In Vitro Antimalarial Activity of Biflavonoids from *Wikstroemia indica*

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Abstract

In our investigation of *in vitro* antimalarial screening of medicinal herbal extracts, the *n*-BuOH extract from the root of *Wikstroemia indica* showed a potent inhibitory effect. Fractionation of the active extract led to the isolation of two biflavonoids, sikokianin B (**1**) and sikokianin C (**2**) with IC_{50} values 0.54 μg/mL and 0.56 μg/mL, respectively, against the chloroquine-resistant strain of *Plasmodium falciparum*. This is the first report of the biological activity of **1** and **2**. As the structure of **l** has remained unsettled, we confirmed the conformation by ^{1}H- and ^{13}C-NMR.

To discover antimalarial substances from medicinal herbs, EtOAc-, *n*-BuOH- and H_2O-soluble fractions were prepared from the initial EtOH/H_2O (1 : 1) extracts. These were then screened *in vitro* against the chloroquine-resistant K1 strain of *Plasmodium falciparum*. This revealed that the *n*-BuOH-soluble fraction of the root of *Wikstroemia indica* (Linne) C. A. Meyer (Thymelaeaceae) had appreciable antimalarial inhibitory activity (Table **1**).

The *n*-BuOH extract was then subjected to activity-guided purification by column chromatography on silica gel, followed by medium pressure liquid chromatography (MPLC), which gave three fractions (fr.1, fr.2 and fr.3). Compounds **1** and **2** were obtained from fr.1 and fr.2, respectively, in a pure form by repeated MPLC. Each purification step enhanced the antimalarial potency, with the IC_{50} values of **1** and **2** being determined as 0.54 μg/mL and 0.56 μg/mL (Table **1**), respectively. Compounds **1** and **2** showed almost similar activity with chloroquine, but they had one fifty-seventh activity compared with artemisinin.

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Table **1** Inhibitory effects of fractions and compounds from *W. indica* for antimalarial activity against K1, FCR3 and cytotoxicity against MRC-5 cells

Fractions and compounds	*Antimalarial activity IC_{50} (μg/mL) for K1*	*IC_{50} (μg/mL) for FCR3*	*Cytotoxicity IC_{50} (μg/mL) for MRC-5 cells*
AcOEt soluble fraction	16.78	–	> 50
BuOH soluble fraction	7.67	–	1.18
H_2O soluble fraction	> 50	–	> 50
Silica gel column chromatography			
Fr.1	> 50	–	35.34
Fr.2	> 50	–	> 50
Fr.3	3.07	.	> 50
Sikokianin B (**1**)	0.54	0.54	22.54
Sikokianin C (**2**)	0.56	0.34	11.21
fr.3	2.81	–	19.82
Chloroquine	0.56	0.014	18.54
Artemisinin	0.0097	0.0068	45.12

Compound **1** was obtained as an amorphous powder with optical activity ($[\alpha]_D^{30}$: + 199.7°), showed a molecular ion in the HR-FAB-MS at *m/z* = 557.1448 $[M + H]^+$, indicating a molecular formula of $C_{31}H_{24}O_{10}$. In the ^{1}H- and ^{13}C-NMR edited by ^{1}H-^{1}H COSY and HMQC experiments (Table **2**), the spectra showed its structural fragments to include two 1,2,3,5-tetrasubstituted benzenes (C-5 to C-10 and C-5″ to C-10″), two 1,4-disubstituted benzenes (C-1′ to C-6′ and C-1‴ to C-6‴), one 1,2,3,4-tetrasubstituted *n*-butyl group (C-2, C-3, C-2″ and C-3″), one methoxy group and two carbonyls (C-4 and C-4″). These structural fragments were connected to form the given carbon framework of **1** by HMBC, NOESY and LSPD (Long Range Selective Proton Decoupling) spectra, and the structure was shown to be a dimer of flavanonol derivatives which were connected C-3 (δ_C = 49.5) to C-3″ (δ_C = 50.8). From the ^{1}H-^{1}H COSY spectra, the stereochemistry at C-2 (δ_H = 5.17, d, *J* = 9.0Hz)/C-3 (δ_H = 3.33, dd, *J* = 9.0, 3.5Hz), C-3/C-3″ (δ_H = 3.23, dd, *J* = 3.5, 3.5Hz) and C-2″ (δ_H = 5.52, d, *J* = 3.5Hz)/ C-3″ positions exhibited *trans*, *cis* and *cis* geometry, respectively. Further from the NOESY spectra, significant spatial conjugations between H-2/H-2‴ (H-6‴) were observed. The relative stereochemistry of compound **1** was confirmed as shown in Fig. **1**.

Since the compound **1** was previously reported as sikokianin B [1] of which the chirality at the C-3/C-3″ position and the detailed assignment by NMR were unsettled, we assigned and confirmed the structure by the spectral data. Analysis of the spectral data showed compound **2** to be sikokianin C [2] (Fig. **1**). No previous reports have appeared for the isolation of **1** and **2** from the root of *W. indica* or of them having antimalarial activity. The root of *W. indica* has been used for the treatment of scrofula, rheumatalgia, carbuncle, traumatic injury, etc. in China [3] but it is not used for malaria.

Compounds **1** and **2** were assayed against the drug-sensitive FCR3 *P. falciparum* strain, with the resulting IC50 values of 0.54 μg/mL and 0.34 μg/mL, respectively. The activity of **1** and **2** against both the K1 and FCR3 strains was similar suggesting no cross-resistance with chloroquine. Compounds **1** and **2** showed the selectivity indexes (cytotoxicity [IC_{50} for the MRC-5 cells]/antimalarial activity [IC_{50} for the K1 strain]) with the ratios of 41.7 and 20.0, respectively. The results discussed above contribute to a growing list of bioactive compounds obtained from natural sources and as such may provide lead compounds for synthesis of more effective antimalarials.

Materials and Methods

Optical rotations were measured with a JASCO polarimeter at 30 °C. ^{1}H- and ^{13}C-NMR spectra were determined on a Varian Unity 400 machine. Mass spectra (MS) were obtained on a JEOL MXA-AM505HA spectrometer.

Table **2** ^{1}H- (400 MHz) and ^{13}C- (100 MHz) NMR spectral data for compound **1** (CD_3OD)

Position	δ_C	δ_H	*Position*	δ_C	δ_H
2	81.4	5.17 (d, *J* = 9.0 Hz)	2″	82.9	5.52 (d, *J* = 3.5 Hz)
3	49.5	3.33 (dd, *J* = 9.0, 3.5 Hz)	3″	50.8	3.23 (dd, *J* = 3.5, 3.5 Hz)
4	196.0		4″	198.5	
5	165.1		5″	165.4	
6	97.1	5.87 (d, *J* = 2.0 Hz)	6″	97.1	5.76 (d, *J* = 2.0 Hz)
7	168.2		7″	168.3	
8	96.0	5.97 (d, *J* = 2.0 Hz)	8″	96.4	5.85 (d, *J* = 2.0 Hz)
9	163.3		9″	165.1	
10	105.0		10″	103.8	
1′	130.1		1‴	128.6	
2′	128.5	7.02 (d, *J* = 9.0 Hz)	2‴	130.2	7.16 (d, *J* = 9.0 Hz)
3′	116.3	6.74 (d, *J* = 9.0 Hz)	3‴	114.7	6.78 (d, *J* = 9.0 Hz)
4′	161.3		4‴	158.6	
5′	116.3	6.74 (d, *J* = 9.0 Hz)	5‴	114.7	6.78 (d, *J* = 9.0 Hz)
6′	128.5	7.02 (d, *J* = 9.0 Hz)	6‴	130.2	7.16 (d, *J* = 9.0 Hz)
–OCH_3	55.7	3.76 (s)			

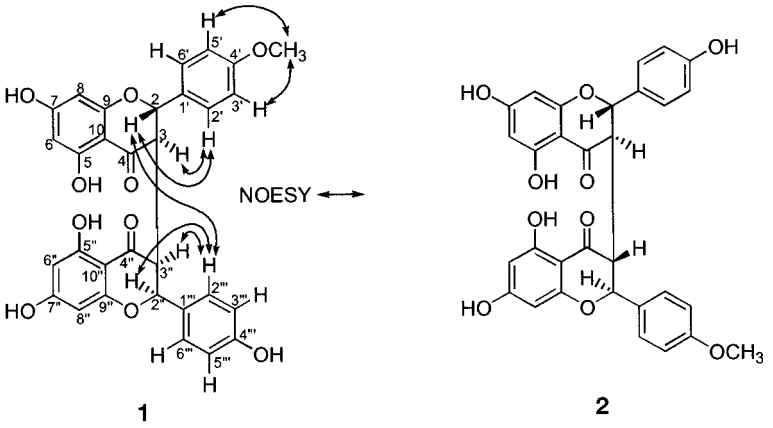

Fig. **1** Biflavonoids from the root of *W. indica*

Plant material: The root of *Wikstroemia indica* was purchased under the herbal name of "Ryokao" in Japanese (Liao-ge-wang in Chinese) from Yamamoto Yakuhin Kogyo Co., Ltd (Tokyo) in April, 2001, and the botanical origin of Ryokao was identified by Dr. Shinyu Nunome in Kitasato Institute for Life Sciences. The voucher specimen (KT-280) has been deposited at the Herbarium of the Kitasato Institute for Life Sciences of Kitasato University.

Extraction and isolation: The root of *W. indica* (100g) was extracted thrice with EtOH/H_2O (1 : 1, 1000 mL) for 1 h under reflux. The solution was concentrated to 400 mL and extracted twice successively with EtOAc (400 mL) and *n*-BuOH (400 mL). The EtOAc-, *n*-BuOH- and H_2O-soluble fractions were evaporated to dryness under vacuum to give each residue of the extract (1.2 g, 1.1 g, 3.3 g), respectively. In three extracts, the antimalarial active *n*-BuOH extract (1.0 g) was chromatographed over a silica gel column (100 g), and was separated by gradient elution with *n*-hexane-EtOAc which yielded after the first 1200 mL of 9 : 1, 0.14 g of Fr.1 (elution after 600 mL of 9 : 1 → 8 : 2), 0.45 g of Fr.2 (8 : 2 → 6 : 4, 900 mL) and 0.16 g of Fr.3 (6 : 4 → 7 : 3, 800 mL). The active fraction Fr.3 (0.15g) was subjected to MPLC on ODS octadecyl silica gel (3.5 × 30 cm) eluting by gradient elution with MeOH-H_2O (5 mL/min, linear gradient, 55 : 45 → 95 : 5, for 3 h) to yield three fractions (fr.1 : 540 – 620 mL, 23 mg, fr.2 : 650 – 710 mL, 21 mg and fr.3 : 730 – 860 mL, 90 mg). Compounds **1** (20 mg) and **2** (20 mg) were obtained from fr.1 and fr.2, respectively, by repeating the purification on MPLC.

Sikokianin B (**1**): Amorphous powder; $[\alpha]_D^{30}$: + 199.7° (*c* 1.0, MeOH); ^{1}H-NMR (CD_3OD, 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz): see Table **2**. HR-FAB-MS: *m/z* = 557.1448 $[M + H]^+$; calcd. for $C_{31}H_{24}O_{10}$: 556.1367.

Sikokianin C **(2):** Amorphous powder; $[\alpha]_D^{30}$: + 3.1° (*c* 1.0, MeOH); ^{1}H-NMR (CD_3OD, 400M Hz): δ = 7.13 (2H, d, *J* = 9 Hz), 7.02 (2H, d, *J* = 9 Hz), 6.94 (2H, d, *J* = 9 Hz), 6.81 (2H, d, *J* = 9 Hz), 5.90 (1H, d, *J* = 2 Hz), 5.89 (1H, d, *J* = 2 Hz), 5.76 (1H, d, *J* = 2 Hz), 5.75 (1H, d, *J* = 2 Hz), 4.90 (1H, br d, *J* = 12 Hz), 4.85 (1H, br d, *J* = 12 Hz), 3.85 (1H, br d, *J* = 12 Hz), 3.76 (3H, s), 3.70 (1H, br d, *J* = 12 Hz); ^{13}C-NMR (CD_3OD, 100 MHz): δ = 196.8, 196.7, 168.5 × 2, 165.5 × 2, 164.4, 164.3, 162.1, 159.8, 130.8 × 4, 130.2, 129.0, 116.6 × 2, 115.2 × 2, 102.8, 102.7, 97.4 × 2, 96.1 × 2, 82.4 × 2, 55.9, 48.2 × 2; HR-FAB-MS: *m/z* = 557.1448 $[M + H]^+$; calcd. for $C_{31}H_{24}O_{10}$: 556.1367.

Antimalarial activity and cytotoxicity: The assays were performed as described in the previous paper [4]. Antimalarial assays were conducted using the drug-resistant K1 strain and the drug-sensitive FCR3 strain of *Plasmodium falciparum.* Chloroquine and artemisinin were used as positive controls. Cytotoxicity was assayed against human diploid embryonic cell line MRC-5.

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