Natural Product Sciences
20(2) : 91-94 (2014)

Phytochemical Constituents of *Bletilla striata* and Their Cytotoxic Activity†

Kyeong Wan Woo[1], Jong Eel Park[1], Sang Un Choi[2], Ki Hyun Kim[1], and Kang Ro Lee[1,*]

[1]*Natural Products Laboratory, School of Pharmacy, Sungkyunkwan University, Suwon 440-746, Korea*
[2]*Korea Research Institute of Chemical Technology, Teajeon 305-600, Korea*

Abstract – Column chromatographic separation of the MeOH extract from the tubers of *Bletilla striata* yielded seven phenolic components including four phenanthrenes, 3,7-dihydroxy-2,4-dimethoxyphenanthrene (**1**), 3,7-dihydroxy-2,4,8-trimethoxyphenanthrene (**2**), 9,10-dihydro-4,7-dimethoxyphenanthrene-2,8-diol (**3**), and 9,10-dihydro-1-(4'-hydroxybenzyl)-4,7-dimethoxyphenanthrene-2,8-diol (**4**) and three stilbenes, gigantol (**5**), 3',4"-dihydroxy-5',3",5"-trimethoxybibenzyl (**6**), and batatasin III (**7**). Their structures were determined on the basis of NMR spectroscopic data. Among them, compound **2**, **3**, and **6** were reported for the first time from this plant. The isolated compounds (**1**-**7**) were tested for cytotoxicity against four human tumor cell lines *in vitro* using a Sulforhodamin B bioassay.
Keywords – *Bletilla striata*, Orchidaceae, Stilbene, Phenanthrene, cytotoxicity

Introduction

The tubers of *Bletilla striata* ($T_{HUNB.}$) Reichb. F. (Orchidaceae) have been used for treatment of tuberculosis and pneumonorrhagia in Chinese traditional medicine.[1] Previous phytochemical investigation on this plant reported the isolation of phenanthrenes, stilbenes, bibenzyls, and flavonoids.[1-4] Pharmacological studies with some of these compounds reported to antimiotic and antimicrobial activities.[3,5] As a part of our continuing search for biologically active compounds from Korean medicinal plants, we investigated the $CHCl_3$ soluble fraction of *B. striata* and recently reported the isolation of spirostane-steroidal derivatives with cytotoxic activity.[6] In this study, we further isolated seven phenolic derivatives, including four phenanthrenes and three stilbenes (**1** - **7**) from the $CHCl_3$ soluble fraction. The identification and structural elucidation of these compounds were based on 1D, 2D NMR and MS data. The isolated compounds (**1** - **7**) were tested for cytotoxicity against four human tumor cell lines *in vitro* using a Sulforhodamin B bioassay. Here, we report the isolation and structural elucidation as well as the cytotoxic effect of phenolic derivatives.

Experimental

General experimental procedures – Silica gel F_{254} plates (Merck) and RP-C_{18} F_{254s} plates (Merck) were used for TLC. Spots on TLC were detected by UV light and/or by heating after spraying with 10% H_2SO_4 in C_2H_5OH (v/v). Si gel 60 (Merck, 70 - 230 and 230 - 400 mesh) and RP-C_{18} silica gel (Merck, 230 - 400 mesh) was used for open column chromatography. Low pressure liquid chromatography was carried out on a Merck Lichroprep Lobar® - A Si 60 (240 × 10 mm) columns with a FMI QSY-0 pump (ISCO). Semi-preparative HPLC used a Gilson 306 pump with Shodex refractive index detector. NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). FAB MS spectra were obtained on a JEOL JMS 700 mass spectrometer. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer.

Plant materials – The tubers of *B. striata* were perchased from Kyungdong herbal market, Seoul, Korea, in June 2011, and were identified by one of the authors (K.R. Lee). A voucher specimen (SKKU-NPL 1106) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and isolation – Dried tubers of *B. striata* (3.6 kg) were extracted three times with 80% MeOH under reflux. The resulting MeOH extracts (568 g) were suspended in distilled water (800 ml × 4) and then

†Dedicated to Prof. Okpyo Zee of the Sungkyunkwan University for his leading works on Natural Products Research.

*Author for correspondence
Kang Ro Lee, Natural Products Laboratory, School of Pharmacy, Sungkyunkwan University, Suwon 440-746, Korea
Tel: +82-31-290-7710; E-mail: krlee@skku.edu

successively partitioned with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH, yielding residues of 3 g, 42 g, 51 g and 159 g, respectively. The $CHCl_3$ soluble extract (40 g) was chromatographed on a silica gel (230 - 400 mesh, 700 g) column eluated with $CHCl_3$: MeOH (50 : 1 – 1 : 1) to obtain seven fractions (A-G). Fraction B (5.6 g) was separated on a silica gel column (Hexane : $CHCl_3$: MeOH = 4 : 5 : 0.1 – $CHCl_3$: MeOH = 50 : 1) to afford ten subfractions (B1-B10). Subfraction B6 (292 mg) was further separated on an RP-C_{18} silica gel column with 50~100% MeOH and purified over an RP-C_{18} semi-prep. HPLC using a 250 mm × 10 mm i.d., 10 μm, Econosil RP-18 column (Alltech) with a solvent system of 70% MeOH (1 L, flow rate; 2 mL/min) to yield compound **2** (12 mg, R_t = 17.0 min). Subfraction B8 (709 mg) was separated on an RP-C_{18} silica gel column with 60~100% MeOH and purified over an RP-C_{18} semi-prep. HPLC (55% MeOH) to yield compound **3** (10 mg, R_t = 15.0 min). Subfraction B9 (439 mg) was separated on an RP-C_{18} silica gel column with 60% MeOH and purified over an RP-C_{18} semi-prep. HPLC (65% MeOH) to yield compounds **1** (23 mg, R_t = 16.0 min), **5** (44 mg, R_t = 13.0 min), and **6** (8 mg, R_t = 22.0 min). Fraction D (4.0 g) was separated on a silica gel column ($CHCl_3$: MeOH = 20 : 1 – 1 : 1) to afford eleven subfractions (D1-D11). Subfraction D5 (392 mg) was further separated on an silica Lobar A®-column ($CHCl_3$: MeOH = 50 : 1) and purified over an RP-C_{18} semi-prep. HPLC (60% MeOH) to yield compounds **4** (10 mg, R_t = 14.0 min) and **7** (5 mg, R_t = 19.0 min).

3,7-Dihydroxy-2,4-dimethoxyphenanthrene (**1**) – Yellow gum; IR ν_{max} cm^{-1}: 3385, 2939, 2835, 1621, 1478, 1354, 1294, 1228, 1164, 1125, 1075, 927 and 678; FAB-MS *m/z* 271 $[M + H]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 9.27 (1H, d, *J* = 9.0 Hz, H-5), 7.52 (1H, d, *J* = 9.0 Hz, H-9), 7.39 (1H, d, *J* = 9.0 Hz, H-10), 7.14 (1H, d, *J* = 2.5 Hz, H-8), 7.12 (1H, s, H-1), 7.09 (1H, d, *J* = 9.0, 2.5 Hz, H-6), 3.97 (3H, s, 4-OCH_3), 3.87 (3H, s, 2-OCH_3); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 154.8 (C-7), 147.7 (C-2), 144.5 (C-4), 139.9 (C-3), 134.2 (C-8a), 128.0 (C-5), 127.0 (C-10), 125.8 (C-10a), 124.3 (C-9), 123.0 (C-4b), 119.1 (C-4a), 116.1 (C-6), 111.1 (C-8), 105.0 (C-1), 58.6 (4-OCH_3), 55.2 (2-OCH_3).

3,7-Dihydroxy-2,4,8-trimethoxyphenanthrene (**2**) – Yellow gum; IR ν_{max} cm^{-1}: 3379, 2943, 2833, 1619, 1481, 1454, 1299, 1216, 1107, 1032, and 676; FAB-MS *m/z* 300 $[M]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 9.16 (1H, d, *J* = 9.0 Hz, H-5), 7.82 (1H, d, *J* = 9.0 Hz, H-9), 7.63 (1H, d, *J* = 9.0 Hz, H-10), 7.30 (1H, d, *J* = 9.0 Hz, H-6), 7.09 (1H, d, *J* = 8.5 Hz, H-1), 4.05 (3H, s, 8-OCH_3), 3.98 (3H, s, 4-OCH_3), 3.94 (3H, s, 2-OCH_3); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 147.9 (C-2), 146.2 (C-4), 144.6 (C-7), 141.3 (C-3), 140.1 (C-8), 127.7 (C-10), 127.0 (C-10a), 125.7 (C-4b), 123.8 (C-8a), 123.2 (C-9), 119.1 (C-4a), 117.6 (C-5), 116.7 (C-6), 104.9 (C-1), 60.2 (OCH_3), 58.6 (OCH_3), 55.2 (OCH_3).

9,10-Dihydro-4,7-dimethoxyphenanthrene-2,8-diol (**3**) – Yellow gum; IR ν_{max} cm^{-1}: 3445, 2940, 2838, 1598, 1482, 1441, 1277, 1158, 1084, 1013, 973, 874, 813, and 542; FAB-MS *m/z* 273 $[M + H]^+$; ^{1}H-NMR (500 MHz, CD_3OD): δ 7.68 (1H, d, *J* = 8.5 Hz, H-5), 6.74 (1H, d, *J* = 8.5 Hz, H-6), 6.39 (1H, d, *J* = 2.5 Hz, H-3), 6.31 (1H, d, *J* = 2.5 Hz, H-1), 3.85 (3H, s, 7-OCH_3), 3.80 (3H, s, 4-OCH_3), 2.72 (2H, m, H-9), 2.59 (2H, m, H-10); ^{13}C-NMR (125 MHz, CD_3OD): δ 158.2 (C-4), 156.4 (C-2), 145.6 (C-7), 141.8 (C-8), 140.9 (C-10a), 126.7 (C-4b), 124.4 (C-8a), 119.3 (C-5), 115.7 (C-4a), 108.0 (C-6), 107.1 (C-1), 98.1 (C-3), 55.2 (OCH_3), 54.7 (OCH_3), 30.2 (C-10), 21.4 (C-9).

9,10-Dihydro-1-(4'-hydroxybenzyl)-4,7-dimethoxyphenanthrene-2,8-diol (**4**) – White amorphous powder; IR ν_{max} cm^{-1}: 3360, 2941, 2838, 1595, 1511, 1490, 1444, 1278, 1022, 877, 826, and 533; FAB-MS *m/z* 379 $[M + H]^+$; ^{1}H-NMR (500 MHz, Pyridine-d_5): δ 8.16 (1H, d, *J* = 8.5 Hz, H-5), 7.41 (2H, d, *J* = 8.5 Hz, H-2', 6'), 7.13 (2H, d, *J* = 8.5 Hz, H-3', 5'), 6.96 (1H, d, *J* = 8.5 Hz, H-6), 6.83 (1H, s, H-3), 4.42 (2H, s, H-1a), 3.73 (6H, s, 4, 7-OCH_3), 3.10 (2H, m, H-9), 2.93 (2H, m, H-10); ^{13}C-NMR (125 MHz, Pyridine-d_5): δ 156.6 (C-4, 4'), 155.9 (C-2), 146.1 (C-7), 143.2 (C-8), 140.5 (C-10a), 132.5 (C-1'), 129.8 (C-2', 6'), 127.8 (C-4b), 125.4 (C-8a), 119.7 (C-5), 118.4 (C-1), 116.6 (C-4a), 115.8 (C-5'), 108.9 (C-6), 99.0 (C-3), 55.6 (4, 7-OCH_3), 30.7 (C-1a), 26.6 (C-10), 22.1 (C-9).

Gigantol (**5**) – Yellow gum; IR ν_{max} cm^{-1}: 3359, 2944, 2833, 1600, 1517, 1457, 1219, 1153, 1117, 1032, and 692; FAB-MS *m/z* 274 $[M]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 6.84 (1H, d, *J* = 8.0 Hz, H-5"), 6.68 (1H, dd, *J* = 8.0, 2.0 Hz, H-6"), 6.63 (1H, d, *J* = 2.0 Hz, H-2"), 6.32 (1H, brd, *J* = 2.0 Hz, H-6'), 6.27 (1H, brd, *J* = 2.0 Hz, H-4'), 6.27 (1H, brd, *J* = 2.0 Hz, H-2'), 3.83 (3H, s, 5'-OCH_3), 3.74 (3H, s, 3"-OCH_3), 2.80 (4H, m, H-1, 2); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 161.0 (C-5'), 156.9 (C-3'), 146.5 (C-3"), 144.7 (C-1'), 143.8 (C-4"), 134.0 (C-1"), 121.2 (C-6"), 114.5 (C-2"), 111.5 (C-5"), 108.4 (C-2'), 107.0 (C-6'), 99.3 (C-4'), 56.1 (OCH_3), 55.5 (OCH_3), 38.4 (C-2), 37.4 (C-1).

3',4''-Dihydroxy-5',3'',5''-trimethoxybibenzyl (**6**) – Yellow gum; IR ν_{max} cm^{-1}: 3358, 2945, 2832, 1452, 1032, and 695; FAB-MS m/z 305 $[M + H]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 6.39 (2H, s, H-2", 6"), 6.35 (1H, m, H-6'), 6.28

(1H, m H-4'), 6.27 (1H, m, H-2'), 3.88 (6H, s, 3", 5"-OCH_3), 3.78 (3H, s, 5'-OCH_3), 2.84 (4H, m, H-1, 2); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 160.8 (C-5'), 156.5 (C-3'), 146.8 (C-3", 5"), 144.4 (C-1'), 132.8 (C-1"), 132.7 (C-4"), 108.0 (C-2'), 106.9 (C-6'), 105.1 (C-2", 6"), 99.0 (C-4'), 56.2 (3", 5"-OCH_3), 55.4 (5'-OCH_3), 38.2 (C-1), 37.9 (C-2).

Batatasin III (**7**) – Yellow gum; IR ν_{max} cm^{-1}: 3358, 2945, 2833, 1698, 1455, 1155, 1116, 1032, and 659; FAB-MS *m/z* 244 $[M]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.14 (1H, m, H-3"), 6.75 (1H, d, *J* = 8.0 Hz, H-2"), 6.65 (1H, dd, *J* = 8.5, 2.0 Hz, H-2'), 6.65 (1H, brs, H-2"), 6.32 (1H, brs, H-6'), 6.25 (1H, brs, H-2'), 3.75 (3H, s, 5'-OCH_3), 2.84 (2H, m, H-1), 2.79 (2H, m, H-2); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 161.1 (C-5'), 156.7 (C-3'), 155.7 (C-3"), 144.6 (C-1'), 143.8 (C-1"), 129.7 (C-5"), 121.2 (C-6"), 115.5 (C-2"), 113.1 (C-4"), 108.1 (C-2'), 107.0 (C-6'), 99.3 (C-4'), 55.4 (5'-OCH_3), 37.8 (C-1), 37.5 (C-2).

Cytotoxicity assay – A sulforhodamine B bioassay (SRB) was used to determine the cytotoxicity of each compound against four cultured human cancer cell lines.[7] The assays were performed at the Korea Research Institute of Chemical Technology. The cell lines used were A549 (non small cell lung adenocarcinoma), SK-OV-3 (ovarian cancer cells), SK-MEL-2 (skin melanoma), and HCT15 (colon cancer cells). Doxorubicin (Sigma Chemical Co., ≥ 98%) was used as a positive control.

Results and Discussion

Compounds **1** - **3**, **5**, and **7** were identified as 3,7-dihydroxy-2,4-dimethoxyphenanthrene (**1**),[8] 3,7-dihydroxy-2,4,8-trimethoxyphenanthrene (**2**),[9] 9,10-dihydro-4,7-dimethoxyphenanthrene-2,8-diol (**3**),[10] gigantol (**5**),[11] and batatasin III (**7**)[11] by comparing the ^{1}H-, ^{13}C-NMR, and MS spectral data with the literature values. Compounds **2**, **3**, and **6** were isolated for the first time from this plant. The following describes the structural elucidation of compounds **4** and **6**, since the ^{13}C-NMR spectral data were not yet reported.

Compound **4** was obtained as a white amorphous powder. FABMS, ^{1}H- and ^{13}C-NMR data gave a molecular formula of $C_{23}H_{22}O_5$. The ^{1}H-NMR spectrum of **4** indicated the presence of seven aromatic protons at δ 8.16 (1H, d, *J* = 8.5 Hz, H-5), 7.41 (2H, d, *J* = 8.5 Hz, H-2', 6') and 7.13 (2H, d, *J* = 8.5 Hz, H-3', 5'), 6.96 (1H, d, *J* = 8.5 Hz, H-6), and 6.83 (1H, s, H-3), two methoxy protons at δ 3.73 (6H, s, 4, 7-OCH_3), and three methylene protons at δ 4.42 (2H, s, H-1a), 3.10 (2H, m, H-9), and 2.93 (2H, m, H-10). The ^{13}C-NMR spectrum demonstrated the presence of 23 carbon signals, consisting of two methoxy carbon signals at δ 55.6, and three methylene carbon signals at δ 30.7, 26.6, and 22.1, and 18 aromatic carbons signals. The ^{1}H- and ^{13}C-NMR spectra implied that compound **4** could be a phenanthrene derivative.[12] In the HMBC spectrum, the correlations between the methylene protons (δ 4.41, H-1a) and the aromatic carbon (δ 118.4, C-1) were observed, indicating that the benzylic –CH_2– moiety was attached at C-1 (Fig. 2). The methoxy position was also confirmed to be C-4, C-7 by HMBC cross peaks of C-4/4-OCH_3 and C-7/7-OCH_3. On the basis of the further comparison with literature values, the structure of **4** was determined to be 9,10-dihydro-1-(4'-hydroxybenzyl)-4,7-dimethoxyphenanthrene-2,8-diol.[13]

Compound **6** was obtained as a yellowish gum. From FAB-MS (*m/z* 305 $[M+H]^+$) and ^{1}H- and ^{13}C-NMR spectral data, the molecular formula of **6** was deduced to be $C_{17}H_{20}O_5$. The ^{1}H-NMR spectrum exhibited the presence of five aromatic protons at δ 6.39 (2H, s, H-2", 6"), 6.35 (1H, m, H-6'), 6.28 (1H, m H-4'), and 6.27 (1H, m, H-2'), three methoxy protons at δ 3.88 (6H, s, 3", 5"-OCH_3) and 3.78 (3H, s, 5'-OCH_3), two methylene protons at δ 2.84

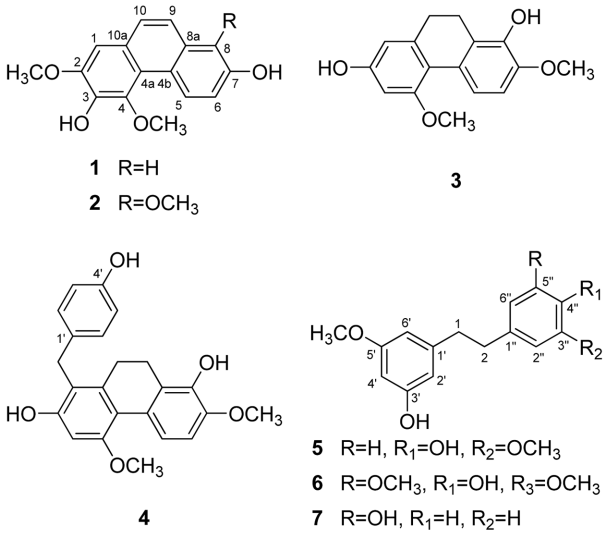

Fig. 1. The structures of **1** - **7** from *B. striata*.

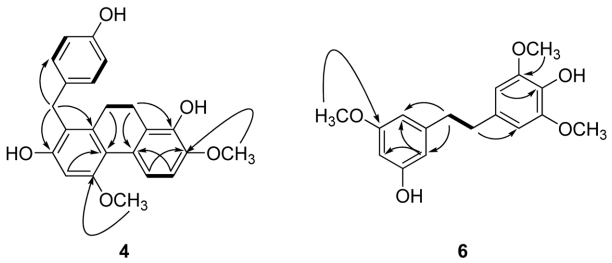

Fig. 2. Key COSY (▬) and HMBC (◠) correlations of compounds **4** and **6**.

Table 1. Cytotoxic activities of compounds (**1** - **7**) isolated from *B. striata*

Compound	IC_{50} (μM)[a]			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	> 30.0	> 30.0	> 30.0	> 30.0
2	> 30.0	> 30.0	> 30.0	> 30.0
3	> 30.0	> 30.0	> 30.0	> 30.0
4	12.65	12.69	10.84	2.16
5	6.69	5.28	7.04	7.14
6	18.94	14.61	14.07	9.86
7	24.51	20.16	17.92	19.43
Doxorubicin	0.0010	0.0083	0.0012	0.0207

[a]IC_{50} value of compounds against each cancer cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*.

(4H, m, H-1, 2). The ^{13}C-NMR spectrum exhibited the presence of 16 carbon signals, including twelve aromatic carbons, two methoxy carbons (δ 56.2 and 55.2), and two methylene carbons (δ 38.2 and 37.2). Above NMR data suggested that **6** was a stilbene.[14] The methoxy positions were confirmed to be C-5', C-3", and 5" by HMBC cross peaks of C-5'/5'-OCH_3, C-3"/3"-OCH_3, and C-5"/5"-OCH_3 (Fig. 2). Based on further comparison with published data, the structure of **6** was identified as 3',4"-dihydroxy-5',3",5"-trimethoxybibenzyl.[13]

The isolated compounds (**1** - **7**) were tested *in vitro* for cytotoxicity against four human tumor cells using the SRB assay. The stilbene type (**5** - **7**) showed considerable cytotoxicity against the tested cells with IC_{50} values of 5.28 – 24-51 μM, but the phenanthrenes type (**1** - **3**) were inactive (IC_{50} > 30.0 μM) except for the compound **4**. According to the substituted pattern in ring B of stilbenes, the cytotoxic activity exhibited different as shown in Table 1.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (2012R1A5A2A 28671869). We are thankful to the Korea Basic Science Institute (KBSI) for the measurements of NMR and MS spectra.

References

(1) Feng, J. Q.; Zhang, R. J.; Zhao, W. M. *Helv. Chim. Acta*. **2008**, *91*, 520-525.
(2) Yamaki, M.; Bai, L.; Kato, T.; Inoue, K.; Takagi, S.; Yamagata, Y.; Tomita, K. I. *Phytochemistry* **1993**, *33*, 1497-1498.
(3) Morita, H.; Koyama, K.; Sugimoto, Y.; Kobayashi, J. *Bioorg. Med. Chem. Lett*. **2005**, *15*, 1051-1054.
(4) Tatsuzawa, F.; Saito, N.; Shigihara, A.; Honda, T.; Toki, K.; Shinoda, K.; Yukawa, T.; Miyoshi, K. *J. Japan. Soc. Hort. Sci.* **2010**, *79*, 215-220.
(5) Takagi, S.; Yamki, M.; Inoue, K. *Phytochemistry* **1983**, *22*, 1011-1015.
(6) Park, J. E.; Woo, K. W.; Choi, S. U.; Lee, J. H., Lee, K. R. *Helv. Chim. Acta*. **2014**, *97*, 56-63.
(7) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst*. **1990**, *82*, 1107-1112.
(8) Leong, Y. W.; Kang, C. C.; Harrison, L. J.; Powell, A. D. *Phytochemistry* **1997**, *44*, 157-165.
(9) Rethy, B.; Kovacs, A.; Zupko, I.; Forgo, P.; Vasas, A.; Falkay, G.; Hohmann, J. *Planta Med*. **2006**, *72*, 767-770.
(10) Kanekar, Y.; Basha, K.; Duche, S.; Gupte, R.; Kapat, A. *Eur. J. Med. Chem*. **2013**, *67*, 454-463.
(11) Chen, Y.; Xu, J.; Yu, H.; Qing, C.; Zhang, Y.; Wang, L.; Liu, Y.; Wang, J. *Food Chem*. **2008**, *107*, 169-173.
(12) Lin, Y. L.; Chen, W. P.; Macabalang, A. D. *Chem. Pharm. Bull*. **2005**, *53*, 1111-1113.
(13) Tuchinda, P.; Udachachon, J.; Khumtaveeporn, K.; Taylor, W. C. *Phytochemistry* **1989**, *28*, 2463-2466.
(14) Kaganda, N. G.; Adesanya, S. A. *J. Nat. Prod*. **1990**, *53*, 1345-1346.

Received May 2, 2014
Revised June 4, 2014
Accepted June 6, 2014