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Inhibitory constituents of the heartwood of *Dalbergia odorifera* on nitric oxide production in RAW 264.7 macrophages

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ABSTRACT

Two new isoflavanones (**1** and **13**), along with 25 known compounds (**2**–**12**, **14**–**27**), were isolated from the EtOAc-soluble fraction of the heartwood of *Dalbergia odorifera* by following their potential to inhibit the LPS-induced nitric oxide production in RAW 264.7 cells. The structures of the isolated compounds were established by spectroscopic data such as ^{1}D, ^{2}D NMR and MS spectrometry. Among the isolated compounds, (2*S*)-pinocembrin (**26**), showed the most potent inhibitory effect with IC_{50} value of 18.1 μM.

The heartwood of *Dalbergia odorifera* T. Chen (Leguminosae), a traditional herbal medicine in North East Asia, has been used to treat blood disorders, swelling, ischemia, necrosis, and rheumatic pain.[1] Previous phytochemical studies on this plant have reported the isolation of various types of phenolic compounds such as isoflavanones, isoflavans, neoflavonoids, and chalcones, which possess various pharmacological effects such as antianalgesic, anti-inflammatory, antibacterial, antiplasmodial, neuroprotective, and antioxidant activities.[1–5] In our continuing search for anti-inflammatory constituents, the MeOH extract of the heartwood of *D. odorifera* showed the inhibitory effect of the nitric oxide production in RAW 264.7 cells. Bioassay-guided fractionation of the EtOAc-soluble fraction of *D. odorifera* led to the isolation of 27 compounds including two new isoflavanones (**1** and **13**)[6] (Fig. 1). Twenty-five known compounds were identified as (3*R*)-4′-methoxy-2′,3′,7-trihydroxyisoflavanone (**2**),[4] (3*R*)-violanone (**3**),[7] (3*R*)-3′-*O*-methylviolanone (**4**),[8] daidzein (**5**),[9] formononetin (**6**),[10] 2′-*O*-methylformononetin (**7**),[11] olibergin A (**8**),[12] (3*R*)-sativanone (**9**),[5] (2*S*)-pinostrobin (**10**),[13] butein (**11**),[14] isoliquiritigenin (**12**),[3,15] (2*S*)-7-methoxy-4′,6-dihydroxyflavanone (**14**),[16] orobol (**15**),[17] (2*S*)-3′,5,5′,7-tetrahydroxyflavanone (**16**),[18] melanettin (**17**),[19] 3′-hydroxymelanettin (**18**),[19] latifolin (**19**),[20] 2,4,5-trimethoxy-3′-hydroxy-dalbergiquinol (**20**),[19] 5-*O*-methyllatifolin (**21**),[20] 4,5-dimethoxy-2-hydroxydalbergiquinol (**22**),[19] α,2′,3,4,4′-pentahydroxydihydrochalcone (**23**),[21] α,2′,4,4′-tetrahydroxydihydrochalcone (**24**),[22] 2-methoxy-3-hydroxyxanthone (**25**),[23] (2*S*)-pinocembrin (**26**),[24] and medicarpin (**27**),[25] by comparison of their UV, NMR, and mass data with the reported values in the literature. This Letter describes the isolation and structural elucidation of the isolated compounds and their inhibitory effects on nitric oxide production in RAW 264.7 cells.

Compound **1**[26] was isolated as a white amorphous powder. The molecular formula was determined as $C_{17}H_{16}O_6$ by HR-ESIMS spectrum (*m*/*z* 339.0842 $[M+Na]^+$, calcd for $C_{17}H_{16}NaO_6$, 339.0839). The UV spectrum showed characteristic absorptions assignable to an isoflavanone skeleton at 229, 276, and 309 nm.[4] The ^{1}H NMR spectrum of **1** (Table 1) showed two sets of ABX-pattern aromatic proton signals [δ_H 7.78 (1H, d, *J* = 9.0 Hz, H-5), 7.58 (1H, d, *J* = 8.5 Hz, H-6′), 6.62 (1H, dd, *J* = 2.5, 8.5 Hz, H-5′), 6.58 (1H, d, *J* = 2.5, 9.0 Hz, H-6), 6.57 (1H, d, *J* = 2.5 Hz, H-3′), and 6.38 (1H, d, *J* = 2.5 Hz, H-8)], an oxygenated methylene proton signal [δ_H 4.75 (1H, d, *J* = 11.5 Hz, H-2a) and 4.13 (1H, d, *J* = 11.5 Hz, H-2b)], and two methoxy group signals [δ_H 3.84 (3H, s, 4′-OCH_3) and 3.68 (3H, s, 2′-OCH_3)]. The ^{13}C NMR and DEPT spectra of **1** (Table 1) exhibited 17 carbon signals including a ketone carbonyl (δ_C 188.5), an oxygenated methylene carbon (δ_C 74.2), and an oxygenated quaternary carbon (δ_C 74.1). These results indicated that **1** was an isoflavanone skeleton lacking a proton at C-3 position. The presence of 3-hydroxyisoflavanone moiety in the molecule

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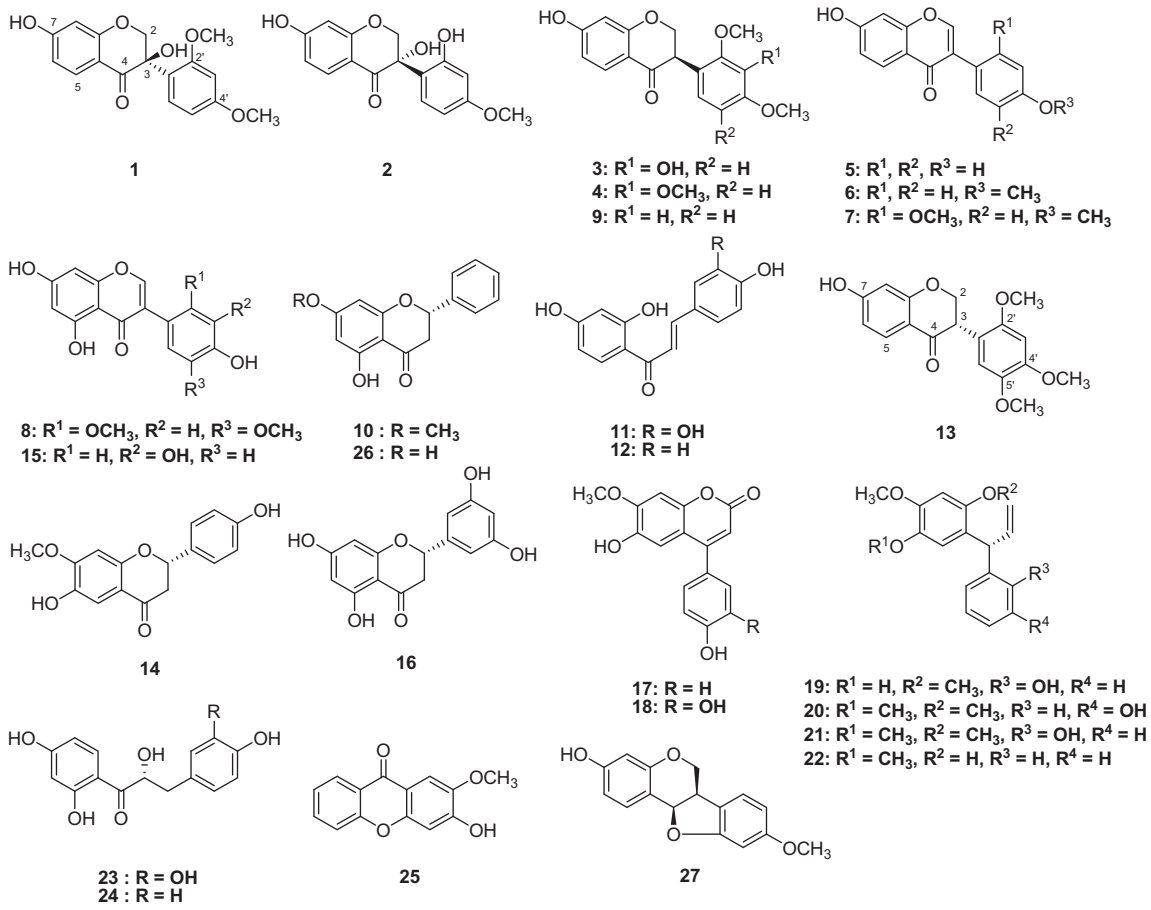

Figure 1. Structures of compounds **1**–**27**.

Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data of compounds **1** and **13**[a]

Carbon no	**1**[b]		**13**[c]	
	δ_H Multi (*J* Hz)	δ_C	δ_H Multi (*J* Hz)	δ_C
2	4.75 (1H, d, 11.5)	74.2	4.60 (1H, dd, 11.0, 11.5)	71.9
	4.13 (1H, d, 11.5)		4.45 (1H, dd, 5.5, 11.0)	
3	–	74.1	4.20 (1H, dd, 5.5, 11.5)	49.6
4	–	188.5	–	194.1
5	7.78 (1H, d, 9.0)	129.5	7.78 (1H, d, 8.5)	130.3
6	6.58 (1H, dd, 2.5, 9.0)	110.8	6.50 (1H, dd, 2.5, 8.5)	112.4
7	–	165.0	–	166.0
8	6.38 (1H, d, 2.5)	102.5	6.33 (1H, d, 2.5)	103.8
9	–	162.9	–	165.9
10	–	112.7	–	115.1
1′	–	121.0	–	116.8
2′	–	157.3	–	153.8
3′	6.57 (1H, d, 2.5)	99.2	6.73 (1H, s)	99.7
4′	–	161.0	–	151.1
5′	6.62 (1H, dd, 2.5, 8.5)	104.8	–	144.5
6′	7.58 (1H, d, 8.5)	128.7	6.78 (1H, s)	116.8
2′-OCH_3	3.68 (3H, s)	55.2	3.78 (3H, s)	56.8
4′-OCH_3	3.84 (3H, s)	54.8	3.88 (3H, s)	56.7
5′-OCH_3	–		3.75 (3H, s)	57.5

[a] The assignments aided by a combination of HSQC, HMBC, and NOESY experiments.
[b] Recorded in a mixture of acetone-d_6 + DMSO-d_6.
[c] Recorded in CD_3OD.

was further confirmed by the observed HMBC correlations from the oxygenated methylenes at δ_H 4.13 and 4.75 to quaternary carbons at C-9 (δ_C 162.9), C-3 (δ_C 74.1), and C-1′ (δ_C 121.0), and from the aromatic proton at δ_H 7.58 to quaternary carbon at C-3 (δ_C 74.1). The location of two methoxy groups were assigned by HMBC correlations between 2′-OCH_3 (δ_H 3.68) and C-2′ (δ_C 157.3) and between 4′-OCH_3 (δ_H 3.84) and C-4′ (δ_C 161.0), respectively (Fig. 2). In the NOESY spectrum, the NOE correlations from 2′-OCH_3 to H-3′, and from 4′-OCH_3 to H-3′ and H-5′ further confirmed the attachment of the methoxy group at C-2′ and C-4′, respectively (Fig. S15, Supplementary data). The circular dichroism (CD) spectrum of compound **1** showed a negative Cotton effect at 341 nm ($n \rightarrow \pi^*$ transition) which was consistent with *S*-configuration at C-3 position of the isoflavanone.[4,27] On the basis of above spectroscopic evidence, the structure of **1** was assigned as (3*S*)-2′,4′-dimethoxy-3,7-dihydroxyisoflavanone. Although, the chiral separation of **1** from the EtOAc fraction of *D. odorifera* was previously analyzed in liquid chromatography mass spectrum by Zeng et al.,[28] the full structural elucidation by spectroscopic data including ^{2}D NMR and CD was suggested for the first time in this Letter.

Compound **13**[29] was isolated as a brownish amorphous powder. Its molecular formula was determined as $C_{18}H_{18}O_6$ by HR-ESIMS

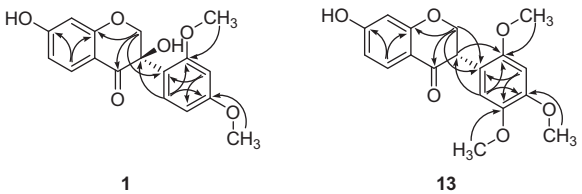

Figure 2. Selected HMBC correlations of compounds **1** and **13**.

spectrum (*m*/*z* 353.0994 $[M+Na]^+$, calcd for $C_{18}H_{18}NaO_6$, 353.0995). The ^1H and ^{13}C NMR spectra of **13** (Table 1) exhibited a ABX-pattern aromatic proton signals [δ_H 7.78 (1H, d, *J* = 8.5 Hz, H-5), 6.50 (1H, dd, *J* = 2.5, 8.5 Hz, H-6), 6.33 (1H, d, *J* = 2.5 Hz, H-8)], a *para*-coupled aromatic proton signals [δ_H 6.78 (1H, s, H-6′), and 6.73 (1H, s, H-3′)], three methoxy proton signals [δ_H 3.88 (3H, s, 4′-OCH_3), 3.78 (3H, s, 2′-OCH_3), 3.75 (3H, s, 5′-OCH_3); δ_C 56.7, 56.8, 57.5], and characteristic proton signals of isoflavanone derivative [δ_H 4.60 (1H, dd, *J* = 11.0, 11.5 Hz, H-2a), 4.45 (1H, dd, *J* = 5.5, 11.0 Hz, H-2b), 4.20 (1H, dd, *J* = 5.5, 11.5 Hz, H-3); δ_C 71.9, 49.6]. The HMBC correlations from 2′-OCH_3 (δ_H 3.78) to C-2′ (δ_C 153.8), from 4′-OCH_3 (δ_H 3.88) to C-4′ (δ_C 151.1), and from 5′-OCH_3 (δ_H 3.75) to C-5′ (δ_C 144.5) indicated the three methoxy groups to be located at C-2′, C-4′, and C-5′, respectively (Fig. 2). This was further corroborated by the observation of NOE correlations from 2′-OCH_3 and 4′-OCH_3 to H-3′ and from 5′-OCH_3 to H-5′ in the NOESY spectrum (Fig. S15, Supplementary data). The absolute configuration at C-3 of **13** was established as *S*-configuration due to a negative Cotton effect at 324 nm ($n \rightarrow \pi^*$ transition) in the CD spectrum.[30] Therefore, the structure of **13** was assigned as (3*S*)-2′,4′,5′-trimethoxy-7-hydroxyisoflavanone.

All isolated compounds **1**–**27** were evaluated for their inhibitory effects on nitric oxide production in LPS-stimulated RAW 264.7 cells, with aminoguanidine as the positive control.[31] Cell viability was determined by the CCK assay and indicated that all the compounds lacked significant cytotoxic effects.[31] Among the active compounds, (2*S*)-pinocembrin (**26**), flavanone derivative lacking the hydroxyl group at the B-ring and having the 5-hydroxy group at the A-ring, showed the most potent inhibitory with IC_{50} value of 18.1 μM. Furthermore, the chalcones such as butein (**11**) and isoliquiritigenin (**12**) and neoflavonoids such as melanettin (**17**) and 3′-hydroxymelanettin (**18**) exhibited inhibitory effects with IC_{50} values of 35.1–72.0 μM. Compounds **19**–**22** which belong to the open-chain neoflavonoids also exhibited the moderate inhibitory activity with IC_{50} values of 70.3–74.0 μM (Table 2). Recently, pinocembrin showed the inhibitory effect of NO production in LPS-induced RAW 264.7 macrophages and oxygen glucose deprivation/reoxygenation injured cortical neurons.[32,33] Moreover, 2′-hydroxy-4′-methoxychalcone and open-chain neoflavonoids such as dalbergione derivatives from the trunk exudates of *Dalbergia sissoo* exhibited significant inhibitory effects on NO production in LPS-induced J774.1 cells.[34] Previous investigation also showed that 6,4′-dihydroxy-7-methoxyflavanone and isoliquiritigenin from *D. odorifera* suppressed the production of NO, TNF-α, and IL-1β.[35,36]

In conclusion, this study and previously reported data suggest that flavonoid derivatives from the heartwood of *D. odorifera* may be worthy of further investigation for their potential as anti-inflammatory diseases associated with the excess production of NO. However, further studies are needed to clarify the action mechanism for the inhibition of NO production.

Table 2
Inhibitory effects of the isolated compounds **1**–**27** on the LPS-induced NO production in RAW 264.7 cells*

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	>100	**10**	53.5	**19**	73.2
2	>100	**11**	35.1	**20**	70.3
3	>100	**12**	72.0	**21**	74.0
4	>100	**13**	>100	**22**	73.9
5	>100	**14**	>100	**23**	>100
6	56.1	**15**	43.7	**24**	>100
7	>100	**16**	>100	**25**	>100
8	45.5	**17**	53.2	**26**	18.1
9	>100	**18**	45.5	**27**	83.7

* Aminoguanidine was used as a positive control (IC_{50} value: 16.6 μM).

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.04.032.

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6. The air-dried heartwood of *D. odorifera* (4 kg) were pulverized and extracted with MeOH (24 L × 3) at room temperature, and then the solution was evaporated under vacuo. The extract was suspended with H_2O and partitioned with *n*-hexane and EtOAc, successively. The EtOAc-soluble extract (80 g) was subjected to column chromatography on silica gel eluting with CH_2Cl_2–EtOAc (100:1 to 100% EtOAc) to EtOAc–MeOH (5:1 to 100% MeOH) to give six subfractions (A–F). The fraction E (14 g) was chromatographed on silica gel and eluted with CH_2Cl_2–MeOH (50:1 to 100% MeOH, step gradient) to yield eight subfractions (E-1–E-8). The subfraction E-1 (1.1 g) was subjected to RP-18 MPLC eluting with H_2O–ACN (30–80% ACN, gradient) to yield compounds **1** (4.1 mg), **3** (14 mg), **4** (18 mg), **6** (11 mg), **8** (6 mg), **9** (140 mg), **10** (28 mg), and **13** (3.2 mg). The fraction E-2 (2.6 g) was chromatographed on RP-18 MPLC eluting with H_2O–ACN (33–70% ACN, gradient) to give seven subfractions (E-2-1–E-2-7). The subfraction E-2-1 was identified as compound **2** (900 mg). The subfraction E-2-4 (0.3 g) and E-2-5 (0.2 g) were combined and were further purified by semi-preparative HPLC eluting with H_2O–ACN (Waters HPLC system, YMC J'sphere ODS-H80, 150 × 20 mm, 24% ACN, isocratic) to yield compounds **7** (9 mg), **14** (21 mg), **17** (38 mg), and **12** (21 mg). The subfraction E-7 (2.4 g) was subjected to column chromatography over RP-18 MPLC eluting with H_2O–ACN (30–70% ACN, gradient) to give seven subfractions (E-7-1–E-7-7) and compound **11** (13 mg). The subfraction E-7-4 (1.8 g) was recrystallized with MeOH to yield compound **18** (1.1 g), and the residue was further purified by semi-preparative HPLC eluting with H_2O–ACN (Waters HPLC system, YMC J'sphere ODS-H80, 150 × 20 mm, 33 to 50% ACN, gradient) to give compounds **5** (9 mg), **15** (4 mg), and **16** (5 mg). The fraction D (6 g) was subjected to column chromatography on silica gel eluting with CH_2Cl_2–MeOH (20:1 to 100% MeOH) to yield six subfractions (D-1–D-6). The subfraction D-1 (1.8 g) was chromatographed over silica gel eluting with *n*-hexane-CH_2Cl_2 (5:1 to 100% CH_2Cl_2) to give four subfractions (D-1-1–D-1-4). The subfraction D-1-1 (0.9 g) was further subjected to column chromatography over RP-18 MPLC eluting with H_2O–ACN (36–80% ACN, gradient) to give compounds **19** (6 mg), **20** (3 mg), **21** (5 mg), **22** (6 mg), **25** (4 mg), **26** (11 mg), and **27** (7 mg). The subfraction D-3 (0.6 g) was purified by semi-preparative HPLC eluting with H_2O–ACN (Waters HPLC system, YMC J'sphere ODS-H80, 150 × 20 mm, 24–42% ACN) to yield compounds **23** (4 mg) and **24** (3 mg).
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26. *Compound* ***1****:* White amorphous powder; $[\alpha]_D^{25} + 8.08^\circ$ (*c* 0.03, MeOH); UV (MeOH) λ_{max} 229*sh* (3.63), 276 (3.58), 309*sh* (3.31) nm; CD (MeOH) nm ($\Delta\varepsilon$): 336 (−1.18), 306 (+0.69), 238 (+3.55); HRESI-MS *m/z* 339.0842 (calcd for $C_{17}H_{16}NaO_6$ 339.0839); ^{1}H NMR (500 MHz, acetone-d_6 + DMSO-d_6) and ^{13}C NMR (125 MHz, acetone-d_6 + DMSO-d_6), see Table 1.
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29. *Compound* ***13****:* Brownish amorphous powder; $[\alpha]_D^{20} + 8.19^\circ$ (*c* 0.02, MeOH); UV (MeOH) λ_{max} 230*sh* (3.90), 278 (3.53), 318*sh* (3.25) nm; CD (MeOH) nm ($\Delta\varepsilon$): 324 (-0.43),273 (+0.53); HRESI-MS *m/z* 353.0994 (calcd for $C_{18}H_{18}NaO_6$ 353.0995); ^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD), see Table 1.
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31. *Determination of NO production and cell viability assay:* The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess method. Briefly, RAW 264.7 cells were seeded into 96-well tissue culture plates at a density of 2×10^5 cells/mL, and stimulated with 1 μg/mL of LPS in the presence or absence of compounds. After incubation at 37 °C and 5% CO_2 atmosphere for 24 h, 100 μL of cell-free supernatant was mixed with 100 μL of Griess reagent containing equal volumes 0.2% (w/v) sulfanilamide in 5% (w/v) phosphoric acid and 0.2% (w/v) of *N*-(1-naphthyl)ethylenediamine solution to determine nitrite production. Absorbance was measured in a microplate reader at 550 nm against a calibration curve with sodium nitrite standards. The remaining cells after Griess assay were used for viability with the CCK (Cell Counting Kit, Dojindo, Tokyo, Japan)-based colorimetric assay.
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