732, 1639, 1455, 1378, 1082, 843; $^1$H- and $^{13}$C-NMR (see Table 1).

Compound (**10**): Amorphous powder, $C_{41}H_{64}O_{13}$; $[\alpha]_D^{21}$ +71° ($c$=0.10, MeOH); FAB-MS $m/z$ 763 $[M-H]^-$, 601 $[M-H-162]^-$, 469 $[M-H-162-132]^-$; HR-FAB-MS $m/z$ 763.42681 $[M-H]^-$; IR (KBr) $\nu_{max}$/cm$^{-1}$: 3440, 2920, 1730, 1630, 1435, 1388, 1086, 863; $^1$H- and $^{13}$C-NMR (see Table 1).

**Acid Hydrolysis** A solution of each compound (5 mg) was heated at reflux at 100 °C in 2 M aqueous $CF_3COOH$ (5 ml) on a water bath for 3 h. The reaction mixture was then diluted with $H_2O$ (15 ml) and extracted with $CH_2Cl_2$ (3×5 ml). The combined $CH_2Cl_2$ extracts were washed with $H_2O$ and then evaporated to dryness *in vacuo*. After evaporation to dryness of the aqueous layer then with MeOH until neutral, the sugars were analyzed by comparison with authentic sample (solvent system b) on silica gel HPTLC. The extract of sugars was derivatized with thiazolidine as reported method.[23] Monosaccharides were detected by GC and conditions: column, SupelcoSPB-1 0.25 mm×27 m; column temperature, 230 °C; carrier gas, $N_2$; $t_R$, L-xylose (7.4 min), D-xylose (7.8 min), L-glucose (13.3 min), D-glucose (13.8 min); D-glucose was detected in **1**, **3**, **4**, **7**, **9**. D-Glucose and D-xylose were detected in **2**, **5**, **6**, **8**, **10**.

**Material Preparations** *Nicotiana tabacum* CV. $K_{326}$ were cultivated in glasshouse without pest. Different compounds were weight precisely with electro-balance, dissolved with a small quantity of DMSO, and added water to dilute to be solutions which the concentration were 0.2 mg/ml, all solutions were in Petri dishes.

**Leaf Disc Method** Leaves of *Nicotiana tabacum* CV., $K_{326}$ were mechanically inoculated with Tobacco mosaic virus (TMV) at 10 $\mu$g/ml 10-mm-diameter. Leaf discs were punched and floated on the solutions of different compounds solutions 7 h after inoculation, and incubated at 25±1 °C for 48 h. Discs were treated with solvent only as the positive control while discs of healthy leaves were used as the negative control. Fourty-eight hours

later, leaf discs 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 microlitre 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 compounds-treated leaf disc)/(virus concentration of leaf disc 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 over night 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). The 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 $\mu$l PNP solution per well was added, and after 10 min, the reaction was stopped by adding 100 $\mu$l 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.

**Acknowledgments** We thank the staff of the analytical group of Kunming Institute of Botany, Chinese Academy of Sciences for measuring the NMR and FAB-MS spectra and this research was supported by National Natural Science Foundation of China (Grant No. 20272015).

**References**

1) Ouyang M. A., Yang C. R., Chen Z. L., Wang H. Q., *Phytochemistry*, **41**, 871—877 (1996).
2) Taketa A. T. C., Schimittmann-Schalanger T., Guillaume D., Gosmann G., Schenkel E. P., *Phytochemistry*, **53**, 901—904 (2000).
3) Tan N. H., Zhou J., Zhao S. X., *Phytochemistry*, **52**, 153—192 (1999).
4) Ouyang M. A., Liu Y. Q., Wang H. Q., Yang C. R., *Phytochemistry*, **49**, 2483—2486 (1998).
5) Pires V. S., Guillaume D., Gosmann G., Schenkel E. P., *J. Agric. Food Chem.*, **45**, 1027—1031 (1997).
6) Martrnez M. A. P., Pelotto J. P., Basualdo N., *Biochem. Syst. Ecol.*, **25**, 619—622 (1997).
7) Saldana M. D. A., Mohamed R. S., Baer M. G., *J. Agric. Food Chem.*, **47**, 3804—3808 (2000).
8) Dong X. W., Zhong L. C., *Phytochemistry*, **41**, 657—659 (1996).
9) Jiang Z. H., Wang J. R., Li M., Liu Z. Q., Chau K. Y., Zhao C., Liu L., *J. Nat. Prod.*, **68**, 397—399 (2005).
10) Fuchino H., Tachibana H., Tanaka N., *Chem. Pharm. Bull.*, **45**, 1533—1535 (1997).
11) Vangenderen H. H., Jaarsma J., *Plant Sci.*, **72**, 165—172 (1990).
12) Ishikura N., *Phytochemistry*, **14**, 743—745 (1975).
13) Vangenderen H. H., Jarsma J., Versluis C., *Plant Sci.*, **55**, 231—238 (1988).
14) Jahromi M. A. F., Gupta M., Manickam M., Ray A. B., Chansouria J. P. N., *Pharm. Biol.*, **37**, 37—41 (1999).
15) Filip R., Lotito S. B., Ferraro G., Fraga C. G., *Nutr. Res.*, **20**, 1437—1446 (2000).
16) Schinella C. R., Troiani G., Davila V., Buschiazzo P. M., Tournier H. A., *Biochem. Biophys. Res. Commun.*, **269**, 357—360 (2000).
17) Ouyang M. A., Wu C. L., *J. Asian Nat. Prod. Res.*, **5**, 89—94 (2003).
18) Ouyang M. A., *Nat. Prod. Res.*, **3**, 183—188 (2003).
19) Zhu M. W., "Chinese Medicinal Handbook of Guangxi," Guangxi People Publishing House, Nanning, 1973, pp. 296—298.
20) Liu X., Cui Y. X., Yu Q., Yu B., *Phytochemistry*, **66**, 1671—1679 (2005).
21) Jiang Z. F., Huang R. X., Qing G. W., Tian Y., Xu R. S., *Zhongcaoyao*, **22**, 291—293 (1991).
22) Mimaki Y., Fukushima M., Yokosuka A., Sashida Y., Furuya S., Sakagami H., *Phytochemistry*, **57**, 773—779 (2001).
23) Miyase T., Saitoh H., Shiokawa K., *Chem. Pharm. Bull.*, **43**, 466—469 (1995).
24) French C. J., Twoers G. H. N., *Phytochemistry*, **31**, 3017—3020 (1992).
25) Chen N. C., "Technology of Biological Mensuration in Pesticides," Beijing Agriculture University Publishing House, Beijing, 1990, pp. 194—196.