Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 69 (2008) 546–552
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Estrogenic constituents of the heartwood of *Dalbergia parviflora*

Kaoru Umehara [a,*], Kiyomitsu Nemoto [a], Kyoko Kimijima [a], Ayako Matsushita [a], Eri Terada [a], Orawan Monthakantirat [b], Wanchai De-Eknamkul [b], Toshio Miyase [a], Tsutomu Warashina [c], Masakuni Degawa [a], Hiroshi Noguchi [a]

[a] *School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan*
[b] *Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*
[c] *Institute for Environmental Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan*

Received 2 September 2006; received in revised form 4 May 2007
Available online 24 August 2007

Abstract

From the heartwood of *Dalbergia parviflora*, five compounds, dalparvin A (**1**), B (**2**), C (**3**), dalparvinol C (**4**), and neokhriol A (**5**), along with 11 known compounds, kenusanone G (**6**), cajanin (**7**), sophorol (**8**), alpinetin (**9**), hesperetin (**10**), 3′-*O*-methylorobol, odoratin, (2*R*)(3*R*)-2,3-*trans* 7-hydroxy-5-methoxydihydroflavonol, (6a*R*, 11a*R*)-3,8-dihydroxy-9-methoxypterocarpan, (6a*R*, 11a*R*)- vesticarpan, and methyl-3,4-dihydroxy-2-methoxybenzoate were isolated and characterized. Isolates were evaluated for their cell proliferation stimulatory activity against MCF-7, T-47D, and BT20 human breast cancer cell lines. Along with **7**–**10**, two compounds **2** and **3** stimulated not only MCF-7, but also T-47D human breast cancer cell proliferation. Compound **6** had activity only against MCF-7 cells, and the activity of **7** was more than equivalent to that of daidzein. On the other hand, none of the isolates had any significant effects on BT20 cell proliferation, and these results indicated that the stimulative activity of these compounds was not general to any cell proliferations. Furthermore, these compounds were tested in the estrogen-responsive transient luciferase reporter assay.

Keywords: *Dalbergia parviflora*; Leguminosae; Heartwood; Estrogenic activity; Luciferase; Flavonoid; Dalparvin A; Dalparvin B; Isodalparvinol A; Dalparvinol C; Neokhriol A

1. Introduction

A methanol extract of the heartwood of *Dalbergia parviflora* R. (Leguminosae) was found to have stimulatory activity against estrogen-responsive MCF-7 human breast cancer cells. This plant species, known as khri in Thai, is a giant liana that develops dark red heartwood, and is found in the Malay Peninsula and the southern part of Thailand (Pichiansunthorn et al., 1999). In Thai folk medicine, the heartwood is used as a blood tonic, to normalize menstruation, as well as finding application as an expectorant, stomachic and cardiotonic (Smitinand, 1983). Oil from the wood is used to heal chronic wounds and as an antipyretic, while its leaves have a mild aphrodisiac effect. Although *Dalbergia* plants are known as rich sources of flavonoids from a number of chemical investigations, very little is known about the constituents of *D. parviflora*. Some arylbenzofurans (Muangnoicharoen and Frahm, 1981) and neoflavanoids (Muangnoicharoen and Frahm, 1982) have been isolated from the heartwood (see Fig. 1).

As part of our continued screening of plant constituents for estrogenic agents (Monthakantirat et al., 2005), an extract of the heartwood of *D. parviflora* was investigated, and five new phenolic compounds (**1**–**5**), together with 11 known compounds, were isolated. This paper deals with the isolation of these compounds, their characterization by spectroscopic methods, and the assessment of their estrogenic activity by measuring the potency of the substances in estrogen-responsive/non-responsive cell lines (Soto et al., 1995).

* Corresponding author. Tel./fax: +81 54 264 5661.
E-mail address: umehara@ys7.u-shizuoka-ken.ac.jp (K. Umehara).

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.07.011

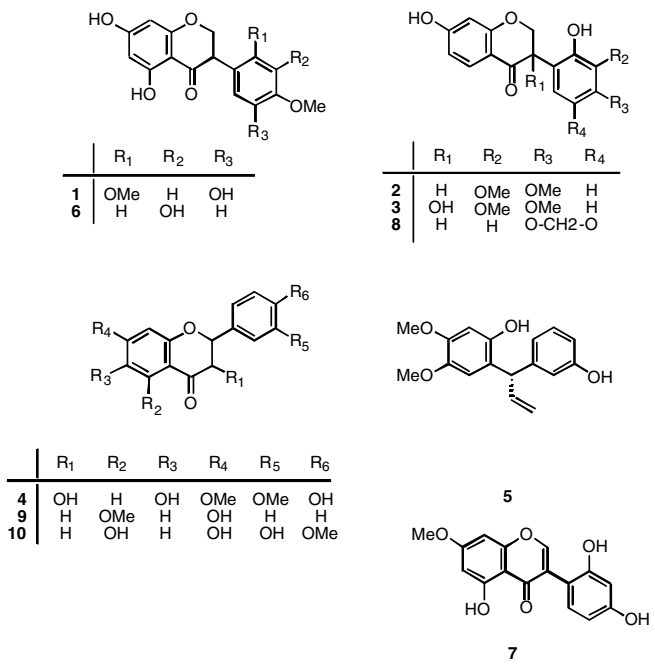

Fig. 1. Chemical structures of isolated compounds (**1**–**10**) from the heartwood of *Dalbergia parviflora*.

2. Results and discussion

Part of the methanolic extract of *D. parviflora* was subjected to an silica gel column chromatography using chloroform–MeOH solvent systems. Fractions were collected and pooled by TLC analysis to afford 26 combined fractions. Purification of fraction 13 (8.9 g, eluted by chloroform–MeOH (96:4)) using HPLC gave 16 phenolic compounds, including five new compounds (**1**–**5**). The 11 known compounds were identified as kenusanone G (**6**) (Iinuma et al., 1993), cajanin (**7**) (Waffoa et al., 2000), sophorol (**8**) (Suginome, 1959), alpinetin (**9**) (Itokawa et al., 1981), hesperetin (**10**) (Shen et al., 1993), 3′-*O*-methylorobol (Tahara et al., 1984), odoratin (Hayashi and Thomson, 1974), (2*R*)(3*R*)-2,3-*trans* 7-hydroxy-5-methoxydihydroflavonol (Bankova et al., 1983), (6a*R*, 11a*R*)-3,8-dihydroxy-9-methoxypterocarpan (Bezuidenhoudt et al., 1987), (6a*R*, 11a*R*)-vesticarpan (Kurosawa et al., 1978), and methyl-3,4-dihydroxy-2-methoxybenzoate (Saito et al., 2004), respectively, by comparison of their spectroscopic data with reported values in the literature.

Dalparvin A (**1**), $[\alpha]_D + 15.1$, a colorless amorphous powder, was assigned the molecular formula $C_{17}H_{16}O_7$, as determined from its molecular ion $[M]^+$ peak at *m/z* 332.0873 in the HRFABMS. The ^1H NMR spectra of **1** showed the characteristic three spin system for an isoflavanone skeleton at δ 4.43 (1H, *dd*, $J = 11$, 6 Hz, H-2), 4.51 (1H, *t*, $J = 11$ Hz, H-2) and 4.24 (1H, *dd*, $J = 11$, 6 Hz, H-3) with a chelated hydroxyl group appearing at δ 12.3. The *meta*-coupled proton signals at δ 5.96 (H-6) and 5.94 (H-8) and two singlet protons at δ 6.67 (H-6′) and 6.74 (H-3′) indicated the presence of a 5,7-dioxygenated A-ring and a 2′,4′,5′-trisubstituted B-ring. The nuclear Overhauser effects (NOEs) were observed at H-3′ [δ 6.74 (*s*)] by irradiation of methoxyl groups δ 3.76 and 3.86. With regard to the absolute configuration of **1** at C-3, the circular dichroism (CD) spectrum showed a positive Cotton effect at 343 nm, which is consistent with *R* configuration (Yenesew et al., 2000). From this spectroscopic evidence, the structure of dalparvin A (**1**) was established to be (3*R*)-5,5′,7-trihydroxy-2′,4′-dimethoxyisoflavanone.

Dalparvin B (**2**) was assigned the molecular formula $C_{17}H_{16}O_6$, as determined from its molecular ion $[M]^+$ peak at *m/z* 316.0931 in the HRFABMS. Analysis of the ^1H NMR spectrum also indicated that this compound was an isoflavanone as evidenced from the spin system [δ 4.50 (1H, *dd*, $J = 9$, 6 Hz, H-2), 4.64 (1H, *t*, $J = 9$ Hz, H-2) and 4.17 (1H, *dd*, $J = 9$, 6 Hz, H-3)]. The *ortho*-coupled proton signal that appeared at lower field [δ 7.77 (1H, *d*, $J = 9$ Hz)] was characteristic for H-5 locating at the peri-position of a carbonyl group, and it was observed to have an ABX spin system with δ 6.58 (1H, *dd*, $J = 9$, 2 Hz, H-6) and 6.40 (1H, *d*, $J = 2$ Hz, H-8) from analysis of the HH-COSY spectrum. Furthermore, the *ortho*-coupled protons at δ 6.47 (*d*, $J = 9$ Hz, H-5′) and 6.75 (*d*, $J = 9$ Hz, H-6′) indicated the presence of a trisubstituted B-ring. A NOE was also observed at δ 6.47 by irradiation of the methoxyl group (δ 3.81) but was not observed when another methoxyl group (δ 3.78) was irradiated. In the HMBC, the correlation of the methoxyl proton signal (δ 3.78) with the carbon resonance at δ 137.1 (C-3′) was observed, and another methoxyl proton resonance (δ 3.81) also showed a correlation with the carbon resonance [δ 153.1 (C-4′)]. From these results, the structure of dalparvin B (**2**) was confirmed as (3*RS*)-2′,7-dihydroxy-3′,4′-dimethoxyisoflavanone. The CD spectrum and $[\alpha]_D$ value indicated **2** was a racemate.

Isodalparvinol A (**3**) was assigned the molecular formula $C_{17}H_{16}O_7$, as determined from its molecular ion $[M+Na]^+$ peak at *m/z* 355.0770 in the HRFABMS. In addition to having ABX pattern signals at δ 6.37 (1H, *d*, $J = 2$ Hz, H-8), 6.58 (1H, *dd*, $J = 9$, 2 Hz, H-6) and 7.76 (1H, *d*, $J = 9$ Hz, H-5), *ortho*-coupled protons at δ 6.52 (*d*, $J = 9$ Hz) and 7.17 (*d*, $J = 9$ Hz) were observed in the ^1H NMR and HH-COSY spectra. In the difference NOE spectrum of **3**, a NOE was observed at δ 6.52 by irradiation of the methoxyl group (δ 3.82), but was again not observed when another methoxyl group (δ 3.75) was irradiated. Two proton signals at δ 4.94 (1H, *d*, $J = 12$ Hz) and 4.22 (1H, *d*, $J = 12$ Hz) were assigned to an oxymethylene at C-2 (δ 74.7) from the HMQC spectrum. In the HMBC spectrum, correlation of the methoxyl proton signal (δ 3.75) with the carbon resonance at δ 137.5 (C-3′) was observed, and another methoxyl proton signal (δ 3.82) also showed a correlation with the carbon resonance [δ 154.0 (C-4′)]. From the above spectroscopic data, the structure of isodalparvinol A (**3**) was established as (3*RS*)-2′,3,7-trihydroxy-3′,4′-dimethoxyisoflavanone. The CD spectrum and $[\alpha]_D$ value also showed that **3** was a racemate, as previously noted for **2**.

Dalparvinol C (**4**), $[\alpha]_D + 27.9$, a colorless amorphous powder, was assigned the molecular formula $C_{17}H_{16}O_7$, as determined from its molecular ion $[M+Na]^+$ peak at *m/z* 355.0772 in the HRFABMS. Analysis of the ^1H NMR spectrum indicated two singlet signals [δ 6.57 (1H, *s*); 7.19 (1H, *s*)] and ABC-type coupled protons [δ 6.87 (1H, *d*, $J = 8$ Hz, H-5′), 7.04 (1H, *dd*, $J = 8$, 2 Hz, H-6′), and 7.22 (1H, *d*, $J = 2$ Hz, H-2′)], in addition to two methoxyl proton resonances [δ 3.88 and 3.92 (each 3H, *s*)]. Two proton signals at δ 5.00 (1H, *d*, $J = 12$ Hz) and 4.57 (1H, *d*, $J = 12$ Hz) also showed correlations to carbon resonances at δ 85.6 (C-2) and 74.2(C-3), respectively, in the HMQC spectrum, and **4** was thus considered to be a flavanonol derivative. Moreover, the NOEs observed at δ 6.57(H-8) and 7.22(H-2′), by irradiating methoxyl signals at δ 3.92 and 3.88, respectively, and the methoxyl groups were assigned to C-7 and C-3′, respectively, from analysis of the HMQC and HMBC spectra. The absolute configuration at C-2 and C-3 of **4** was then determined as 2(*R*), 3(*R*) by a positive Cotton effect ($[\theta]_{350}$ 16,200) and a negative Cotton effect ($[\theta]_{312} - 21{,}900$) in the CD spectrum (Rensburg et al., 1997). Based on the above spectroscopic evidence, dalparvinol C (**4**) was (2*R*,3*R*)-2,3-*trans*-4′,6-dihydroxy-3′,7-dimethoxyflavonol.

Neokhriol A (**5**), $[\alpha]_D - 5.0$, a colorless amorphous powder, was assigned the molecular formula, $C_{17}H_{18}O_4$, as determined from its molecular ion $[M]^+$ peak at *m/z* 286.1201 in the HRFABMS. The ^1H NMR spectra of **5** showed two aromatic ring systems from two singlet signals (δ 6.46 and 6.60) and 1,3-disubstituted benzene ring protons [δ 6.68 (*t*, $J = 2$ Hz), 6.72 (*dd*, $J = 8$, 2 Hz), 6.81 (brd, $J = 8$ Hz), and 7.20 (*t*, $J = 8$ Hz)], and the structure was indicative of a neoflavone with characteristic resonances for the vinyl group [δ 5.04 (*dt*, $J = 17$, 1.5 Hz), 5.30 (*dt*, $J = 10$, 1.5 Hz), and 6.28 (*ddd*, $J = 17$, 10, 7 Hz)]. The methoxyls were indicated to be attached at C-4 (δ 149.0) and C-5 (δ 143.3) from the NOE enhancements of H-3 (δ 6.46) and H-6 (δ 6.60) by irradiating signals at δ 3.83 and 3.77, respectively, and they were further confirmed from analysis of the HMBC spectrum. The CD curve of the compound showed a negative Cotton effect at 236 nm, which is consistent with the *S* configuration at C-3 (Muangnoicharoen and Frahm, 1982). From the above spectroscopic data, neokhriol A (**5**) was confirmed as (3*S*)-3-(2-hydroxy-4,5-dimethoxyphenyl)-3-(3′-hydroxyphenyl) prop-1-ene.

Effects on MCF-7 cell proliferation were also tested with increasing concentrations of isoflavones ranging from 10 nM to 100 μM, and their EqE_{10} and EqE_{100} values were determined for the required concentrations against cell proliferation equivalent to 10 and 100 pM of estradiol (E2) treatment, respectively (Table 3). Compounds **2** and **10** were found to have stimulatory activities (EqE_{10}: 5.7 μM for **2** and 7.0 μM for **10**) against the cell proliferation and showed high potency, as effective as 100 pM of E2 (EqE_{100}: 83.1 μM for **2** and 86.7 μM for **10**). Although **3**, **6**, **8**, and **9** were observed to enhance cell proliferation (EqE_{10}: 8.3 μM for **3**, 19.2 μM for **6**, 2.7 μM for **8**, and 5.4 μM for **9**), their EqE_{100} values could not be assessed since their stimulatory activities were not high enough, even at a concentration of 100 μM. Compound **7** showed more than an equivalent level of activity to that of daidzein (EqE_{10}: 0.5 μM for **7** and 0.6 μM for daidzein, EqE_{100}: 1.9 μM for **7** and 10.6 μM for daidzein), whereas cytotoxicity of this compound was observed at a concentration of 10 μM. The remaining compounds did not show any significant activities against the cell line at these concentrations, and their EqE_{10} values could not be evaluated.

The effects of **1**–**10** on breast cancer cell proliferation were tested in T47D cells (Table 3). Almost all compounds

Table 1
^1H NMR spectroscopic data of **1**–**5** (δ in ppm)

Position	**1**	**2**	**3**	**4**	Position	**5**
2	4.43 (*dd*, $J = 11,6$)	4.50 (*dd*, $J = 9,6$)	4.22 (*d*, $J = 12$)	5.00 (*d*, $J = 12$)	2	
	4.51 (*t*, $J = 11$)	4.64 (*t*, $J = 9$)	4.94 (*d*, $J = 12$)		3	6.46 *s*
3	4.24 (*dd*, $J = 11,6$)	4.17 (*dd*, $J = 9,6$)		4.57 (*d*, $J = 12$)	4	
					5	
5		7.77 (*d*, $J = 9$)	7.76 (*d*, $J = 9$)	7.19 *s*	6	6.60 *s*
6	5.96 (*d*, $J = 2$)	6.58 (*dd*, $J = 9$, 2)	6.58 (*dd*, $J = 9$, 2)		7	4.79 (*d*, $J = 7$)
7					8	6.28 (*ddd*, $J = 17$, 10, 7)
8	5.94 (*d*, $J = 2$)	6.40 (*d*, $J = 2$)	6.37 (*d*, $J = 2$)	6.57 *s*	9	5.04 (*dt*, $J = 17$, 1.5)
						5.30 (*dt*, $J = 10$, 1.5)
2′				7.22 (*d*, $J = 2$)		
3′	6.74 *s*				2′	6.68 (*t*, $J = 2$)
					3′	
4′						
5′		6.47 (*d*, $J = 9$)	6.52 (*d*, $J = 9$)	7.41 (*d*, $J = 8$)	4′	6.72 (*dd*, $J = 8,2$)
6′	6.67 *s*	6.75 (*d*, $J = 9$)	7.17 (*d*, $J = 9$)	7.04 (*dd*, $J = 8,2$)	5′	7.20 (*t*, $J = 8$)
					6′	6.81 (brd, $J = 8$)
MeO	3.76 *s*	3.78 *s*	3.75 *s*	3.88 *s*		
	3.86 *s*	3.81 *s*	3.82 *s*	3.92 *s*	MeO	3.77 *s*
						3.83 *s*

Spectra were recorded at 400 MHz in acetone-d_6 solution.

Table 2
^{13}C NMR spectroscopic data of compounds **1**–**5**

Position	**1**	**2**	**3**	**4**	Position	**5**
2	71.3	71.5	74.7	85.6	1	119.6
3	47.3	48.0	75.2	74.2	2	147.7
4	198.2	190.9	189.8	193.6	3	102.0
5	165.7	129.9	130.6	110.9	4	149.0
6	97.0	111.1	111.5	158.1	5	143.3
7	167.2	164.8	165.1	156.2	6	113.7
8	95.7	103.3	103.4	101.0	7	49.2
9	164.6	164.5	164.0	143.0	8	139.2
10	103.7	115.7	114.1	112.5	9	117.2
1′	116.6	116.4	119.8	130.0	1′	143.6
2′	152.0	149.3	148.9	112.5	2′	115.5
3′	99.5	137.1	137.5	148.1	3′	155.9
4′	148.3	153.1	154.0	148.2	4′	113.9
5′	141.4	104.3	104.1	115.5	5′	130.0
6′	117.5	125.0	122.8	122.2	6′	121.0
MeO	57.0	60.6	60.7	56.7	MeO	56.0
	56.7	56.0	56.2	56.5		56.8

Spectra were recorded at 100.4 MHz in acetone-d_6 solution.

Table 3
Cell proliferation stimulatory activities against MCF-7 and T47D cells[a]

Compound	MCF-7		T47D	
	EqE_{10} (μM)	EqE_{100} (μM)	EqE_{10} (μM)	EqE_{100} (μM)
Daidzein	0.6	10.6	0.6	11.7
1	ND	ND	79.3	ND
2	5.6	83.1	7.8	ND
3	8.3	ND	5.9	ND
4	ND	ND	ND	ND
5	ND	ND	ND	ND
6	19.2	ND	ND	ND
7	0.5	1.9	0.5	6.9
8	2.7	ND	9.5	ND
9	5.4	ND	32.3	ND
10	7.0	86.7	3.2	ND

[a] EqE_{10} and EqE_{100} represent the concentration of the compound that stimulated the cell proliferations equivalent to estradiol 10 pM and 100 pM, respectively. These values were determined by linear regression analysis using at least five different concentrations in quadruplicate. 3′-*O*-Methylorobol, odoratin, (2*R*, 3*R*)-2,3-*trans* 7-hydroxy-5-methoxydihydroflavonol, (6a*R*, 11a*R*)-3,8-dihydroxy-9-methoxypterocarpan, (6a*R*, 11a*R*)-vesticarpan, and methyl-3,4-dihydroxy-2-methoxybenzoate were not determined (ND).

showed similar activities to those exhibited against MCF-7 cell proliferation. Compound **6** showed significant activity against this cell line at concentrations of more than 10 μM, but the EqE_{10} value could not be evaluated as a result of its low activity. The stimulatory effects of the isolates on cell proliferation were examined using another human breast cancer cell line BT20, which was not responsive to E2. No compounds displayed any significant effects on cell proliferation (data not shown), and results obtained indicated that the stimulatory effect on cell proliferation of these compounds was not general to any cell lines.

To determine the estrogenic characteristics of the constituents further, eight compounds possessing cell proliferation stimulatory activities in either MCF-7 or T47D cells were selected, and a luciferase reporter assay was carried out at concentrations ranging from 0.1 to 100 μM (Table 4). In general, compounds possessing cell proliferation stimulative effects showed luciferase induction. Compound **7**, which only showed EqE_{100} values for both MCF-7 and T47D cell proliferations, was observed to have luciferase inducing activity in both luciferase transfected-MCF-7 (MCF-7/Luc) and luciferase transfected-T47D (T47D/Luc) cells at low concentrations. Furthermore, compound **8** induced luciferase activities in both cell lines. Although **1** was not observed to stimulate cell proliferation activity against MCF-7, it showed strong luciferase induction in MCF-7/Luc cells at low concentrations. On the other hand, compound **6** did not show significant luciferase induction against MCF-7/Luc cells whereas the stimulative effect on MCF-7 cell proliferation was recognized.

Table 4
Luciferase reporter assay using luciferase transfected-human breast cancer cells[a]

Compound	MCF-7		T47D	
	EqE_{10} (μM)	EqE_{100} (μM)	EqE_{10} (μM)	EqE_{100} (μM)
1	0.04	0.1	ND	ND
2	0.8	3.8	0.2	ND
3	2.5	ND	ND	ND
6	ND	ND	ND	ND
7	0.6	2.7	1.0	3.3
8	2.8	54.0	3.8	13.5
9	6.6	ND	2.7	ND
10	0.2	1.3	0.7	ND

[a] EqE_{10} and EqE_{100} represent the concentration of the compound that stimulated the luciferase induction equivalent to estradiol 10 pM and 100 pM, respectively. These values were determined by linear regression analysis using at least five different concentrations in quadruplicate. Dalparvinol C(4), neokhriol A(5), 3′-*O*-methylorobol, odoratin, (2*R*, 3*R*)-2,3-*trans* 7-hydroxy-5-methoxydihydroflavonol, (6a*R*, 11a*R*)-3,8-dihydroxy-9-methoxypterocarpan, (6a*R*, 11a*R*)-vesticarpan, and methyl-3,4-dihydroxy-2-methoxybenzoate were not determined (ND).

2.1. Concluding remarks

Recently, much interest has been paid to phytoestrogens for their potential health benefits in counteracting menopausal symptoms and in lowering incidence of hormone dependent diseases (Adlercreutz, 2002). Such diverse actions of phytoestrogens may involve non-estrogen mediated mechanisms, a major mechanism of their action is thought to result from their ability to interact directly with estrogen receptors (ERs) (Makela et al., 1999). Some phytoestrogens displayed higher binding affinity to ERβ than to ERα and to recruit coregulators necessary for transcription of target genes selectively to ERβ (Kuiper et al., 1998). The effects of phytoestrogens on health benefits can be related to their selective estrogen receptors modulators (SERMs)-like activity. Though estrogenic compounds associate a number of undesirable side effects and increased relative risk for breast and uterine cancer, several *in vitro* assays using constructs with different gene promoters and

variant forms of ER can distinguish between phytoestrogens and lead to unique tissue specific SERMs.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a JASCO DIP-360 digital polarimeter, whereas CD spectra were recorded on a JASCO J-20A spectropolarimeter. UV spectra were obtained using a Hitachi U3410 spectrometer, while ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded in acetone-d_6 solution on a JEOL JNM-α 400 instrument with chemical shifts in δ (ppm) using tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained using JEOL JMS-SX 102 and JMS-700 mass spectrometers. HPLC was carried out with a JASCO model 887-PU pump and an 875-UV variable wavelength detector with a reversed-phase column (Capcell Pak ODS, 5 μm, 2 × 25 cm, Shiseido Fine Chemicals Co. Ltd., at 6 mL/min with detection at 205 nm and Develosil-Lop-ODS, 10–20 μm, 5 × 100 cm, Nomura Chemical Co., Ltd., at 45 mL/min with detection at 205 nm).

3.2. Chemicals

Eagle's MEM and RPMI media were purchased from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY). Antibiotics were purchased from Meiji Seika Kaisha Ltd (Tokyo, Japan). L-Glutamine was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 17β-Estradiol and dextran-coated charcoal (DCC) were purchased from Sigma Chemicals (St. Louis, MO).

3.3. Plant material

Commercially available heartwood of *Dalbergia parviflora* was purchased from a Thai medicinal herb store in Bangkok, in July 2004. The plant was identified by Dr. C. Niyomdham of The Forest Herbarium, Bangkok. A voucher specimen was deposited at the herbarium of The Forest Herbarium (68143), Bangkok, Thailand.

3.4. Extraction and isolation

The dried heartwood of *D. parviflora* (2 kg) was extracted three times with MeOH (3 × 20 L) at room temperature for 6 h. The extracts were combined and concentrated under reduced pressure at 60 °C to yield a viscous mass (910 g). Part of this concentrated extract (150 g) was subjected to silica gel CC and fractionated using eluants chloroform–MeOH (98:2, 96:4, 94:6, 90:10, 15 L each). Fractions were collected and pooled by TLC analysis to afford 26 combined fractions. Purification of fraction 13 (8.9 g, eluted by chloroform–MeOH (96:4)) using HPLC on Develosil-Lop-ODS (5 × 100 cm, flow rate 45 mL/min with detection at 205 nm) with MeOH–H_2O (50:50) as eluent gave **1** (57 mg), **2** (45 mg), **3** (3 mg), **4** (3 mg), **5** (2 mg), **6** (20 mg), **7** (80 mg), **8** (1 mg), **9** (100 mg), **10** (2 mg), 3′-*O*-methylorobol (18 mg), odoratin (3 mg), (2*R*)(3*R*)-2,3-*trans* 7-hydroxy-5-methoxydihydroflavonol (3 mg), (6a*R*, 11a*R*)-3,8-dihydroxy-9-methoxypterocarpan (1 mg), (6a*R*, 11a*R*)-vesticarpan (9 mg), and methyl-3,4-dihydroxy-2-methoxybenzoate (30 mg) (t_R = 264, 236, 109, 88, 314, 228, 430, 246, 400, 256, 297, 252, 224, 171, 218, and 84 min, respectively). Compounds **6**–**10**, 3′-*O*-methylorobol, odoratin, (2*R*)(3*R*)-2,3-*trans* 7-hydroxy-5-methoxydihydroflavonol, (6a*R*, 11a*R*)-3, 8-dihydroxy-9-methoxypterocarpan, (6a*R*, 11a*R*)-vesticarpan, and methyl-3,4-dihydroxy-2-methoxybenzoate were identified by comparison of their spectroscopic data with published data.

3.5. Dalparvin A (*1*)

Amorphous powder. UV λ^{max}_{MeOH} nm (log ε): 228 (4.36), 292 (4.39), 323 (sh) (3.73) nm. $[\alpha]^{25}_D$ + 15.1 (MeOH; *c* 0.51). CD (MeOH; *c* 0.001): $[\theta]_{343}$ + 1890, $[\theta]_{300}$ − 1140, $[\theta]_{281}$ + 8830, $[\theta]_{245}$ 0, $[\theta]_{237}$ + 5050. For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HRFABMS *m*/*z* $[M]^+$ 332.0873 ($C_{17}H_{16}O_7$ calcd. as 332.0896).

3.6. Dalparvin B (*2*)

Amorphous powder. UV λ^{max}_{MeOH} nm (log ε): 228 (sh) (4.23), 275 (4.15), 311 (3.89) nm. For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HRFABMS *m*/*z* $[M]^+$ 316.0931 ($C_{17}H_{16}O_6$ calcd. as 316.0947).

3.7. Isodalparvinol A (*3*)

Amorphous powder. UV λ^{max}_{MeOH} nm (log ε): 229 (4.12), 277 (4.04), 311 (3.79) nm. For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HRFABMS *m*/*z* $[M+Na]^+$ 355.0770 ($C_{17}H_{16}O_7Na$ calcd. as 355.0794).

3.8. Dalparvinol C (*4*)

Amorphous powder. UV λ^{max}_{MeOH} nm (log ε): 238 (4.39), 277 (4.15), 347(3.88) nm. $[\alpha]^{25}_D$ + 27.9 (MeOH; *c* 0.3). CD (MeOH; *c* 0.001): $[\theta]_{350}$ + 16,200, $[\theta]_{312}$ − 21,900, $[\theta]_{267}$ + 5240, $[\theta]_{239}$ 14,800. For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HRFABMS *m*/*z* $[M+Na]^+$ 355.0772 ($C_{17}H_{16}O_7Na$ calcd. as 355.0794).

3.9. Neokhriol A (*5*)

Amorphous powder. UV λ^{max}_{MeOH} nm (log ε): 291 (3.88) nm. $[\alpha]^{25}_D$ − 5.0 (MeOH; *c* 0.2). CD (MeOH; *c* 0.002): $[\theta]_{290}$ − 2860, $[\theta]_{236}$ − 7730. For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HRFABMS *m*/*z* $[M]^+$ 286.1201 ($C_{17}H_{18}O_4$ calcd. as 286.1205).

3.10. Cell culture

MCF-7, T47D, and BT20 human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA). MCF-7 cells were grown in MEM supplemented with 6 ng/mL insulin, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 2 mM glutamine, 10% FBS, and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin), under a 5% CO_2 humidified atmosphere at 37 °C. T47D and BT20 cells were grown in RPMI-1640 supplemented with 1 mM sodium pyruvate, 1 mM non-essential amino acids, 2 mM glutamine, 10% FBS, and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin), under a 5% CO_2 humidified atmosphere at 37 °C.

3.11. Cell proliferation assay

Cells were seeded into 96-well tissue culture plates in 5% DCC-treated, FBS-supplemented RPMI phenol red-free medium at a density of 1×10^4 cells/well. Test compounds at concentrations ranging from 0.01 to 100 μM were added in DMSO solution (control contained 1% DMSO) and incubated at 37 °C with 5% CO_2 for 96 h. In all experiments, serial dilutions of estradiol at concentrations ranging from 1 to 1000 pM were added as a positive control. To evaluate relative cell concentrations, Alamar Blue reagent was used. After 3 h, fluorescence was measured at 590 nm with excitation at 530 nm using a Wallac 1420 ARVOsx multilabel counter (Perkin–Elmer Inc., Wellesley, MA).

3.12. Luciferase reporter gene assay

The luciferase reporter gene assay was conducted according to the procedure reported previously (Umehara et al., 2004). MCF-7 or T47D cells were placed in phenol red-free MEM supplemented with 5% DCC FBS for 48 h prior to plating. The cells were transfected for 12 h in OPTI-MEM with 2 μg of EREx3-pGV-P2 plasmid (containing three copies of the ERE linked to the luciferase gene) using LIPOFECTAMINE in a 12:1 ratio (24 μL of lipid/2 μg of DNA) according to the manufacturer's instructions. The medium was changed for phenol red-free MEM with 5% DCC-FBS, and the cells were incubated for 12 h. After 12 h, luciferase transfected-MCF-7 (MCF-7/Luc) or luciferase transfected-T47D (T47D/Luc) cells (2×10^4/well) were seeded in 96 well plates with the same medium (90 μL), and after culturing for 24 h, a test compound dissolved in the medium (10 μL) was added to each well, and further incubated for 12 h. In all experiments, serial dilutions of estradiol were added as positive control at concentrations ranging from 1 to 1000 pM. After treatment with test compounds, the cells were washed twice with PBS and lysed by treatment with 20 μL lysis buffer (Pica gene assay kit; Toyo Ink Co. Tokyo) per well for 10 min at room temperature. Luciferase activities of the cell extracts were determined using Luciferase Substrate (Pica gene assay kit; Toyo Ink Co. Tokyo) in a luminometer (Labsystems Luminoskan RS).

3.13. Data and statistical analysis

Statistical differences were determined by analysis of variance followed by Dunnett's multiple comparison test. Statistical significance was established at the $p < 0.05$ level.

Acknowledgements

This work was supported by The Royal Golden Jubilee Ph.D. Program from The Thailand Research Fund, Thailand and the National Center for Genetic Engineering and Biotechnology, Bangkok. We thank the staff of the Central Analytical Laboratory of the University of Shizuoka for MS measurements.

References

Adlercreutz, H., 2002. Phytoestrogen and breast cancer. J. Steroid Biochem. Mol. Biol. 83, 113–118.

Bankova, V.S., Popov, S.S., Marekov, N.L., 1983. A study on flavonoids of propolis. J. Nat. Prod. 46, 471–474.

Bezuidenhoudt, B.C.B., Brandt, E.V., Ferreira, D., 1987. Flavonoid analogues from *Pterocarpus* species. Phytochemistry 26, 531–535.

Hayashi, T., Thomson, R.H., 1974. Isoflavones from *Dipteryx odorata*. Phytochemistry 13, 1943–1946.

Iinuma, M., Ohyama, M., Tanaka, T., Mizuno, M., Hong, S.K., 1993. Five flavonoid compounds from *Echinosophora koreensi*. Phytochemistry 33, 1241–1245.

Itokawa, H., Morita, M., Mihashi, S., 1981. Phenolic compounds from the rhizomes of *Alpinia speciosa*. Phytochemistry 20, 2503–2506.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139, 4252–4263.

Kurosawa, K., Ollis, W.D., Redman, B.T., Sutherland, I.O., Gottlieb, O.R., 1978. Vestitol and vesticarpan, isoflavonoids from *Machaerium vestitum*. Phytochemistry 17, 1413–1415.

Makela, S., Hyder, S.M., Stancel, G.M., 1999. Environmental estrogens. In: Oettel, M., Schillinger, E. (Eds.), Estrogens and Antiestrogens II, Handbook Exp Pharrm, vol. 135 (II). Springer-Verlag, Berlin, pp. 613–663.

Monthakantirat, O., De-Eknamkul, W., Umehara, K., Yoshinaga, Y., Miyase, T., Warashina, T., Noguchi, H., 2005. Phenolic constituents of the rhizomes of the Thai medicinal plant *Belamcanda chinensis* with proliferative activity for two breast cancer cell lines. J. Nat. Prod. 68, 361–364.

Muangnoicharoen, N., Frahm, A.W., 1981. Arylbenzofurans from *Dalbergia parviflora*. Phytochemistry 20, 291–293.

Muangnoicharoen, N., Frahm, A.W., 1982. Neoflavanoids of *Dalbergia parviflora*. Phytochemistry 21, 767–772.

Pichiansunthorn, C., Chavalit, M., Jeerawong, V., 1999. Text Book of Medicinal Plants. Amarin Printing and Publishing, Bangkok.

Rensburg, H., Heerden, P.S., Bezuidenhoudt, B.C.B., Ferreira, R., 1997. Stereoselective synthesis of flavonoids. Part 4. *Trans*-and *cis*-dihydroflavonols. Tetrahedron 53, 14141–14152.

Smitinand, T., 1983. Valuable Economic Plants in Thailand Part III. The Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

Shen, C.C., Chang, Y.S., Hott, L.K., 1993. Nuclear magnetic resonance studies of 5,7-dihydroxyflavonoids. Phytochemistry 34, 843–845.

Saito, S., Okamoto, Y., Kawabata, J., 2004. Effects of alcoholic solvents on antiradical abilities of protocatechuic acid and its alkyl esters. Biosci. Biotechnol. Biochem. 68, 1221–1227.

Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., Serrano, F.O., 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ. Health Perspect. 103, 113–122.

Suginome, H., 1959. Oxygen Heterocycles. A new isoflavanone from *Sophora japonica*, L. J. Org. Chem. 24, 1655–1662.

Tahara, S., Ingham, J.L., Nakahara, S., Mizutani, J., Harborne, J.B., 1984. Fungitoxic dihydrofuranoisoflavones and related compounds in white lupin, *Lupinus albus*. Phytochemistry 23, 1889–1900.

Umehara, K., Nemoto, K., Ohkubo, T., Miyase, T., Degawa, M., Noguchi, H., 2004. Isolation of a new 15-membered macrocyclic glycolipid lactone, cuscutic resinoside A from the seeds of *Cuscuta chinensis*: a stimulator of breast cancer cell proliferation. Planta Med. 70, 299–304.

Yenesew, A., Midiwo, J.O., Heydenreich, M., Schanzenbach, D., Peter, M.G., 2000. Two isoflavanones from the stem bark of *Erythrina sacleuxii*. Phytochemistry 55, 457–459.

Waffoa, A.K., Azebazea, G.A., Nkengfacka, A.E., Fomuma, Z.T., Meyerb, M., Bodob, B., Heerdenc, F.R., 2000. Indicanines B and C, two isoflavonoid derivatives from the root bark of *Erythrina indica*. Phytochemistry 53, 981–985.