Isoflavones and Rotenoids from the Leaves of *Millettia brandisiana*

Orasa PANCHAROEN,*[,a] Anan ATHIPORNCHAI,[a] Ampai PANTHONG,[b] and Walter Charles TAYLOR[c]

[a] *Department of Chemistry, Faculty of Science, Silpakorn University; Nakorn Pathom 73000, Thailand:* [b] *Department of Pharmacology, Faculty of Medicine, Chiangmai University; Chiangmai 52000, Thailand: and* [c] *School of Chemistry, University of Sydney; NSW 2006, Australia.*

Received November 16, 2007; accepted February 23, 2008; published online March 17, 2008

A new isoflavone, 4′-γ,γ-dimethylallyloxy-5,7,2′,5′-tetramethoxyisoflavone, brandisianin A (1), was isolated from the leaves of *Millettia brandisiana*, along with one synthetically known isoflavone, 7,4′-di-*O*-prenylgenistein (2) and twelve known compounds. The structures were elucidated on the basis of extensive spectroscopic analysis. Two isolated compounds were tested for anti-inflammatory activity; 12a-hydroxy-α-toxicarol (11) showed significant anti-inflammatory activity.

Key words *Millettia brandisiana*; isoflavone; rotenoid; anti-inflammatory activity

Millettia brandisiana KURZ, a member of the Leguminosae, is a medium-sized tree growing throughout Thailand;[1] however, its medicinal use is unknown. Chemical investigations of a few species of genus *Millettia* in Thailand resulted in the isolation of several flavonoids, some of which showed antiviral and anti-inflammatory activities.[2] There have been no previous studies on the chemical composition of the leaves of *M. brandisiana*. In an earlier report, we described the isolation of four rotenoids from the hexane extract of the flowers of *M. brandisiana*.[3] In the present investigation, the ethyl acetate extract of the leaves yielded a new isoflavone, 4′-γ,γ-dimethylallyloxy-5,7,2′,5′-tetramethoxyisoflavone (**1**), as well as a synthetically known isoflavone, 7,4′-di-*O*-prenylgenistein (**2**),[4] three known isoflavones, eight known rotenoids, and a sterol. The structures of the known compounds were determined to be robustigenin (**3**),[5] toxicarol isoflavone (**4**),[6] viridiflorin (**5**),[7] sermundone (**6**),[8] 6-deoxyclitoriacetal (**7**),[9] 6a,12a-dehydrosermundone (**8**),[10,11] stemonal (**9**),[12] α-toxicarol (**10**),[13] 12a-hydroxy-α-toxicarol (**11**),[14] 6a,12a-dehydro-α–toxicarol (**12**),[15] 6-hydroxy-6a,12a-dehydro-α-toxicarol (**13**),[16] and β-sitosterol (**14**)[17] by analysis of physical and spectroscopic evidence. In addition, the anti-inflammatory activity using ethyl phenylpropiolate-induced ear edema in rats of two pure compounds isolated was also studied. TLC screening assay of free radical scavenging activity of the isolated flavonoids is also described.

Results and Discussion

Purification of the ethyl acetate extract of the leaves of *M. brandisiana* gave a new isoflavone, 4′-γ,γ-dimethylallyloxy-5,7,2′,5′-tetramethoxyisoflavone (**1**). The molecular formula of **1** was determined to be $C_{24}H_{26}O_7$ by HR-ESI-MS (*m*/*z* 427.1818 $[M+H]^+$, Calcd 427.1757). The UV spectrum showed maxima absorptions at λ 290 and 256 nm. It showed a strong IR absorption band at 1651 cm^{-1}. The ^{1}H-NMR spectrum (Table 1) exhibited a signal of a characteristic isoflavone proton at δ_H 7.79 (s, H-2). The ^{13}C, Distortionless Enhancement by Polarization Transfer (DEPT), and HMQC spectra showed 24 carbon signals, corresponding to four methoxyls, two methyls, one methylene, six methines, and eleven quarternary carbons. Two methoxyls were attached to ring A, as indicated by signals at δ_H 3.90 and 3.94 (3H each, s) which were confirmed by ^3J correlations between 5-OMe (δ_H 3.90) to C-5 (δ_C 163.7) and 7-OMe (δ_H 3.94) to C-7 (δ_C 161.4) in the HMBC spectrum (Table 1). This was supported by an AM spin system at δ_H 6.38 (d, J=2.1 Hz, H-6) and δ_H 6.47 (d, J=2.1 Hz, H-8). In addition, the signals for a γ,γ-dimethylallyloxy group [δ_H 1.78, 1.80 (6H, 2×Me), δ_H 4.64 (2H, d, J=6.6 Hz, H_2-1″), and δ_H 5.54 (1H, t, J=6.6 Hz, H-2″)], as well as two methoxyls at δ_H 3.84 and 3.74 (3H each, s) were observed. For ring B, the appearance of two aromatic proton singlets at δ_H 6.62 and 6.95 indicated that the *para*-correlation of two protons was deduced. The HMBC spec-

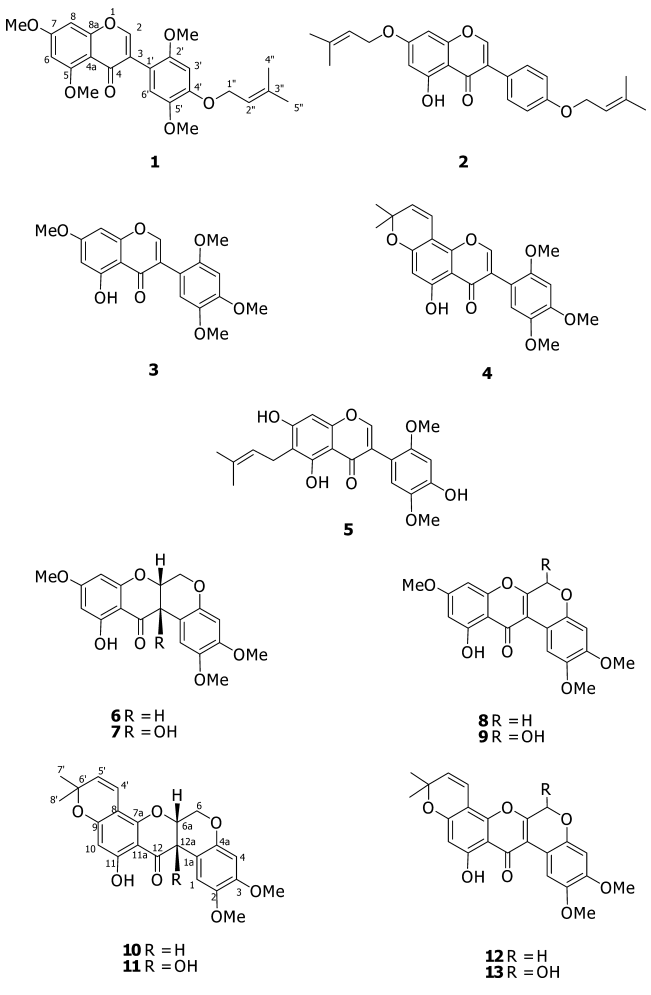

Fig. 1. Compounds Isolated from Leaves of *M. brandisiana*

* To whom correspondence should be addressed. e-mail: orasa@su.ac.th

trum of **1** exhibited ^3J correlations of the singlets at δ_H 6.62 to C-1′ (δ_C 112.6) and δ_H 6.95 to C-3 (δ_C 122.6) suggesting that the two protons were H-3′ and H-6′, respectively. From the NOE difference experiment, irradiation of H-6′ (δ_H 6.95) enhanced the signal of the methoxyl at δ_H 3.84 (2.6%) indicating that this methoxyl group was located at C-5′ which was confirmed by the HMBC correlation of the signal at δ_H 3.84 to C-5′ (δ_C 143.4). Irradiation of H-3′ (δ_H 6.62) enhanced the signals of the methoxyl at δ_H 3.74 (3.8%) and the methylene (H_2-1″) at δ_H 4.64 (5.8%); while irradiation of 5′-OMe (δ_H 3.84) enhanced the signal of H-6′ (11.6%) and irradiation of H_2-1″ (δ_H 4.64) enhanced the signal of H-3′ (14.4%). Irradiation of the methoxyl at δ_H 3.74 enhanced the signal of H-3′ (12.1%) but no NOE effect to the signal of 5′-OMe was observed. The placement of the second methoxyl group at C-4′ was unlikely; the γ,γ-dimethylallyloxy unit was therefore placed at C-4′. This was supported by ^3J correlations of H_2-1″ (δ_H 4.64) to C-4′ (δ_C 148.8) and 2′-OMe (δ_H 3.74) to C-2′ (δ_C 151.7) in the HMBC spectrum. Structure **1** was thus concluded to be 4′-γ,γ-dimethylallyloxy-5,7,2′,5′-tetramethoxyisoflavone, now named brandisianin A.

Table 1. The ^{1}H- (300 MHz) and ^{13}C-NMR (75 MHz) Data of **1** ($CDCl_3$)

Position	δ_H	δ_C	HMBC	
			^2J	^3J
2	7.79 (1H, s)	152.3	3	4, 8a, 1′
3		122.6		
4		175.3		
4a		110.1		
5		163.7		
6	6.38 (1H, d, J=2.1 Hz)	96.1	5	8, 4a
7		161.4		
8	6.47 (1H, d, J=2.1 Hz)	92.6	8a	6, 4a
8a		159.9		
1′		112.6		
2′		151.7		
3′	6.62 (1H, s)	100.1	2′, 4′	1′, 5′
4′		148.8		
5′		143.4		
6′	6.95 (1H, s)	115.9	1′, 5′	3, 2′, 4′
1″	4.64 (2H, d, J=6.6 Hz)	66.1	2″	4′, 3″
2″	5.54 (1H, t, J=6.6 Hz)	120.1		
3″		137.6		
4″	1.80 (3H, s)[a]	25.9[a]	3″	2″, 5″
5″	1.78 (3H, s)[a]	18.3[a]	3″	2″, 4″
5-OMe	3.90 (3H, s)	55.7		5
7-OMe	3.94 (3H, s)	56.4		7
2′-OMe	3.74 (3H, s)	56.9		2′
5′-OMe	3.84 (3H, s)	56.6		5′

a) Interchangeable values.

α-Toxicarol (**10**), 12a-hydroxy-α-toxicarol (**11**), and 6a,12a-dehydro-α-toxicarol (**12**) were previously evaluated for their potential chemopreventive properties in a mouse mammary organ culture (MMOC) model; only α-toxicarol (**10**) was found to exhibit inhibition of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced pre-neoplastic lesions (80% at a dose of 10 μg/ml).[13] 6-Deoxyclitoriacetal (**7**) was reported to possess strong cytotoxic activity against culture P-338 lymphocytic leukemia cells.[9] Compound **7** also showed smooth muscle relaxant activity through a non-specific mechanism by interference with intracellular calcium metabolism.[18]

Two rotenoids, 6-deoxyclitoriacetal (**7**) and stemonal (**9**), were studied for anti-inflammatory activity. Compound **7** was shown to possess higher anti-inflammatory activity than **9** using a capillary permeability assay, a typical model of first-stage inflammatory reactions.[19]

In our earlier report, 6-deoxyclitoriacetal (**7**) and 12a-hydroxy-α-toxicarol (**11**) showed no activity against the tested bacteria and yeasts at the highest concentration (128 μg/ml).[3] Since several compounds were isolated in the present investigation in only low yields, the compound quantities available did not permit any biological assays.

In our present study, compounds **7** and **11**, which were isolated as the major components, were tested for anti-inflammatory activity; the results are shown in Table 2. Application of ethyl phenylpropiolate (EPP) topically on rat ears produced marked edema formation. At all assessment times compounds **7** and **11** at the dose of 1 mg per ear significantly inhibited the ear edema formation. When the intensity of the inhibitory effect of the two test compounds was compared, compound **11** showed highest intensity. Phenylbutazone at the dose of 1 mg per ear, used as positive control, significantly reduced the edema formation at all assessment times. The results of the present study revealed the anti-inflammatory activity of compounds **7** and **11** on acute phase of inflammation. Edema caused by topically applied EPP is due to vasodilatation and increased vascular permeability. This event is caused by the release of various inflammatory mediators such as histamine, 5-HT, and PGs.[20] Our test compounds markedly inhibited ear edema formation induced by EPP. It is suggested that compounds **7** and **11** probably exerted anti-inflammatory activity through the inhibition of the above inflammatory mediators of acute inflammation.

All isolated flavonoids were also evaluated for free radical scavenging activity using TLC screening assay. Only three compounds, robustigenin (**3**), viridiflorin (**5**), and 12a-hy-

Table 2. Inhibitory Effect of **7**, **11** and Phenylbutazone on Ethyl Phenylpropiolate (EPP)-Induced Ear Edema in Rats

Group	Dose mg/ear	Time after topical application of EPP							
		15 min		30 min		1 h		2 h	
		ED (μm)	ED (%)	ED (μm)	ED (%)	ED (μm)	ED (%)	ED (μm)	ED (%)
Control acetone	—	80±29.4	—	205±9.6	—	285±9.6	—	225±9.6	—
Phenylbutazone	1	15±5.0	81.3	65±15.0**	65.9	135±20.7*	52.6	135±20.6*	47.1
7	1	10±5.8	87.5	95±5.0**	53.7	125±9.6**	56.1	135±5.0**	47.1
11	1	10±5.8	87.5	75±5.0**	63.4	95±5.1**	66.7	80±8.2**	68.6

Values are expressed as mean±S.E.M. (n=4). Statistically significant from control group: * $p<0.05$, ** $p<0.001$.

droxy-α-toxicarol (**11**), revealed DPPH radical scavenging activity and appeared as yellow spots against a purple background.

Two isoflavones, robustigenin (**3**) and toxicarol isoflavone (**4**), were isolated in small amounts from the hexane-soluble part of the EtOAc extract. From both the hexane-soluble and hexane-insoluble parts of the extract a number of corresponding rotenoids and dehydrorotenoids were obtained with 6-deoxyclitoriacetal (**7**) and 12a-hydroxy-α-toxicarol (**11**) as the main components. The isolation of these metabolites is in accordance with the biosynthetic pathway of these compounds. Although 7,4′-di-*O*-prenylgenistein (**2**) was earlier synthesized, this is the first time it has been found as a naturally occurring compound. It is interesting to note that *M. brandisiana* is the first Thai *Millettia* species to produce isoflavones and rotenoids; other species of *Millettia* in Thailand have yielded only flavones and chalcones.[2,22] In addition, 12a-hydroxy-α-toxicarol (**11**) seems worthy of additional testing further to evaluate its potential as an anti-inflammatory agent.

Experimental

General Experimental Procedures Melting points were determined by a Kofler hot stage apparatus. UV spectra were obtained on a Hewlett Packard 8453 UV–VIS spectrophotometer. IR spectra were recorded on a Perkin-Elmer GX FT-IR spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ solutions on a Bruker AVANCE (300 MHz) spectrometer using TMS as internal standard. HMQC and HMBC spectra were acquired using the standard Bruker software. Optical rotations were measured on a JASCO P1010 digital polarimeter. Mass spectra were obtained from a Bruker MicroTOF spectrometer. Vacuum liquid chromatography (VLC) and column chromatography (CC) were performed on silica gel 60 (70—230 mesh, Merck). TLC and preparative TLC (PLC) were conducted on silica gel plates ($60F_{254}$, Merck).

Plant Material The leaves of *M. brandisiana* were collected at Silpakorn University, Nakorn Pathom in June 2003. A voucher specimen (SUMB603) was identified by Assoc. Prof. Nijsiri Ruangrungsi and deposited at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Extraction and Isolation The dried leaves of *M. brandisiana* (1.27 kg) were extracted with EtOH (5 l×3) at room temperature. The combined extract was concentrated to dryness to give 186.7 g of residue. The EtOH extract was sequentially partitioned between organic solvents and water to yield EtOAc extract (79.4 g), *n*-butanol extract (30.6 g), and water extract (76.5 g). The EtOAc extract was further dissolved in hexane to afford a hexane-soluble fraction (22.3 g) and a hexane-insoluble fraction (57.1 g). Separation of the hexane-soluble fraction by VLC using gradient hexane–EtOAc gave 12 fractions (A1—A12). The combined A3 and A4 (5.06 g) was subjected to CC with gradient hexane–acetone (9 : 1, 8 : 2, 7 : 3) to give 4 fractions (B1—B4). B1 (284.5 mg) was purified by PLC (hexane–CH_2Cl_2, 1 : 9) to afford **11** (117.1 mg). B2 (177.1 mg) was purified by PLC (hexane–CH_2Cl_2–EtOAc, 4 : 1 : 1) to yield **13** (10.1 mg). B3 (143.4 mg) and B4 (46.6 mg) were purified by PLC (CH_2Cl_2–MeOH, 30 : 1) to give **7** (34.3 mg) and **9** (3.9 mg). The combined A5 and A6 (1.79 g) was purified by CC with gradient hexane–acetone (9 : 1, 8 : 2, 7 : 3) to afford 2 fractions (C1, C2) that were further purified by PLC (hexane–acetone, 3 : 1) to yield **10** (21 mg) and **11** (178.8 mg). A7 (1.70 g) was purified by CC with gradient hexane–acetone (8 : 2, 7 : 3) to give **7** (373.6 mg). Repetitive PLC of A8 (1.54 g) with hexane–acetone (3 : 1) afforded **4** (2.6 mg), **3** (7.2 mg), **11** (309.2 mg), and **7** (186.8 mg). The combined A9 and A10 (1.86 g) was subjected to CC with gradient CH_2Cl_2–MeOH (40 : 1, 30 : 1, 20 : 1, 10 : 1) to give 5 fractions (D1—D5). D3 (86.1 mg) was purified by PLC (hexane–acetone–MeOH, 3 : 1 : 0.4) to afford **9** (3.4 mg). Purification of D4 (303.5 mg) by repeated PLC (hexane–acetone–MeOH, 3 : 1 : 0.8) gave **1** (7 mg).

Separation of the hexane-insoluble fraction (57.1 g) by VLC using gradient hexane–EtOAc gave 17 fractions (E1—E17). The combined E1, E2, and E3 (1.96 g) was subjected to CC with gradient hexane–EtOAc and further purified by PLC (hexane–acetone, 3 : 1) to yield **12** (18.3 mg) and **10** (7.2 mg). E6 (10.09 g) was separated on CC with gradient hexane–EtOAc to afford 11 fractions (F1—F11). F3 (311.3 mg) was purified by CC with CH_2Cl_2 and further crystallized from acetone to give **14** (50.2 mg). F6 (3.36 g) was purified by CC with gradient hexane–EtOAc to yield 6 fractions (G1—G6). G3 (874 mg) was purified by PLC (hexane–CH_2Cl_2–EtOAc, 6 : 1 : 1) to afford **10** (31.5 mg). Repetitive PLC of G4 (411 mg) using hexane–CH_2Cl_2–EtOAc (6 : 1 : 1) and hexane–acetone (3 : 1) gave **6** (2.7 mg). F9 (1.15 g) was purified by CC using CH_2Cl_2 to yield **7** (890.4 mg). Purification of F10 (617 mg) by CC with CH_2Cl_2 afforded **11** (49.1 mg), **7** (251.6 mg), and **5** (10 mg). The combined E4, E5, and E7 (10.9 g) was separated on CC using gradient hexane–acetone (8 : 2, 7 : 3) to give 10 fractions (H1—H10). Repeated PLC of H1 (590.5 mg) with hexane–acetone (3 : 1) yielded **2** (17.4 mg). H2 (292.5 mg) was purified by CC with CH_2Cl_2 and PLC (hexane–acetone, 3 : 1) to afford **12** (49.2 mg) and **10** (125.6 mg). Repetitive PLC of H3 (741.3 mg) using CH_2Cl_2 gave **6** (2.3 mg), **8** (23.7 mg), and **9** (3 mg). Purification of H5 (345.3 mg) by CC with CH_2Cl_2 yielded a mixture that afforded **6** (41.6 mg) and **11** (273 mg) after crystallization from acetone. The combined H6 and H7 (2.24 g) was purified by CC using CH_2Cl_2 to give **11** (1.92 g). Purification of H8 (1.97 g) by CC with CH_2Cl_2 yielded **7** (1.94 g). The combine E11 and E12 (3.74 g) was purified by CC using gradient hexane–acetone (8 : 2, 7 : 3) to give 5 fractions (I1—I5). Addition of acetone to I2 (107.6 mg) gave **12** (77.5 mg). Purification of I4 (406.7 mg) by CC with CH_2Cl_2 and further crystallization from acetone yielded **1** (119 mg).

4′-γ,γ-Dimethylallyloxy-5,7,2′,5′-tetramethoxyisoflavone, Brandisianin A (**1**): White needles; mp 152—154 °C. UV λ_{max} (EtOH) nm (log ε) 226 (5.00), 256 (5.50), 290 (4.85). IR (acetone film) cm^{-1} 2936, 2852, 1651, 1571, 1511, 1463, 1285, 1214, 1154, 1042. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) data: see Table 1. EI-MS *m*/*z* (%): 426 $[M]^+$ (3), 358 (29), 343 (9), 327 (23), 181 (8), 149 (11), 69 (100). HR-ESI-MS *m*/*z*: 427.1818 $[M+H]^+$ (Calcd for $C_{24}H_{26}O_7$+H 427.1757).

Anti-inflammatory Assay. Animals Male Sprague-Dawley rats weighing 40—60 g purchased from National Laboratory Center, Nakorn Pathom, Thailand, were used. The experiment was conducted according to the Ethical Principles and Guidelines for the Use of Animals prepared by the National Research Council of Thailand. The young rats were used in this study as described by Brattsand *et al.*[20] because of the thin skin of the ear and being easy to induce edema by irritant.

Ethyl Phenylpropiolate (EPP)-Induced Ear Edema in Rats Topical anti-inflammatory activity of compounds **7** and **11** was assessed by the method described by Brattsand *et al.*[20] Male rats of 40—60 g body weight were used.

The inflammogen EPP was dissolved in acetone and ear edema was induced by topical application of EPP at a dose of 1 mg/20 μl/ear to the inner and outer surfaces of both ears using an automatic microliter pipette. Test substances also dissolved in acetone (20 μl/ear) were administered topically just before the inflammogen. The thickness of ear was measured with vernier calipers before and at 15, 30, 60, and 120 min after edema induction. The effect of the test substances on the ear edema was compared with that of the vehicle-control group, and the percent inhibition was calculated.

Statistical Analysis The data from the experiment were expressed as mean±S.E.M. Statistical comparison between groups was analyzed by one-way analysis of variance (ANOVA) and *post hoc* least-significant difference (LSD) test. p values, <0.05 were considered significant.

Evaluation of Free Radical Scavenging Activity TLC Screening Assay Test samples were spotted on a TLC plate, and development was performed with a suitable solvent. After drying, the TLC plate was sprayed with 0.3% solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in MeOH and left 30 min before visual examination.[21] Constituents possessing free radical scavenging activity appeared as yellow spots against a purple background.

Acknowledgements We are grateful to Assoc. Prof. Nijsiri Ruangrungsi, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand, for identification of the plant material.

References

1) Smitinand T., "Thai Plant Names," Revised ed., The Forest Herbarium, Royal Forest Department, Bangkok, 2001, p. 359.
2) Phrutivorapongkul A., Lipipun V., Ruangrungsi N., Kirtikara K., Nishikawa K., Maruyama S., Watanabe T., Ishikawa T., *Chem. Pharm. Bull.*, **51**, 187—190 (2003).
3) Pancharoen O., Petveroj S., Phongpaichit S., *Songklanakarin J. Sci. Technol.*, **29**, 151—156 (2007).
4) Jain A. C., Sharma B. N., *J. Org. Chem.*, **39**, 2515—2517 (1974).
5) Chibber S. S., Sharma R. P., *Phytochemistry*, **18**, 1082 (1979).

6) Dagne E., Mammo W., Sterner D., *Phytochemistry*, **31**, 3662—3663 (1992).
7) Gomez F., Calderon J. S., Quifano L., Dominguez M., Rios T., *Phytochemistry*, **24**, 1126—1128 (1985).
8) Nakatani N., Ohta H., Matsui M., *Agric. Biol. Chem.*, **36**, 2433—2439 (1972).
9) Lin L.-J., Ruangrungsi N., Cordell G. A., Shieh H.-L., You M., Pezzuto J. M., *Phytochemistry*, **31**, 4329—4331 (1992).
10) Bai L., Jiang H., Kang T., Zhang H., Jiang Z., Zhao Z., *J. Nat. Med.*, **58**, 275—277 (2004).
11) Baran-Marszak M., Massicot J., Molho D., *Bull. Soc. Chim. France*, No. 1, 191—198 (1971).
12) Shiengthong T., Donavanik T., Uaprasert V., Roengsumran S., *Tetrahedron Lett.*, **23**, 2015—2018 (1974).
13) Jang D. S., Park E. J., Kang Y.-H., Hawthrone M. E., Vigo J. S., Graham J. G., Cabieses F., Fong F. H. H., Mehta R. G., Pezzuto J. M., Kinghorn A. D., *J. Nat. Prod.*, **66**, 1166—1170 (2003).
14) Andrei C. C., Vieira P. C., Fernandes J. B., Silva M., Fo E. R., *Phytochemistry*, **46**, 1081—1085 (1997).
15) Lin Y.-L., Kuo Y.-H., *Chem. Pharm. Bull.*, **41**, 1456—1458 (1993).
16) Somleva T., Ognyanov I., *Planta Med.*, **51**, 219—221 (1985).
17) Moghaddam F. M., Farimani M. M., Salahvarzi S., Amin G., *Evid. Based Complement. Alternat. Med.*, **4**, 95—98 (2007).
18) Itthipanichpong C., Ruangrungsi N., Saibundasak K., *J. Med. Assoc. Thai*, **84** (Suppl. 1), S208—S215 (2001).
19) Silva B. P., Parente J. P., *Phytother. Res.*, **16**, S87—S88 (2002).
20) Brattsand R., Thalen A., Roempke K., Kallstrom L., Gruvstad E., *J. Steroid Biochem.*, **16**, 779—786 (1982).
21) Takao T., Kitatani F., Watanabe N., Yagi N., Sakata K., *Biosci. Biotech. Biochem.*, **58**, 1780—1783 (1994).
22) Srituralak B., Likhitwitayawuid K., *Phytochemistry*, **67**, 812—817 (2006).