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A novel flavonoid from *Lespedeza virgata* (Thunb.) DC.: Structural elucidation and antioxidative activity

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Abstract—Bioactivity guided fractionation of the ethanolic extract of the whole plants of *Lespedeza virgata* (Thunb.) DC. resulted in the isolation of a novel flavonoid (**1**) along with five known compounds **2**–**6**. The molecular and structural formula as well as the stereochemistry of compound **1** were determined using data obtained from ^{1}H and ^{13}C NMR spectra, DEPT135 and by 2D HSQC, HMBC, ^{1}H–^{1}H correlated spectroscopy (^{1}H–^{1}H COSY), and nuclear overhauser effect spectroscopy (NOESY) experiments. The superoxide anion scavenging activities of all isolated compounds were evaluated by the hypoxanthine nitro blue tetrazolium and ESR methods and the new compound **1** showed the strongest antioxidative activity 95.79% (IC_{50} = 0.14 mg/ml).

Antioxidants are the chemical substances that reduce or prevent oxidation. They have the ability to counteract the damaging effects of free radicals in tissues and thus are believed to protect against cancer, arteriosclerosis, heart disease, and several other diseases.[1]

Many low-toxic antioxidants, such as flavonoids, desired to be used as food additives and medical substances are usually obtained from the *Lespedeza*. As previously reported, the stem of *Lespedeza homoloba* yields 23 flavonoids with strong antioxidative activity against lipid peroxidation in the rat brain homogenate test.[2,3] Two prenylated isoflavanones were isolated from the stem bark of *Lespedeza bicolor*.[4] *Lespedeza* also has other biological properties used in treatment of nephritis, azothemia, and diuresis.[5]

To search for some new flavonoid-type compounds with more effective antioxidative property from *Lespedeza virgata* (Thunb.) DC., the ethanolic extract of the whole plant was isolated with the help of antioxidative filtration. As a result, we obtained a totally new flavonoid-type compound: 5-hydroxy-6-methyl-2′-methoxy-[6″,6″-dimethylpyrano(2″,3″:7,8)] [6‴,6‴-dimethylpyrano(2‴,3‴:4′,5′)]-(2*S*) flavonone (**1**). Besides this, five other known flavonoids were isolated and identified according to their NMR spectra, including Kaempferol-3,7-*O*-α-L-dirhamnoside (**2**), Quercetin-7-*O*-β-D-glucoside (**3**), Quercetin-3-*O*-α-L-rhamnoside (**4**), Kaempferol-3-*O*-β-D-glucoside (**5**), and Kaempferol-3-*O*-α-L-rhamnoside (**6**) (Fig. 1). It is first time compounds **3**, **4**, and **6** were discovered from the *Lespedeza*. Proton and carbon chemical shifts of these compounds were obtained by one- and two-dimensional NMR spectrum assignments. The antioxidative activities of all isolated compounds were evaluated by superoxide anion radical assay, and compound **1** showed the strongest effect among the six flavonoids.

The whole plants of *Lespedeza virgata* (Thunb.) DC. were collected in Enshi region of Hubei province in June 2005 and authenticated by Dr. Zhenyu Li in the Institute of Botany, the Chinese Academy of Sciences. A voucher specimen (Liu 0503) was deposited at State Key Laboratory for Structural Chemistry of Unstable and Stable Species, Center for Molecular Science, Institute of Chemistry, Chinese Academy of Sciences.

The whole plants (10 kg) were extracted for three times with 95% ethanol under reflux for 3 h. The ethanol extract was concentrated under reduced pressure (405 g).

Keywords: *Lespedeza virgata*; Flavonoid; NMR; Antioxidative activity.
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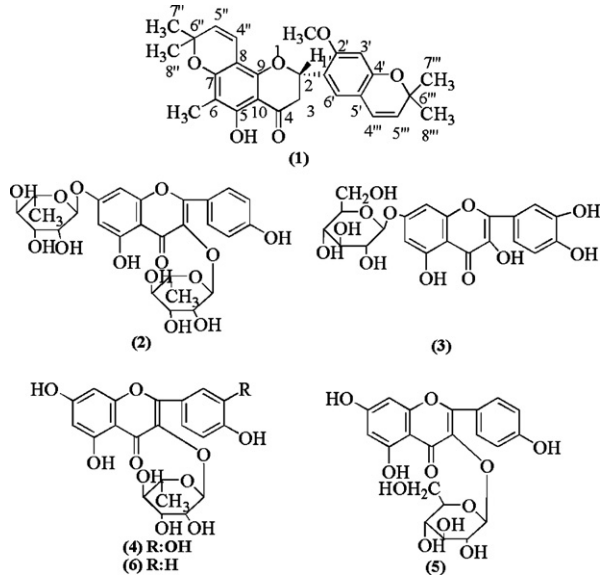


Figure 1. Chemical structures of flavonoids from *Lespedeza virgata* (Thunb.) DC.

After that, the residue was partitioned by petroleum ether, chloroform, *n*-butanol, and water. The chloroform extract (140.5 g) was chromatographed on a silica column by using petroleum ether–ethyl acetate (9:1)–(1:9) as a solvent to give 10 fractions. After the petroleum ether–ethyl acetate (9:1) fraction having been loaded on a silica column using cyclohexane–ethyl acetate (99:1)–(1:99) as a solvent to give 16 fractions, the cyclohexane–ethyl acetate (95:5) fraction was chromatographed on a silica column using chloroform as a solvent. With the above process, seven fractions were obtained. Fraction 5 was purified by TLC using petroleum ether–ethyl acetate (30:1) as a mobile phase. Yield of compound **1** is 9 mg.

The *n*-butanol extract (160 g) which was loaded on a silica column using chloroform–methanol (4:1) as a solvent gives 13 fractions. The seventh fraction (11 g) was then subjected to a silica column using chloroform–methanol (99:1)–(1:99) as a solvent to give 100 fractions. Fractions 15–25 showed similar profiles in a TLC analysis and gave a brown material (4.5 g) after eluent evaporation. This material was chromatographed on a silica column using chloroform–methanol–water–acetic acid (80:20:3:0.1) as a solvent to give nine fractions mainly. Fraction 3 (58 mg) was separated by TLC using chloroform–methanol–water–acetic acid (80:20:2:0.2) as a mobile phase to yield compound **5** (7 mg) and compound **6** (5 mg); fraction 4 (241 mg) was loaded on a polyamide column using ethanol–water (3:7) as a solvent and then purified by TLC using chloroform–methanol–water–acetic acid (80:20:2:0.2) as a mobile phase to yield compound **4** (8 mg); fraction 7 (154 mg) was separated by polyamide column chromatograph using ethanol–water (3:7) as a solvent to yield compound **2** (15 mg) and compound **3** (12 mg).

Compound **1**, a pale yellow powder, gave a positive HCl–Mg test. The IR spectra of **1** showed absorption band at 1630, 2970, 1498, 1450, 1378 cm^{-1}. The UV spectra of **1** showed two strong absorption bands at 300 nm and 365 nm respectively. These results strongly suggest that the compound **1** is a flavonoid.

The high resolution TOF mass spectra of compound **1** had molecular ion peak at 448.1890 *m*/*z*, in agreement with the masses calculated for the molecular formula, $C_{27}H_{28}O_6$.

The ^{13}C NMR spectrum revealed a carbonyl carbon signal at δ 196.9, which was characteristic of flavonone. In the ^{1}H NMR spectrum, two double doublets (J = 17.4 and 3.6 Hz, and J = 17.4 and 13.2 Hz) at δ 2.84 and δ 2.91 (due to βH and αH, respectively) and a double doublets (J = 13.2 and 3.6 Hz) at δ 5.65 ppm were assignable to H-3 and H-2, respectively. The ^{1}H NMR spectrum also showed the presence of two 2,2-dimethylpyran units as four methyls at δ 1.46 (12, m) and four methine protons at δ 5.48 (1H, d, J = 10.2 Hz), δ 5.52 (1H, d, J = 10.2 Hz), δ 6.34 (1H, d, J = 10.2 Hz), δ 6.60 (1H, d, J = 10.2 Hz) due to H-5″, H-5‴, H-4‴, H-4″, respectively.[6] In an aromatic proton region, two singlet proton signals at δ 6.41 (1H, s) and δ 7.20 (1H, s) were observed. In NOE experiments, irradiation of the methoxyl proton signal at δ 3.80 (3H, s) enhanced the aromatic proton signal at δ 6.41 (1H, s, H-3′) and a correlation between δ 3.80 (3H, s) and δ 156.9 (C-2′), all these inferred methoxyl group substituted on C-2′. The methyl proton signal at δ 2.03 (3H, s) had correlation peaks with carbon signals at δ 105.5 and δ 161.4 in the HMBC spectrum. The latter carbon signals were also correlated with a hydrogen-bonded hydroxyl proton signal at δ 12.47 (1H, s). So the position of the hydroxyl group was deduced to be C-5 and the vinyl methyl group was deduced to be C-6.

In the ^{1}H–^{1}H COSY spectrum, correlations were observed between the proton signal from H-2 (δ 5.65) and the protons signal at δ 2.84 and δ 2.91 which were assigned to H-3 correlating to the carbon signal at δ 42.7 in the HSQC spectrum, and between the proton signal from H-4″ (δ 6.60) and the proton signal at δ 5.48 which was assigned to H-5″ correlating to the carbon signal at δ 125.9 in the HSQC spectrum, and between the proton signal from H-4‴ (δ 6.34) and the proton signal at δ 5.52 which was assigned to H-5‴ correlating to the carbon signal at δ 128.1 in the HSQC spectrum. In the HMBC spectrum, correlations were observed between the aromatic singlet proton signal at δ 6.41 (1H, s) due to H-3′ and the carbons at δ 114.1, δ 119.6, δ 154.2, and δ 156.9 due to C-5′, C-1′, C-4′, and C-2′, respectively, and between the aromatic singlet proton signal at δ 7.20 (1H, s) due to H-6′ and the carbons at δ 74.1, δ 121.8, δ 154.2 and δ 156.9 due to C-2, C-4‴, C-4′ and C-2′, respectively. In NOE experiments, irradiation at the methyl proton signal at δ 2.03 (3H, s) enhanced the methyl proton signal at δ 1.46 due to H-8″, and aromatic singlet proton signal at δ 7.20 (1H, s) from H-6′ in the B moiety on irradiation on a pyran proton signal at δ 6.34 due to H-4‴.

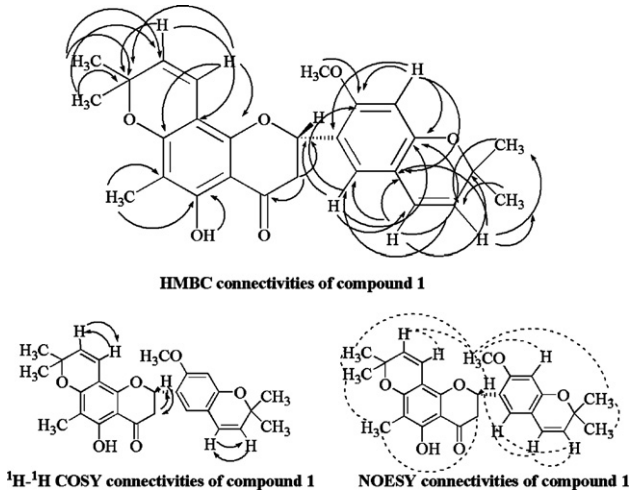
Since the specific optical rotation of compound **1** had minus (−) sign ($[\alpha]_D^{20}$: -267°(c 0.03, $CHCl_3$)), and the

^{1}H NMR spectrum showed trans diaxial coupling J = 13.2 Hz between C_2–H and C_3–H, like those other natural flavanones,[7,8] we believe that compound **1** has (*S*)-configuration at C_2. Consequently, the structure of compound **1** was established as 5-hydroxy-6-methyl-2′-methoxy-[6″,6″-dimethylpyrano(2″,3″:7,8)][6‴,6‴-dimethylpyrano(2‴,3‴:4′,5′)]-(2*S*)flavonone (**1**).

The ^{1}H and ^{13}C NMR assignments for compound **1** are listed in Table 1. The numbering scheme and 2D NMR (^{1}H–^{1}H COSY, HMBC and NOESY) connectivities of the molecular structure are illustrated in Scheme 1.

From the first structure of dimethylpyrano-flavonone being found in 1982 till now,[6,9–14] only seven dimethylpyrano-flavonone compounds were isolated from the nature products. More importantly, the structural skeleton of the dimethylpyrano-flavonone was found for the first time among all kinds of flavonoids isolated from *Lespedeza*.[2–4,15]

The latter five flavonoids (**2–6**) were identified by comparison of their spectroscopic data with the literature data as Kaempferol-3,7-*O*-α-L-dirhamnoside,[16] Quercetin-7-*O*-β-D-glucoside,[17] Quercetin-3-*O*-α-L-rhamnoside,[18] Kaempferol-3-*O*-β-D-glucoside[19], and Kaempferol-3-*O*-α-L-rhamnoside,[19], respectively. Although structures of the compounds**3**, **4**, and **6** are not new, they are firstly discovered from *Lespedeza*.



Scheme 1. 2D NMR connectivities of compound **1**.

Superoxide anion radical scavenging activity was surveyed by the hypoxanthine nitro blue tetrazolium method.[2,3] Briefly, the NBT method is based on the reduction of NBT by reaction with $O_2^{\cdot-}$ yielding to the generation of a formazan blue dye (λ_{max}: 560 nm). DTPA (1 mM) and hypoxanthine (0.4 mM) were added into phosphate buffer (pH 7.4, 10 mM), and the mixture was incubated in oxygen for 2 min. 0.5 mg/ml flavonoid or trolox C and 0.1 mg/ml nitro blue tetrazolium (NBT) and

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data for compound **1** (δ in ppm, **1** in $CDCl_3$, TMS as internal standard)

Number	δ (ppm)		^{1}H–^{1}H COSY	HMBC	NOE
	^{1}H	^{13}C			
2	5.65, dd, J = 3.6, 13.2	74.1	3	5″	5″, 5‴
3	α 2.91, dd, J = 13.2, 17.4	42.7	2	2	6-Me
	β 2.84, dd, J = 3.6, 17.4			4	3
4		196.9			
5		161.4			
6		105.5			
7(**2″**)		159.7			
8(**3″**)		101.5			
9		155.3			
10		102.4			
6-Me	2.03, s	6.7		5, 6	8″, 3α
1′		119.6			
2′		156.9			
3′	6.41, s	99.6		1′, 2′, 4′, 5′	2′-OMe
4′(**2‴**)		154.2			
5′(**3‴**)		114.1			
6′	7.20, s	124.2		2, 2′, 4′, 4‴	4‴
4″	6.60, d, J = 10.2	116.2	5″	7, 9, 6″	5″
5″	5.48, d, J = 10.2	125.9	4″	8, 6″	4″
6″		77.8			
7″	1.46, m	28.6		5″, 6″, 8″	8″, 2′-OMe
8″	1.46, m	28.3		5″, 6″, 7″	7″, 6-Me
4‴	6.34, d, J = 10.2	121.8	4‴	4′, 5′, 6′, 6‴	6′, 5‴
5‴	5.52, d, J = 10.2	128.1	3‴	5′, 6′, 7‴, 8‴	4‴
6‴		76.7			
7‴	1.46, m	28.2		5‴, 8‴	8‴, 2′-OMe
8‴	1.46, m	28.1		5‴, 7‴	7‴
2′-OMe	3.80, *s*	55.5		2′	3′, 7″, 7‴
6-OH	12.47, s			5	

0.06 μ/ml XOD, an initiator of the production of superoxide anion radical, were incubated at 25 °C. Formed NBT diformazan was determined by a spectrophotometer at 560 nm with the passage time for 14 min. The decreasing absorption rate of the reaction mixture with an antioxidant was compared with that without the antioxidant and superoxide anion radical scavenging activity was calculated as an inhibition ratio (%).

Potential superoxide anion radical scavenging activities of all the compounds **1**–**6** isolated from an ethanolic fraction are presented in Figure 2. The well-known trolox C, a water soluble analogue of Vitamin E, was used as a comparable reference. The $O_2^{\cdot-}$ radical scavenging activity of trolox C was 70.02%. As expected, compounds **3** and **4**, which contain an *ortho*-dihydroxy group, showed stronger antioxidative activity (71.65% and 88.89%) than that for compounds **2**, **5**, and **6** (17.62%, 51.72%, and 29.89%, respectively). The title compound **1** unexpectedly had the strongest antioxidative effect 95.79% in NBT experiment. Although the novel scavenging effect of **1** on the superoxide anion radical cannot be simply explained by usual H atom abstraction[20,21] or electron transfer/proton transfer[22,23] mechanism, our undergoing studies include theoretically analyzing possible reaction pathway through seeking transient-states.

To evaluate the antioxidative effect of the title compound **1**, ESR technique in combination with the spin trapping of 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was used for determining superoxide anion inhibiting ability. Superoxide radicals were generated in vitro by irradiated riboflavin/NADH system. The riboflavin (0.1 mM) was oxidated by irradiation with visible light under molecular oxygen (incubated for 2 min) in the presence of 1 mM NADH and 1 mM desferal and the produced $O_2^{\cdot-}$ was trapped by adding 20 mM DEPMPO. After incubation for 5 min, the ESR spectra obtained from the trapped DEPMPO-OOH adduct were collected. The decreasing peak values of the first peak with the various concentrations of the title compound **1** were compared with that without the compound. The antioxidant data as percentages of inhibition were calculated at five different concentrations of compound **1**.

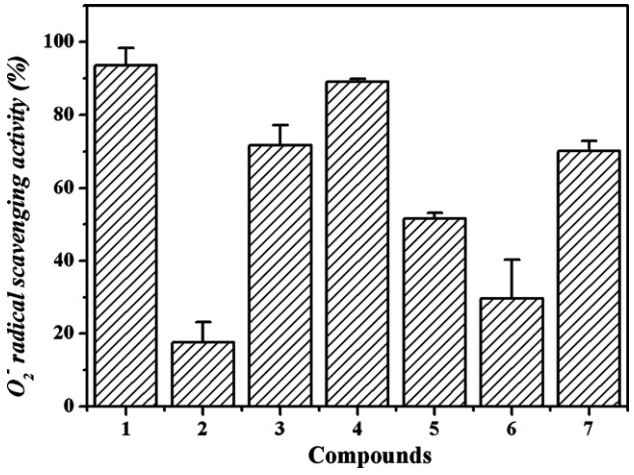


Figure 2. Antioxidative activities of compounds **1**–**6** from *Lespedeza virgata* (Thunb.) DC. **7**, Trolox C.

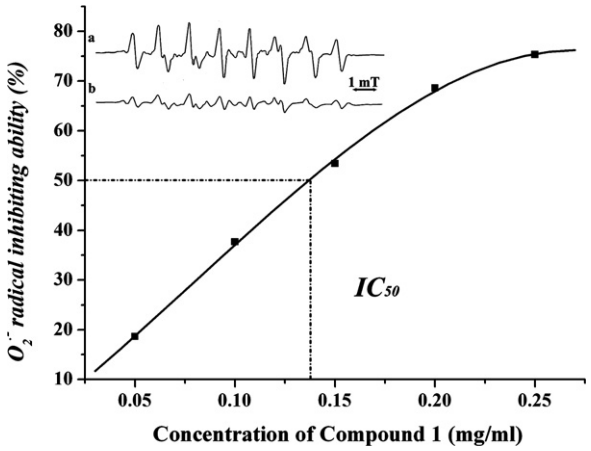


Figure 3. $O_2^{\cdot-}$ radical inhibiting activities of different concentration of compound **1** (a, ESR spectra obtained from the trapped DEPMPO-OOH adduct by illuminating riboflavin; b, pre-incubated by adding compound **1** with concentration 0.20 mg/ml).

ESR experimental data (Fig. 3a and b) revealed that the title compound **1** (0.20 mg/ml) exhibited strong scavenging activities against superoxide radical. As illustrated in Figure 3, crescent $O_2^{\cdot-}$ radical inhibiting ability was gained as concentration of compound **1** doubled each time. By plotting $O_2^{\cdot-}$ radical inhibiting ratio which range from 18.63% to 75.32% against concentration of compound **1**, IC_{50} value of compound **1** can be obtained which is equal to 0.14 mg/ml.

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