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Antioxidants from *Lespedeza homoloba*. (I)

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Abstract

The stems of *Lespedeza homoloba* yielded eight new and three known phenolic compounds. Their structures have been elucidated on the basis of their spectral data. These compounds had strong antioxidative activity against lipid peroxidation in the rat brain homogenate test. 3,9-Dihydroxypterocarp-6a-en and lespedezol A_2 showed significant antiallergic activity in allergic (type I) mice.

Keywords: *Lespedeza homoloba*; Leguminosae; Isoflav-3-en; Pterocarp-6a-en; 2-Arylbenzofuran; Isoflavone; Antioxidative activity; Antiallergic activity

1. Introduction

Non-toxic antioxidants are desired for use as food additives and medical substances. In our studies of lipophilic natural antioxidants, the methanolic extract of the stems of *Lespedeza homoloba* Nakai (Leguminosae) showed a strong antioxidative activity against lipid peroxidation in the rat brain homogenate assay. The root of *L. bicolor* Turez. var. *japonica* Nakai has dimethyl triptamine, which contracts the uterus and has been used to cure giddiness in women (Mitsuhashi, 1988; Morimoto & Matsumoto, 1966; Morimoto & Oshio, 1965). The antioxidative activity of the methanolic extract of the stems of *L. homoloba* was highest in the random screening of more than one hundred plant extracts. Our previous studies on the constituents of *Lespedeza* spp. (Ueno, Ichikawa, Miyase et al., 1973; Ueno, Ichikawa & Fukushima, 1973; Ueno, Ichikawa, Fukushima et al., 1973; Miyase, Ueno, Noro & Fukushima, 1980, 1981) revealed that this genus was rich in isoflavonoids like isoflav-3-en and pterocarp-6a-en. Isoflav-3-en having two hydroxyl groups at C-7 and C-4′ and pterocarp-6a-en having two hydroxyl groups at C-3 and C-9 are easily oxidized to the conjugated quinone. Active compounds of this plant were assumed to be these type of isoflavonoids because the *Lespedeza* species were easily hybridized with each other and had similar constituents. Tea catechins, strong antioxidants, have also been demonstrated to have antiallergic effects, though the relationships between antioxidative activity and the antiallergic activity was not clear until now (Shiozaki, Sugiyama, Nakazato & Takeo, 1997; Ohmori et al., 1995). Here we have tested the antioxidative and antiallergic activity of the eight new and three known compounds isolated from *L. homoloba*.

2. Results and discussion

The methanolic extract was partitioned between ether and water. The ether layer was concentrated and the residue was partitioned between hexane–benzene

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Table 1
Antioxidative activities of the fractions from *L. homoloba*

Fraction	Antioxidative activity inhibition ratio (%)[a]
1	40.4
2	37.3
3	21.5
4	7.9
5	64.8
6	−5.1
7	71.9
8	0.6

[a] The final concentration of all fractions was 0.227 μg/ml.

(1:1) and methanol–water (4:1). The water layer was passed through a porous polymer gel (Diaion HP-20) column and the column was washed with water, with the adsorbed material eluted with 50% methanol and methanol. Antioxidant activity of the fractions (Frs 1–8) from *L. homoloba* is shown in Table 1. Fr. 5, contains lipophilic compounds, and fr. 7, containing polar and hydrophilic compounds showed strong antioxidative activity against lipid peroxidation in the rat brain homogenate assay. The methanol–water layer was concentrated and the residue was chromatographed on a silica gel column using a chloroform–methanol system to afford 21 frs (Frs A–U). Frs C–P were separated by preparative HPLC on a reversed phase column (ODS, PhA). Eight new (**1**, **4**–**6**, **8**–**11**) and three known (**2**: haginin D (Miyase et al., 1981), **3**: 3,9-dihydroxypterocarp-6a-en (Miyase et al., 1980), **7**: 2-(2,4-dihydroxyphenyl)-6-hydroxybenzofuran (Miyase et al., 1981) phenolic compounds were obtained.

Haginin E (**1**) showed a molecular ion peak at *m/z*: 240 in the FAB-MS. The UV and the ^{1}H NMR spectra suggested that **1** had an isoflav-3-en skeleton showing absorption maxima at 245.5 and 333 nm and characteristic ^{1}H-NMR signals at δ 5.07 (2H, *d, J* = 1.5 Hz) and 6.74 (1H, *br s*) due to H_2-2 and H-4, respectively (Miyase et al., 1980, 1981). The ^{1}H NMR spectrum revealed A_2B_2-type proton signals at δ 6.87 (2H, *d, J* = 8.5 Hz); 7.36 (2H, *d, J* = 8.5 Hz) and ABC-type proton signals at δ 6.33 (1H, *d, J* = 2 Hz); 6.41 (1H, *dd, J* = 8, 2 Hz); 6.94 (1H, *d, J* = 8 Hz). Irradiation of the signal at δ 5.07 (H_2-2) enhanced the proton signal at δ 7.36 due to H-2′ and H-6′. In the HMBC spectrum, a correlation was observed between the proton signal from H-4 (δ 6.74) and the carbon signal at δ 128.4 which was assigned to C-5 correlating to the proton signal at δ 6.94 (1H, *d, J* = 8 Hz) in the HMQC spectrum. Based on this spectroscopic evidence, the structure for haginin E was established as **1**.

The ^{1}H NMR spectrum of lespedezol A_1 (**4**) was very similar to that of 3.9-dihydroxypterocarp-6a-en (**3**) except for the presence of a methoxyl proton signal at δ 3.80 (3H, *s*). Irradiation of the methoxyl proton signal enhanced the proton signals at δ 6.49 (1H, *d, J* = 2.5 Hz) and 6.57 (1H, *dd, d, J* = 8.5, 2.5 Hz) and irradiation of a methylene proton signal at δ 5.57 (2H, *s*) enhanced the proton signal at δ 7.29 (1H, *d, J* = 8.5 Hz) from H-7 which had no correlation to the former two proton signals in the ^{1}H–^{1}H COSY. These observations led us to conclude the structure of lespedezol A_1 to be **4**.

Lespedezol A_2 (**5**) was one of the major compounds, and the FAB-MS showed a molecular ion peak at *m/z*: 406. Its ^{1}H NMR spectrum showed the presence of a pterocarp-6a-en skeleton and a geranyl side chain (Ueno et al., 1973). ABC-type aromatic proton signals from A-ring were almost the same as those of 3,9-dihydroxypterocarp-6a-en (**3**) and irradiation of the proton signal at δ 5.48 (2H, *s*) from H_2-6 enhanced the aromatic proton signal at δ 6.77 (1H, *s*) suggesting that the proton signal at δ 6.77 was due to H-7. On acetylation, compound **5** afforded triacetate **5a**. The H-7 proton signal at δ 6.77 was correlated to carbon signals at δ 106.9 (C-6a), 142.3 (C-9), 143.0 (C-8) and 149.7 (C-10a) and a proton signal at δ 3.65 (2H, *br d, J* = 7.5 Hz) from H_2-1 of the geranyl side chain was correlated to carbon signals at δ 113.1 (C-10), 142.3 (C-9) and 149.7 (C-10a) in the HMBC spectrum of **5**. These correlations led us to conclude the structure of lespedezol A_2 to be **5**.

The FAB-MS of lespedezol A_3 (**6**) showed a molecular ion peak at *m/z*: 404 which was two mass units smaller than that of **5**. The ^{1}H NMR spectrum of **6** revealed AX-type olefinic proton signals at δ 5.86 (1H, *d, J* = 10 Hz) and 6.87 (1H, *d, J* = 10 Hz) suggesting that **6** was an oxidatively-cyclized derivative of **5** (Miyase et al., 1980) as shown in Fig. 1.

Lespedezol B_1 (**8**) showed a molecular ion peak at *m/z*: 408 in the FAB-MS and its ^{1}H NMR spectrum showed a methyl proton at δ 2.15 (3H, *s*), an aromatic proton at δ 6.82 (1H, *s*), a set of ABX-type aromatic protons at δ 6.48 (1H, *dd, J* = 8.5, 2.5 Hz); 6.52 (1H, *d, J* = 2.5 Hz); 7.25 (1H, *d, J* = 8.5 Hz) and geranyl side chain proton signals. Irradiation of the methyl proton signal enhanced two aromatic protons at δ 6.82 and 7.25 and a hydroxyl proton signal at δ 8.03 (1H, *s*). After assignment of ^{13}C NMR signals by HMQC and HMBC spectra, the structure of lespedezol B_1 was established as **8**.

The FAB-MS of lespedezol B_2 (**9**) showed a molecular ion peak at *m/z*: 508 and the ^{13}C NMR spectrum revealed two sp^3 carbon signals at δ 25.1 and 66.0 and 28 sp^2 carbon signals. By comparing these carbon signals with those of 3,9-dihydroxypterocarp-6a-en (**3**) and 2-(2,4-dihydroxyphenyl)-6-hydroxybenzofuran (**7**), compound **9** was assumed to be a complex composed of a pterocarpen and a 2-arylbenzofuran derivative. In the difference NOE spectrum, NOEs were observed at an aromatic proton signal at δ 7.12 (1H, *s*) from H-7

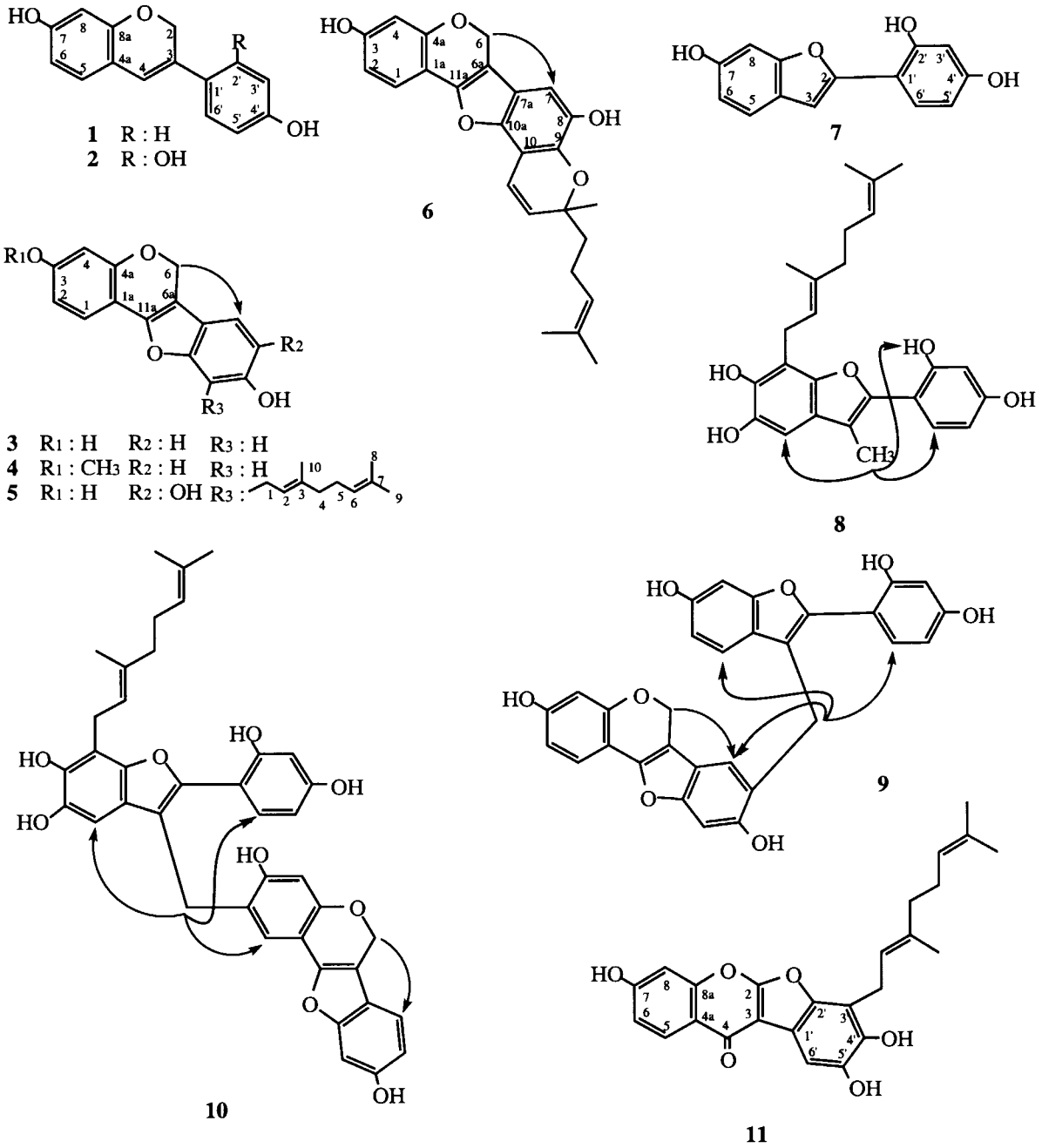

Fig. 1

of the pterocarp-6a-en on irradiation of a methylene proton signal at δ 5.39 (2H, *s*) from H_2-6 of the pterocarp-6a-en, and at three aromatic proton signals at δ 7.11 (1H, *d*, J = 8.5 Hz) from H-5 of the 2-arylbenzofuran; 7.12 (1H, *s*) from H-7 of the pterocarp-6a-en; 7.27 (1H, *d*, J = 8.5 Hz) from H-6′ of the 2-arylbenzofuran on irradiation on a methylene proton signal at δ 4.10 (2H, *s*). Therefore, the methylene proton signal at δ 4.10 was assigned to H_2-4 of 2-arylbenzofuran. In the HMBC spectrum, correlations were observed between the methylene proton signal at δ 4.10 and C-2, C-3, C-4a (upper moiety); C-7a, C-8, C-9 (lower moiety) and between the aromatic proton signal at δ 7.12 from H-7 of the pterocarp-6a-en and C-6a, C-9 (lower moiety). These data led us to conclude the structure of lespedezol B_2 was **9**.

Lespedezol B_3 (**10**) showed a molecular ion peak at *m/z*: 660 in the FAB-MS and its ^{13}C NMR spectrum revealed 40 carbon signals suggesting that compound **10** was also a complex composed of a pterocarp-6a-en, a 2-arylbenzofuran and a geranyl side chain. The ^{1}H and ^{13}C NMR spectra were very similar to those of **8**

in the 2-arylbenzofuran moiety and to those of 3,9-dihydroxypterocarp-6a-en (**3**) except for the signals due to an A-ring in the pterocarp-6a-en moiety. To determine the attachment position, we used NOE and HMBC spectral data. On irradiation of a methylene proton signal at δ 5.50 (1H, *s*) from H_2-6 of lower moiety enhanced the proton signal at δ 7.24 (1H, *d*, $J = 8.5$ Hz) from H-7 of lower moiety and on irradiation of a methylene proton signal at δ 3.97 (2H, *s*) from H_2-4 of upper moiety enhanced three aromatic proton signals at δ 6.62 (1H, *s*) from H-5 of upper moiety; 7.17 (1H, *s*) from H-1 of lower moiety; 7.30 (1H, *d*, $J = 8.5$ Hz) from H-6′ of upper moiety. HMBC correlations were observed between an aromatic proton signal at δ 6.50 (1H, *s*) from H-4 of lower moiety and carbon signals at δ 24.7 from C-4 of upper moiety; 120.6 from C-2 of lower moiety; 147.7 from C-11a; 154.2 from C-4a; 157.0 from C-3 of lower moiety, between a methylene proton signal at δ 5.50 (2H, *s*) from H_2-6 of lower moiety and a carbon signal at δ 154.2 from C-4a of lower moiety, between a methylene proton signal at δ 3.97 (2H, *s*) and carbon signals at δ 115.1 from C-3; 121.9 from C-4a; 150.0 from C-2 of upper moiety, 120.6 from C-2; 122.0 from C-1; 157.0 from C-3 of lower moiety. The structure of lespedezol B_3 was therefore established as **10**.

Lespedezol C_1 (**11**) showed absorption maxima at 229 *sh* (4.37), 259 (4.35), 289 (4.31) and 324 *sh* (3.92) nm (low ϵ) in the UV spectrum and an upfield shifted carbonyl carbon signal at δ 173.5 and geranyl side chain carbon signals in the ^{13}C NMR spectrum. Furthermore, a downfield shifted aromatic proton signal at δ 8.14 (1H, *d*, $J = 9$ Hz) was observed in the ^1H NMR spectrum. These spectral data indicated **11** was an isoflavone derivative. A coumaronochromone skeleton was indicated by the presence of the carbonyl carbon (δ 173.5) due to the C-4 of the 5-deoxycoumaronochromone (Marques, Gottlieb & Maia, 1992) and the absence of a characteristic singlet proton signal (H-2) for isoflavone in the lower field. An aromatic proton signal at δ 7.45 (1H, *s*) was correlated to carbon signals at δ 99.9 (C-3); 114.8 (C-1′); 143.0 (C-4′); 144.0 (C-2′) and a methylene proton signal at δ 3.65 (2H, *br d*, $J = 7$ Hz, H_2-1 of geranyl) was correlated to carbon signals at δ 143.0 (C-4′); 144.0 (C-2′). Consequently, the structure of lespedezol C_1 was concluded to be **11**.

Relative antioxidant potentials of phenolic compounds isolated from a lipophilic fraction are shown in Table 4. Compounds **5**, **6** and **8** had strong antioxidative activity in three experimental systems. These activities, however, were lower than those of (-)-epigallocatechin-3-*O*-gallate (EGCg). These compounds had unique skeletons and compounds **9** and **10** were the first examples of a complex composed of a 2-arylbenzofuran and a pterocarp-6a-en. Tea leaf is rich in antioxidants such as epicatechins, vitamins C and E. Recently, several studies have demonstrated the antiallergic effect of tea catechins and tea extracts (Shiozaki et al., 1997; Ohmori et al., 1995). EGCg, which is the major catechin in tea, has shown an antiallergic effect in ovalbumin (OVA)-induced allergic model mice. The effects of compounds **3** and **5** were much higher than that of EGCg. Since compounds **3** and **5** are lipophilic and potent antioxidants, these compounds may be localized into the allergy-related cell membranes and exhibit protective effects against oxidative stress and allergic disease but further investigation is required.

3. Experimental

The instruments used in this work were: a JASCO DIP-1000 digital polarimeter for $[\alpha]_D$ at 23°C; a JEOL α-400 FT-NMR spectrometer for NMR spectra (^1H: 400 MHz, ^{13}C: 100 MHz, in acetone-d_6 at 35°C); JEOL JMS-SX102 spectrometer for positive mode FAB-MS; a Hitachi U-3410 spectrophotometer for UV spectra in methanol; a Hitachi F-3010 fluorescence spectrophotometer for fluorescence.

3.1. Plant material

The stems of *L. homoloba* were collected in Shizuoka, Japan in January 1997. A voucher specimen was deposited at the Herbarium of School of Pharmaceutical Sciences, University of Shizuoka.

3.2. Extraction and isolation

Dried stems (11.2 kg) of *L. homoloba* were extracted twice with methanol under reflux for 3 h. The methanolic extract (Fr. 1) was concentrated under reduced pressure and the residue was partitioned between ether and water. The ether layer (Fr. 2) was partitioned between hexane–benzene (1:1) and methanol–water (4:1) after evaporation of the solvent. Each layer was concentrated to give brown residue (upper layer 41 g, Fr. 4; lower layer 85 g, Fr. 5). The water layer (Fr. 3) was chromatographed on a porous polymer gel Diaion HP-20 (9×40 cm) and the adsorbed material was eluted with water (Fr. 6), 50% MeOH (52.7 g, Fr. 7) and methanol (18.5 g, Fr. 8), successively. The methanol–water (4:1) layer (85 g) was chromatographed on a silica gel column using chloroform–methanol (97:3)–(4:1) as a solvent to give 21 fractions (Frs A–U). Frs C–P were separated by preparative HPLC using reversed phase columns (ODS, PhA). Yields: **1** (66 mg), **2** (375 mg), **3** (1440 mg), **4** (80 mg), **5** (2431 mg), **6** (147 mg), **7** (577 mg), **8** (2 mg), **9** (29 mg), **10** (8 mg) and **11** (51 mg).

Table 2
^{1}H NMR spectral data of compounds **1**, **4**–**6**, **8**–**11**[a]

	1	**4**	**5**	**6**	**8**[b]	**9**	**10**[c]	**11**
Upper moiety								
2	5.07 *d* (1.5)							
4	6.74 *br s*				2.15 *s*	4.10 *s*	3.97 *s*	
5	6.94 *d* (8)				6.82 *s*	7.11 *d* (8.5)	6.62 *s*	8.14 *d* (9)
6	6.41 *dd* (8, 2)					6.66 *dd* (8.5, 2)		7.06 *dd* (9, 2)
8	6.33 *d* (2)					6.93 *d* (2)		7.08 *d* (2)
2′	7.36 *d* (8.5)							
3′	6.87 *d* (8.5)				6.52 *d* (2.5)	6.56 *d* (2)	6.54 *d* (2)	
5′	6.87 *d* (8.5)				6.48 *dd* (8.5, 2.5)	6.47 *dd* (8.5, 2)	6.48 *dd* (8.5, 2)	
6′	7.36 *d* (8.5)				7.25 *d* (8.5)	7.27 *d* (8.5)	7.30 *d* (8.5)	7.45 *s*
Lower moiety								
6		5.57 *s*	5.48 *s*	5.51 *s*		5.39 *s*	5.50 *s*	
1		7.35 *d* (8.5)	7.30 *d* (8.5)	7.32 *d* (8.5)		7.26 *d* (8.5)	7.17 *s*	
2		6.57 *dd* (8.5, 2.5)	6.50 *dd* (8.5, 2)	6.51 *dd* (8.5, 2)		6.48 *dd* (8.5, 2)		
4		6.49 *d* (2.5)	6.42 *d* (2)	6.43 *d* (2)		6.40 *d* (2)	6.50 *s*	
7		7.29 *d* (8.5)	6.77 *s*	6.79 *s*		7.12 *s*	7.24 *d* (8.5)	
8		6.84 *dd* (8.5, 2.5)					6.78 *dd* (8.5, 2)	
10		7.02 *d* (2.5)				7.06 *s*	6.90 *d* (2)	
OMe		3.80 *s*						
Side chain								
1			3.65 *br d* (7.5)	6.87 *d* (10)	3.61 *br d* (7.5)		3.64 *br d* (7)	3.65 *br d* (7)
2			5.44 *m*	5.86 *d* (10)	5.44 *m*		5.55 *m*	5.44 *m*
4			1.99 *br t* (7.5)	1.78 *m*	1.98 *m*		2.00 *m*	2.01 *m*
5			2.08 *br t* (7.5)	2.17 *m*	2.07 *m*		2.09 *m*	2.09 *m*
6			5.06 *m*	5.12 *m*	5.07 *m*		5.09 *m*	5.07 *m*
8			1.57 *br s*	1.63 *d* (1)	1.59 *d* (1)		1.60 *d* (1)	1.57 *d* (1)
9			1.52 *br s*	1.56 *br s*	1.53 *d* (1)		1.55 *br s*	1.53 *br s*
10			1.90 *br s*	1.45 *s*	1.82 *d* (1)		1.85 *d* (1)	1.87 *d* (1)

[a] Assigned by ^{1}H–^{1}H COSY, HMQC, HMBC and NOE.
[b] $C_{2'}$-OH (8.03, *s*); $C_{4'}$-OH (8.43, *s*); C_6-OH (8.12, *s*); C_7-OH (7.04, *s*).
[c] Upper moiety: $C_{2'}$-OH (8.30, *s*); $C_{4'}$-OH (8.48, *s*); C_6-OH (8.03, *s*); C_7-OH (7.00, *s*), lower moiety: C_3-OH (8.63, *s*); C_9-OH (8.44, *s*).

3.3. Haginin E (*1*)

Amorphous powder, UV λ_{max} nm (log ϵ): 245.5 (4.20), 333 (4.39). FAB-MS *m*/*z*: 240 $[M]^+$. ^{1}H and ^{13}C NMR spectra: Tables 2 and 3.

3.4. Lespedezol A_1 (*4*)

Amorphous powder, UV λ_{max} nm (log ϵ): 229 sh (4.17), 289.5 sh (3.86), 326 (4.22). FAB-MS *m*/*z*: 268 $[M]^+$. ^{1}H and ^{13}C NMR spectra: Tables 2 and 3.

3.5. Lespedezol A_2 (*5*)

Amorphous powder, UV λ_{max} nm (log ϵ): 246.5 (4.17), 324.5 (4.24). FAB-MS *m*/*z*: 406 $[M]^+$. ^{1}H and ^{13}C NMR spectra: Tables 2 and 3.

3.6. Lespedezol A_3 (*6*)

Amorphous powder, UV λ_{max} nm (log ϵ): 288 (4.10), 302 (4.08), 357 (4.08). $[\alpha]_D$ −10.7° (MeOH; *c* 0.79). FAB-MS *m*/*z*: 404 $[M]^+$. ^{1}H and ^{13}C NMR spectra: Tables 2 and 3.

3.7. Lespedezol B_1 (*8*)

Amorphous powder, UV λ_{max} nm (log ϵ): 272 (3.93), 315 (4.02). FAB-MS *m*/*z*: 408 $[M]^+$. ^{1}H and ^{13}C NMR spectra: Tables 2 and 3.

3.8. Lespedezol B_2 (*9*)

Amorphous powder, UV λ_{max} nm (log ϵ): 209 sh (4.28), 320 (4.38). FAB-MS *m*/*z*: 508 $[M]^+$. ^{1}H and ^{13}C NMR spectra: Tables 2 and 3.

3.9. Lespedezol B_3 (*10*)

Amorphous powder, UV λ_{max} nm (log ϵ): 245 sh (4.39), 325 (4.43). FAB-MS *m*/*z*: 660 $[M]^+$. ^{1}H and ^{13}C NMR spectra: Tables 2 and 3.

3.10. Lespedezol C_1 (*11*)

Amorphous powder, UV λ_{max} nm (log ϵ): 229 sh (4.37), 259 (4.35), 289 (4.31), 324 sh (3.92). FAB-MS *m*/*z*: 421 $[M+H]^+$. ^{1}H and ^{13}C NMR spectra: Tables 2 and 3.

Table 3
^{13}C NMR spectral data of compounds **1**–**11**

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Upper moiety											
2	67.7	68.8					153.1	148.9	150.1	150.0	166.9
3	128.9	129.6					103.9	122.4	115.3	115.1	99.9
4	118.3	121.0						9.3	25.1	24.7	173.5
4a	116.5	117.2					123.5	112.4	123.4	121.9	117.7
5	128.4	128.3					121.4	101.9	121.0	102.8	128.3
6	109.5	109.3					112.6	142.4	112.1	142.3	115.5
7	159.1	158.3					156.0	142.2	156.1	142.2	163.0
8	103.4	103.4					98.3	112.2	98.4	112.2	104.0
8a	155.3	155.6					155.6	148.3	156.4	148.9	155.8
1	129.3	118.4					111.1	111.5	110.7	111.1	114.8
2	126.6	156.8					156.2	157.0	157.4	157.1	144.0
3	116.4	103.9					104.1	104.1	104.0	104.1	113.6
4	158.1	159.1					159.2	160.0	160.4	160.2	143.0
5	116.4	108.2					108.3	108.3	108.3	108.4	143.0
6	126.6	129.9					128.0	131.9	132.7	132.2	104.7
Lower moiety											
6			65.8	66.1	66.1	66.0			66.0	65.8	
6a			106.4	107.1	106.9	106.8			106.5	106.6	
11a			147.6	147.5	147.4	148.1			147.4	147.7	
1a			109.6	110.6	110.2	109.8			109.8	109.6	
1			121.5	121.5	121.5	121.7			121.6	122.0	
2			109.3	107.9	109.4	109.4			109.4	120.6	
3			159.6	162.0	159.5	159.8			159.6	157.0	
4			104.4	103.3	104.5	104.6			104.5	104.3	
4a			156.0	156.0	156.0	156.2			155.6	154.2	
7a			119.2	119.3	117.9	119.3			124.1	119.3	
7			119.6	119.9	101.7	104.2			119.1	119.6	
8			113.0	113.2	143.0	138.7			119.8	113.1	
9			156.2	156.5	142.3	143.7			153.8	156.2	
10			99.0	99.2	113.1	107.4			98.6	99.1	
10a			157.2	157.4	149.7	145.7			156.0	157.1	
OMe				55.7							
Side chain											
1					23.7	117.1		23.7		23.8	23.6
2					122.8	131.4		123.1		123.1	122.3
3					135.9	80.9		135.8		135.8	136.5
4					40.5	41.4		40.5		40.5	40.4
5					27.4	23.5		27.4		27.4	27.3
6					125.1	125.0		125.1		125.2	125.0
7					131.7	132.1		131.7		131.7	131.7
8					25.7	25.8		25.7		25.8	25.7
9					17.7	17.7		17.7		17.7	17.7
10					16.3	26.1		16.4		16.4	16.4

*3.11. Acetylation of lespedezol A_2 (**5**)*

Compound **5** (8 mg) was acetylated by pyridine and acetic anhydride in the usual manner to give triacetate (**5a**). ^1H NMR ($CDCl_3$) δ: geranyl side chain; 1.55 (3H, *br s*, H_3-9), 1.62 (3H, *br s*, H_3-8), 1.83 (3H, *br s*, H_3-10), 1.99 (2H, *m*, H_2-4), 2.04 (2H, *m*, H_2-5), 3.58 (2H, *br d*, $J = 7$ Hz, H_2-1), 5.04 (1H, *m*,, H-6), 5.25 (1H, *m*, H-2); 2.29, 2.30, 2.32 (each 3H, *s*), 5.56 (2H, *s*, H_2-6), 6.69 (1H, *d*, $J = 2$ Hz, H-4), 6.73 (1H, *dd*, $J = 8$, 2 Hz, H-2), 7.06 (1H, *s*, H-7), 7.48 (1H, *d*, $J = 8$ Hz, H-1).

3.12. Antioxidative activity in rat brain homogenate assay

Suppression of autooxidation of rat brain homogenates was measured by the method of Stocks, Gutteridge, Sharp and Dormandy (1974). Eight week old male Wistar rats were purchased from SLC. Rats were sacrificed and the brain removed. The brain was homogenized in 9 volumes of 40 mM phosphate buffer (pH 7.4) and centrifuged at 3000 rpm for 10 min at 4°C. The supernatant (0.5 ml) was added to 10% ethanolic solution of isoflavonoid (0.1 ml). The mixture was incubated at 37°C for 60 min. After the autooxi-

Table 4
Antioxidative activities of isoflavonoids from *L. homoloba*

Compounds	Antioxidative activity IC_{50} (μM)	Fe^{2+}-complex % vs EGCg[a]	O_2^- radical scavenging activity (%)[b]
1	0.3	–[c]	52.4
2	0.2	–[c]	38.0
3	0.2	–[c]	76.1
4	0.2	6.0	61.4
5	0.2	22.0	51.3
6	0.3	31.8	64.8
7	0.2	–[c]	39.7
8	0.3	46.9[c]	64.4
9	0.2	–[c]	69.8
10	0.3	14.6	61.4
11	0.3	16.9	55.5
EGCg	0.07	100.0	83.7

[a] Final concentration of all compounds was 0.033 mg/ml.
[b] Final concentration of all compounds was 0.020 mg/ml.
[c] No complexes were formed.

dation of rat brain homogenate, the content of thiobarbituric acid reactive substance (TBARS) in the solution was measured by the colorimetric method of Masugi and Nakamura (1977). Three percent sodium dodecyl sulfate (0.5 ml), 0.1 N hydrochloric acid (2.0 ml), 10% phosphotungstic acid (0.3 ml) and 0.7% thiobarbituric acid (1.0 ml) was added to TBARS. The mixture was boiled at 100°C for 45 min and cooled in an ice water bath for 5 min. TBARS was extracted with *n*-butanol (5.0 ml). The absorption of the *n*-buthanol layer at Ex 515, Em 555 nm was measured. The inhibitory ratio was calculated by the comparison of the contents of TBARS with or without antioxidant. The concentration of the samples giving 50% inhibition (IC_{50}) was determined from a dose response curve (Table 4).

3.13. Formation and determination of the complexes of Fe^{2+} with phenolic compounds

Iron-chelating activity was estimated by the ferrous tartrate method of Iwasa and Torii (1962). 6.96 mM ferrous sulfate (0.25 ml), 14.2 mM sodium tartrate (0.25 ml) and 99.0 μg/ml phenolic compounds (1.0 ml) were added into 25 mM phosphate buffer (pH 7.5) (1.5 ml). Immediately, blue color of formed complexes of Fe^{2+} with phenolic compounds was determined by a spectrophotometer at 540 nm. For a comparable reference, EGCg was used and the values of Fe^{2+}-phenolic compound complexes were expressed at that of EGCg in Table 4.

3.14. Determination of superoxide anion radical scavenging activity of phenolic compounds

Superoxide anion radical scavenging activity was measured by the phenazine methosulfate nitro blue tetrazolium method of Nishikimi, Rao and Yagi (1972). 15 μM phenazine methosulfate (0.5 ml), 200 μM nitro blue tetrazolium (NBT) (0.5 ml), 100 μg/ml isoflavonoid or EGCg (0.5 ml) and 750 μM nicotinamide adenine dinucleotide (0.5 ml), an initiator of the production of superoxide anion radical, were added into 20 mM phosphate buffer (pH 7.4) (0.5 ml) and the mixture was incubated at 25°C. Formed NBT diformazan was determined by a spectrophotometer at 560 nm with the passage of time. The increasing absorption rate of the reaction mixture with an antioxidant were compared with that without the antioxidant and superoxide anion radical scavenging activity was calculated as an inhibition ratio (%) (Table 4).

3.15. Antiallergic activity of phenolic compounds

Antiallergic activity of compounds **3** and **5** were evaluated by the method of Kataoka et al. (1997) using the mouse abdominal wall (AW method) (Table 5. Male *dd*Y mice (Japan SLC, Hamamatsu, Japan) at five weeks of age were sensitized intraperitoneally with a mixture (1:1) of OVA (Sigma, St Louis, MO, USA) (2 mg/ml N-saline) and Freund's incomplete adjuvant (FIA) (Wako Pure Chemical Co. Ltd, Osaka, Japan). The natural compounds (50 mg/kg bw) was administered orally into a mouse nine days after OVA initial exposure. Sixty minutes after the administration, 1%

Table 5
Antiallergic activities of compounds **3** and **5**

Compounds	Dose (mg/kg bw)	Area (% of control)	Inhibition %
3	50	50.4 ± 19.9	49.6
5	50	60.1 ± 14.7	39.9
EGCg	50	87.2 ± 12.6	12.8

Evance blue dye was injected intravenously and then the abdominal skin of the mice was detached without injury to the abdominal wall under ether anaesthesia. Five minutes after injection of the dye, 50 μl OVA solution (5 μg/site) was administered on the exposed abdominal wall (5 μg/50 μl/site). The mice were killed by cervical dislocation 7 min after the challenge. Antiallergic activity was determined by measuring the area of blue dye permeated on the abdominal wall.

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