—Original— Natural Medicines 57 (1), 18-22 (2003)

Glycosides and Their Quantitative HPLC Analysis of Commercial Persicae Semen

Toshiyuki FUKUDA, Hideyuki ITO, and Takashi YOSHIDA*

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700-8530, Japan

(Received November 21, 2002)

The minor constituents of a Chinese medicine, Persicae semen (Tounin), were investigated and four newly isolated compounds were characterized as mandelic acid glycosides (gentiobioside and glucopyranoside) and benzyl glycosides (gentiobioside and glucopyranoside) based on their spectral analysis. These glycosides were quantified by HPLC along with the cyanogenic components, amygdalin and prunasin, of commercial samples bought from Japanese and Chinese markets. Although the composition of commercial Persicae semen from different markets did not differ remarkably, the amygdalin content varied significantly. The composition and quantity of glycosides in Persicae semen differed slightly from that of a related medicine, Armeniacae semen (Kyounin), which contained much less benzyl gentiobioside than any of the crude Persicae semen, and no benzyl glucopyranoside.

Keywords: Persicae semen; *Prunus persica*; mandelic acid glycoside; benzyl glycoside; quantitative analysis; Armeniacae semen

A Chinese medicine, Persicae semen (Tounin, Taoren in Chinese), which consists of the dried seeds of *Prunus persica* (L.) Batsch or *P. persica* Batsch var. *davidiana* Maxim. (Rosaceae), is an important ingredient in various Kampo prescriptions that are used to treat women's diseases, such as Toukaku-jouki-to (Tao he cheng qi tang) and Keishi-bukuryo-gan (Gui zhi fu ling wan). Persicae semen is frequently used to treat "Oketsu" syndrome (a syndrome caused by blood stagnation). The extract of Persicae semen has various pharmacological activities. For example, it inhibits the hind paw edema induced by carrageenan in rats,[1] it inhibits granuloma formation induced by felt-pellet implantation in rats,[2] it has anticoagulant effects,[3] and it inhibits platelet aggregation.[4] In spite of its popularity in Chinese medicine, little is known of its chemical constituents other than that it contains two cyanogenic glycosides, amygdalin (**1**) and prunasin (**2**), and sterols.[5] Here, we report the isolation and characterization of four minor compounds (**3**-**6**) from this traditional medicine. The quantitative analysis by HPLC of those compounds along with **1** and **2** in Persicae semen and related traditional medicines (Persicae flos (Hakutouka, Baitaohua), dried leaves of *P. persica* (Momonoha), and Armeniacae semen (Kyounin, Xingren)) bought from Chinese and Japanese markets is also described.

RESULTS AND DISCUSSION

Extraction and Characterization of the Components

Commercial Persicae semen was homogenized in 70% aqueous EtOH. HPLC analysis of the homogenate revealed several minor peaks besides the two peaks attributable to **1** and **2** (Fig. 1). The concentrated homogenate was extracted successively with hexane, EtOAc, and *n*-BuOH. The *n*-BuOH soluble fraction, which had an HPLC profile similar to that of the homogenate, was then separated by column chromatography over Diaion HP-20 with water and aqueous EtOH followed by preparative HPLC, and

yielded four minor components (**3-6**).

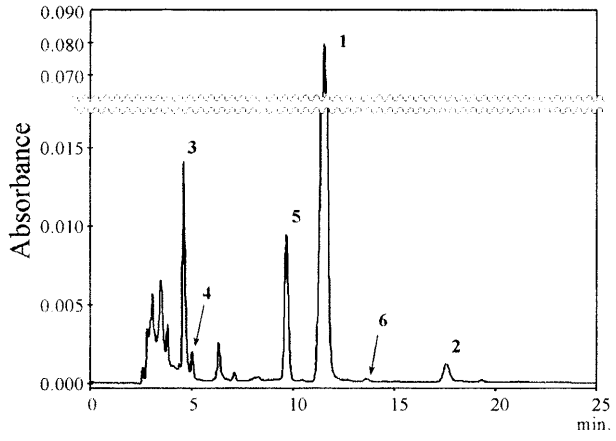


Fig. 1. HPLC Profile of the aqueous EtOH extract from Persicae Semen (No.18)

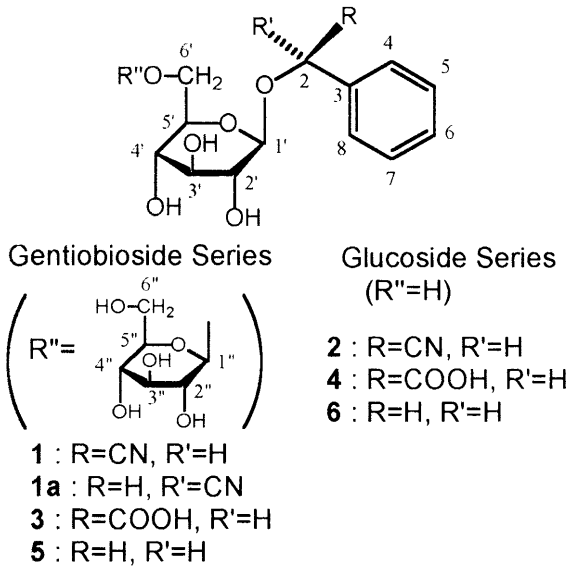


Fig. 2. Structures of the Constituents of Persicae Semen

Compound **3** was shown to have the molecular formula $C_{20}H_{28}O_{13}$ by high-resolution mass spectroscopy (HRMS){*m/z* 499.1451 $(M+Na)^+$}. The ^{1}H- and ^{13}C-NMR spectra of **3** were very similar to those of **1**, except for the presence of a carboxyl group instead of the nitrile group in **1**, identifying **3** as amygdalinic acid (mandelic acid gentiobioside) (**3**).[6] Similarly, compound **4** was identified as mandelic acid β-D-glucopyranoside (**4**)[7] by spectral analyses. The *R* configuration at the C-2 of both **3** and **4** was confirmed by the formation of *R*-(-)-mandelic acid on acid hydrolysis. Compounds **5** and **6** were identified as benzyl β-gentiobioside (**5**)[8] and benzyl β-D-glucopyranoside (**6**),[9] respectively, by comparing their physicochemical data with data reported in the literature (Fig. 2). Of the four, compound **3** has been reported as a synthetic product[5] and **5** was known only as a metabolite from cell cultures of *Lycopersicon esculentum*[8]. This is the first report on the isolation and characterization of glycosides **3-6** from Persicae semen. Since the physicochemical data of **3** and **4**, including the ^{13}C-NMR spectral assignments, in the literature are incomplete, we describe these data in the Experimental section, not only to provide evidence for the identification of each compound, but also for full characterization.

Interestingly, when Persicae semen was extracted with boiling water (Method 3), amygdalin (**1**) was partly converted to neoamygdalin (*S*-mandelonitrile-β-gentiobioside) (**1a**) to form a mixture with a 1:1 ratio. The ^{1}H-NMR spectrum of **1a**, which was separated by preparative HPLC, could nearly be superimposed on that of **1**; the only feature distinguishing **1a** was a lower field shift of the H-2 signal by 0.15 ppm compared with that of **1** (δ 5.81→ 5.96). This is reported to be diagnostic of the configuration at C-2 of amygdalin.[6] The identity of **1a** was confirmed by comparing the NMR data with data reported for synthetic **1a**.[6] This result indicates that amygdalin exists as a mixture of diastereoisomers at C-2 in decoctions of Kampo prescriptions containing Persicae semen.

Quantitative Analysis of Commercial Persicae Semen

Usually, glycosides in plants are hydrolyzed enzymatically when the plants are harvested or extracts are prepared. Before analyzing the amount of each component in commercially available traditional medicines bought from several markets, we first examined the difference in the quantities of the constituents in extracts prepared with and without heat (methods 2 and 1, respectively). As expected, twice the

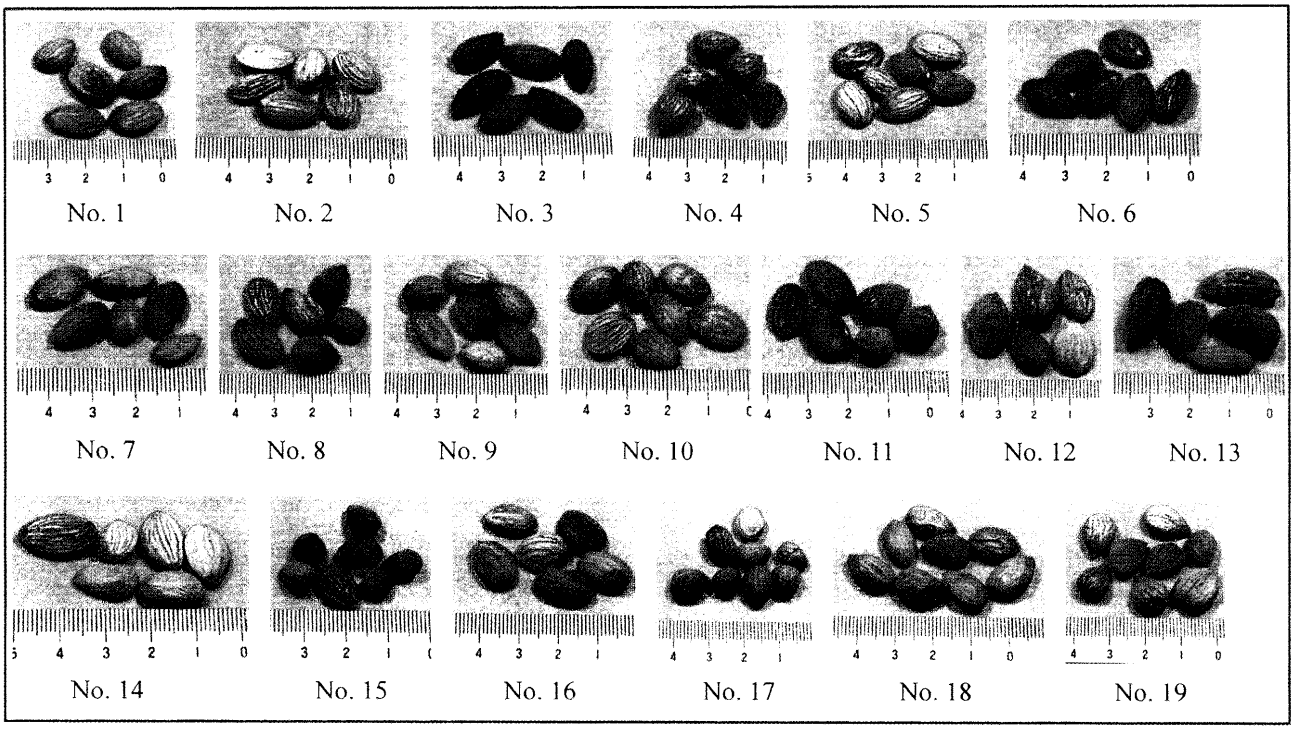
Table 1. Quantities of Glycosides in Persicae Semen (No. 18) extracts prepared by Methods 1 and 2

Compound	Method 1	Method 2
1	1.257%	2.840%
2	0.091%	0.039%
3	0.134%	0.360%
4	0.013%	0.032%
5	0.145%	0.322%
6	0.006%	0.004%

Table 2. Quantities of Glycosides **1-6** in Persicae Semen Samples from Japanese and Chinese Markets (%)

No.	Market Location	**1**	**2**	**3**	**4**	**5**	**6**
1	Sichuan 四川	2.266	0.022	0.455	0.085	0.286	0.004
2	Sichuan 四川 (Jinchuan 金川)	5.853	0.047	0.674	0.148	0.192	0.013
3	Sichuan 四川 (Pingwu 平武)	2.685	0.046	0.168	0.016	0.147	0.009
4*	Sichuan 四川 (Songpan 松藩)	5.479	0.062	0.811	0.147	0.189	0.015
5	Sichuan 四川 (Xichang 西昌)	3.385	0.040	0.319	0.045	0.227	0.005
6	Sichuan 四川 (Shimian 石棉)	2.966	0.070	0.203	0.142	0.289	0.031
7	Yunnan 雲南 (Luoping 羅平)	1.568	0.031	0.581	0.083	0.166	0.004
8	Yunnan 雲南 (Dali 大理)	2.371	0.025	0.324	0.042	0.293	0.010
9	Yunnan 雲南 (Xiaguan 下関)	2.182	0.033	0.175	0.045	0.169	0.011
10	Yunnan 雲南 (Lingang 臨港)	2.519	0.046	0.234	0.073	0.241	0.023
11	Xinjiang Uygur 新疆	3.176	0.025	0.306	0.044	0.269	0.004
12	Xinjiang Uygur 新疆 (Qingshuihe 清水河)	3.134	0.052	0.313	0.053	0.235	0.007
13	Xinjiang Uygur 新疆 (Yili 伊犁)	3.859	0.018	0.259	0.017	0.184	0.003
14	Guizhou 貴州 (Anshun 安順)	1.966	0.018	0.519	0.080	0.230	0.002
15*	Shaanxi 陝西 (Yanan 延安)	3.060	0.027	0.629	0.089	0.266	0.004
16	Xizang 西蔵 (Bomi 波密)	2.330	0.012	0.774	0.072	0.150	0.001
17*	Shanxi 山西	3.712	0.020	0.198	0.036	0.202	0.005
18	Tokyo, Japan	2.842	0.041	0.362	0.032	0.322	0.004
19†	Tokyo, Japan	5.179	0.058	0.253	0.011	0.150	-
20‡	Tokyo, Japan	0.325	0.302	-	1.759	-	-
21#	Tokyo, Japan	-	-	-	-	-	-

* *P. persica* Batsch var. *davidiana* Maxim., † Armeniacae Semen, ‡ Persicae Flos, # Momonoha



amount of all the components except for prunasin (**2**) was extracted using method 2 versus method 1 (Table 1). Therefore, for quantitative HPLC analysis, extracts of the commercial traditional medicines (Table 2) were prepared by method 2. Using the calibration curves shown in Fig. 3, glycosides **1-6** were subjected to quantitative analysis by HPLC. The results are summarized in Table 2. Glycosides **1-6** were detected in all samples of Persicae semen. The amygdalin (**1**) content varied from 1.57-5.85%. However, there was almost no difference in the

content of **1** in Tounin and San-tounin (seeds of *Prunus persica* Batsch var. *davidiana* Maxim.; samples 4, 15, and 17 in Table 2), although the latter has smaller size with more round shape than the former.

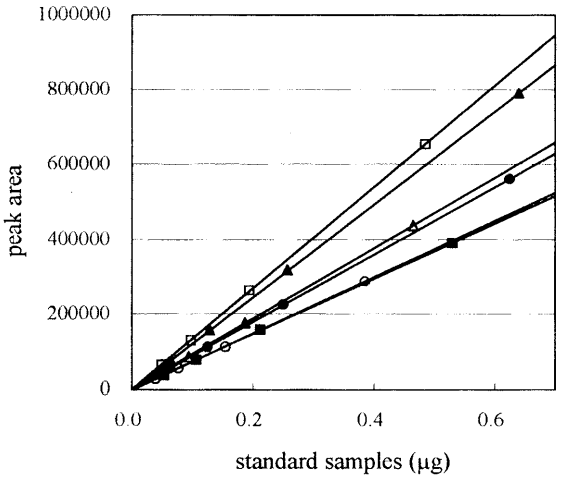


Fig. 3. Calibration curves for **1-6**: ● **1**; ▲ **2**; ■ **3**; ○ **4**; △ **5**; □ **6**

The amygdalin content of Armeniacae semen (Kyounin) was twice as high as that of Persicae semen from the Tokyo Market. This is consistent with earlier reports.[10] Of note is that, all of the minor components except for **6** were also detected in Armeniacae semen. Interestingly, compounds **1**, **2**, and **4** were detected in extracts of commercial Persicae flos (Hakutouka), and the yields of **2** and **4** were over ten times higher than in Persicae semen (Table 2). The leaves of *P. persica* contained no glycosides detected in Persicae semen. These results suggest that a comparison with more samples of Armeniacae semen, Persicae flos and Momonoha should be further examined.

This study detected four minor glycosides, which are regarded as metabolites of the cyanogenic glycosides amygdalin and prunasin, in Persicae semen, and most of them were also detected in Armeniacae semen. The quantitative analysis of the components of samples obtained from various markets showed that the amygdalin content varied significantly. This should be considered if amygdalin is the pharmacologically active substance in Persicae semen. The biological activity of the minor glycosides of Persicae semen is now under investigation.

EXPERIMENTAL

General: Optical rotations were measured on a JASCO DIP-370 digital polarimeter. ^{1}H and ^{13}C-NMR spectra were recorded on a Varian VXR-500 or BRUKER DRX-500 (500 MHz for ^{1}H and 126 MHz for ^{13}C), and the chemical shifts are given in δ (ppm) values relative to that of the solvent [acetone-d_6 (δ_H 2.04; δ_C 29.8)] on a tetramethylsilane scale.

Extraction and Isolation: Persicae semen (500 g) (Uchida Wakanyaku Co., Ltd., Tokyo) was homogenized in 70% aqueous EtOH (5 l). The concentrated homogenate was successively extracted with hexane (3 l), EtOAc (3 l), and *n*-BuOH (3 l). The *n*-BuOH extract (5.0 g) was passed through a porous polymer gel, Diaion HP-20 (Mitsubishi Chemical Co.) column, and the adsorbed materials were eluted successively with H_2O, 10 and 20% aqueous EtOH, and EtOH. These eluates were concentrated *in vacuo* to give residues (yields: 2.0, 1.1, 1.4, 0.1 g, respectively). The eluates using 10% (0.98 g) and 20% (0.48 g) aqueous EtOH were purified by preparative HPLC [Waters μ-Bondasphere C-18 (5 μ) 100 Å, *i.d.* 19 × 150 mm; CH_3CN-H_2O (13:87, 10:90 or 5:95) or MeOH-H_2O (15:85)] to give **1** (671.8 mg), **2** (173.7 mg), **3** (32.5 mg), **4** (37.0 mg), **5** (74.4 mg), and **6** (13.8 mg).

Amygdalinic acid (3): Amorphous powder, $[\alpha]_D^{23}$ −108° (*c* 0.6, MeOH). HRMS: *m/z* 499.1451(M+Na)$^+$:Calcd for $C_{20}H_{28}O_{13}$+Na, 499.1428. ^{1}H-NMR (500 MHz, D_2O+acetone-d_6) δ: 3.17-3.24 (2H, m, H-3', 2"), 3.26-3.31 (2H, m, H-2', 4"), 3.31-3.38 (4H, m, H-4', 5', 3", 5"), 3.58 (1H, dd, *J*=12, 5.5 Hz, H-6"), 3.73 (1H, dd, *J*=12, 4 Hz, H-6'), 3.78 (1H, dd, *J*=12, 2 Hz, H-6"), 4.06 (1H, dd, *J*=12, 2 Hz, H-6'), 4.15 (1H, d, *J*=8 Hz, H-1'), 4.39 (d, *J*=8 Hz, H-1"), 5.24 (1H, s, H-2), 7.32-7.36 (5H, m, aromatic-H). ^{13}C-NMR (126 MHz, D_2O+acetone-d_6) δ: 61.0 (C-6"), 68.6 (C-6'), 69.6 (C-4'), 69.9 (C-4"), 73.1 (C-2'), 73.4 (C-2"), 75.4 (C-5'), 75.6 (C-3'), 76.0 (C-3"), 76.3 (C-5"), 79.0 (C-2), 99.4 (C-1'), 103.2 (C-1"), 128.5 (C-4, 8), 129.4 (C-5, 7), 129.9 (C-6), 135.4 (C-3), 176.0 (C-1).

Mandelic acid β-D-glucopyranoside (4): Amorphous powder, $[\alpha]_D^{23}$ −83° (*c* 0.5, C_5H_5N). HRMS: *m/z* 337.0899 (M+Na)$^+$: Calcd for $C_{14}H_{18}O_8$+Na, 337.0899. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 3.79 (1H, m, H-5'), 4.12-4.20 (2H, m, H-2', 3'), 4.30 (1H, t, *J*=9 Hz, H-4'), 4.38 (1H, dd, *J*=12, 5, H-6'), 4.50 (1H, dd, *J*=12, 2 Hz, H-6'), 4.96 (1H, d, *J*=8 Hz, H-1'), 5.96 (1H, s, H-2), 7.30 (1H, d, *J*=8 Hz), 7.35 (2H, m), 8.00 (2H, dd, *J*=8, 1.5 Hz). ^{13}C-NMR (126 MHz, pyridine-d_5) δ:62.4 (C-6'), 71.3 (C-4'), 75.3 (C-2'), 78.3 (C-3'), 78.7 (C-5'), 79.8 (C-2),

101.8 (C-1'), 128.5 (C-6), 128.6 (C-4, 8), 128.7 (C-5, 7), 138.4 (C-3), 173.6 (C-1).

Acid hydrolysis of 3 and 4: Solutions of **3** and **4** (each 10 mg) in 1 M H_2SO_4 were heated in a water bath for 3 h. The reaction mixtures had a peak identical with that of *R*-(-)-mandelic acid, on reversed-phase HPLC with a chiral column [column, Chiralpak WH, (*i.d.* 4.6 × 250 mm, Daicel Chemical Ind., Ltd.); mobile phase, 0.25 mM $CuSO_4$; flow rate, 1 ml/min; detection, UV 220 nm; room temp.; retention time, *R*-(-)-mandelic acid =24.3 min., *S*-(+)-mandelic acid=19.5 min.] .

Materials for HPLC analysis: Persicae semen, Persicae flos, Persicae folium, and Armeniacae semen from Japanese markets were purchased from Uchida Wakanyaku Co., Ltd., Tokyo. Persicae semen from Chinese markets was obtained from the Ministries of Sichuan, Yunnan, Guizhou and, Shanxi Provinces and Xinjiang Uygur and Xizang Autonomous region in China, in 2001. Voucher specimens (No. 1～21) are deposited in the Herbal Garden, Faculty of Pharmaceutical Sciences, Okayama University.

Preparation of samples for quantitative analysis

Method 1: Exactly 5 g of sample were directly homogenized in 70% aqueous EtOH (100 ml) at room temperature and filtered. After evaporating the solvent, the residue was dissolved in 50% aqueous methanol (100 ml). One milliliter of the solution was applied to a Sep-Pak C_{18} cartridge (Waters), and the column was eluted with 50% aqueous MeOH (2 ml). The eluate was diluted to exactly 5 ml with 50% aqueous MeOH, and an aliquot (5 μl) was subjected to HPLC analysis.

Method 2: The sample (5 g) was first put in hot 70% aqueous EtOH (100 ml) and refluxed for ten minutes, and then homogenized. After filtering and evaporating the solvent, the residue was dissolved in 50% aqueous methanol (100 ml) and treated in a way similar to Method 1 to prepare an HPLC sample.

Method 3: Five grams of finely chopped kernels were refluxed in water (100 ml) for 2 hours. A sample for HPLC analysis was prepared and analyzed similarly.

HPLC conditions for quantitative analysis: The HPLC was performed on a Waters HPLC apparatus with a model 600 controller and a model 486 detector (Waters) [column, Develosil RPAQUEOUS (C-30) (*i.d.* 4.6 x 250 mm, Nomura Chemical Co. Ltd.); mobile phase, water-acetonitrile-MeOH=10:1:1; flow rate, 1 ml/min; detection, UV 210 nm; column temperature, 40 °C].

Calibration curves: Calibration curves were prepared by the absolute calibration method using standard solutions of **1-6** (0.05-0.50 μg per injection) **1**, $y = 898921x+400$ ($r^2 = 0.99998$); **2**, $y = 1238145x-1290$ ($r^2 = 0.99993$); **3**, $y = 753347x-2182$ ($r^2 = 0.99996$); **4**, $y = 941262x-132$ ($r^2 = 0.99997$); **5**, $y = 738168x-323$ ($r^2 = 0.99996$); **6**, $y = 1350555x+80$ ($r^2 = 0.99997$) where y is the peak area and x is the μg per injection; n=3 (Fig. 3).

Acknowledgments: The authors thank Mr. Hidetoshi Tanaka of Alps Pharmaceutical Ind. Co., Ltd., and Prof. Xin-Min Chen and Associate Prof. Kai-Ze He, of the Division of Phytochemistry Chengdu Institute of Biology, Chinese Academy of Sciences, for providing the Persicae semen from Chinese markets. The Varian VXR 500 used in this study is the property of the SC-NMR Laboratory of Okayama University, which is gratefully acknowledged.

REFERENCES

1) Arichi S., Kubo M., Tani T., Nakamura H., Motoyoshi S., Ishii K., Imazu C., Seto Y., Kadokawa T., Nagamoto N., Namba K., Nishimura H., *Yakugaku Zasshi*, **105**, 886—894 (1985).
2) Arichi S., Kubo M., Tani T., Nanba K., Nagamoto N., Noguchi H., Nakamura H., Uno H., Nishimura H., *Shoyakugaku Zasshi*, **40**, 129—138 (1986).
3) Kosuge T., Ishida H., Ishii M., *Chem. Pharm. Bull.*, **33**, 1496—1498 (1985).
4) Yun-Choi H. S., Kim S. O., Kim J. H., Lee J. R., *J. Nat. Prod.*, **48**, 363—370 (1985).
5) Morishige H., Ida Y., Shoji J., *Shoyakugaku Zasshi*, **37**, 46—51 (1983).
6) Turczan J. W., Medwick T., Plank W. M., *J. Assoc. Off. Anal. Chem.*, **61**, 192—207 (1978).
7) Kitajima J., Tanaka Y., *Chem. Pharm. Bull.*, **41**, 2007—2009 (1993).
8) De Rosa S., De Giulio A., Tommonaro G., *Phytochemistry*, **42**, 1031—1034 (1996).
9) Miyase T., Ueno A., Takizawa N., Kobayashi H., Karasawa H., *Chem. Pharm. Bull.*, **35**, 1109—1117 (1987).
10) The Japanese Pharmacopoeia 14th Edition, Ed. By Ministry of Health, Labour and Welfare, Tokyo, Japan, 2001, pp. D-803—806, pp.D-284—287.