

Chikara Ichino¹
Hiroaki Kiyohara^{1, 2}
Noppamas Soonthornchareonnon³
Wongsatit Chuakul³
Aki Ishiyama⁴
Hitomi Sekiguchi⁴
Miyuki Namatame⁴
Kazuhiko Otoguro⁴
Satoshi Omura¹
Haruki Yamada^{1, 2}

Antimalarial Activity of Biflavonoids from *Ochna integerrima*

Abstract

During the screening of antimalarial substances, the 80% EtOH extract from the outer bark of *Ochna integerrima* Merr. (Ochnaceae) was shown to have a good anti-malarial activity (IC₅₀ value: 6.5 µg/mL) whereas extracts from the inner barks of *O. integerrima* showed no antimalarial activity. Biflavanone (**1**), which had not been found previously from a natural plant

source, was isolated as a potent antimalarial active ingredient (IC₅₀ value: 80 ng/mL) from the extract of the outer barks. The stereoisomer of **1** (= compound **2**) was also isolated from this plant; however, its activity was significantly lower than that of **1**.

Key words

Antimalarial · Ochnaceae · *Ochna integerrima* Merr. · biflavanones

Introduction

Malaria is the major parasitic infection in many tropical and sub-tropical regions, leading to more than one million deaths (principally among African children) out of 400 million cases each year [1] and to major consequent impacts on economic productivity and livelihood [2]. The incidence of malaria is now increasing because of the appearance of multi-drug resistant *Plasmodium falciparum*, therefore new and more effective antimalarial drugs are urgently required.

Ochna integerrima Merr. (Ochnaceae) is a tree that is widely distributed in Thailand [3], and the bark of *O. integerrima* Merr. has been used for digestive disorders as a folk medicine in Thailand [4]. In phytochemical studies on *O. integerrima* Merr, many flavonoids have been isolated [5], [6], [7]. During a screening for anti-malarial active sources among Thai plants, it was found that the 80% EtOH extract from the outer bark of *O. integerrima* Merr.

showed significant anti-malarial activity. The active ingredients in the outer bark were clarified in the present study.

Materials and Methods

Apparatus

Optical rotations were measured on a JASCO polarimeter. ¹H- and ¹³C- NMR spectra were determined on a Varian Mercury-300. Mass spectra (MS) were obtained on JEOL MXA-AM505HA and JMS-700 MStation spectrometers. Chromatographic separations were carried out by column chromatography on Wakogel C-200 (75–150 µm, Wako; Osaka, Japan). Preparative reverse phase HPLC was carried out on PEGASIL (250×20 mm i.d., Senshu Co. Ltd.; Tokyo, Japan).

Affiliation

¹ Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan

² Oriental Medicine Research Center, The Kitasato Institute, Tokyo, Japan

³ Faculty of Pharmacy, Mahidol University, Bangkok, Thailand

⁴ Research Center for Tropical Diseases, Institute for Basic Research, The Kitasato Institute, Tokyo, Japan

Correspondence

Prof. Dr. Haruki Yamada · Kitasato Institute for Life Sciences · Kitasato University · 5-9-1 Shirokane · Minato-ku · Tokyo 108-8641 · Japan · Phone: +81-3-3444-6164 · Fax: +81-3-3445-1351 · E-mail: yamada@lisci.kitasato-u.ac.jp

Received December 1, 2005 · Accepted January 17, 2006

Bibliography

Planta Med 2006; 72: 611–614 © Georg Thieme Verlag KG Stuttgart · New York
DOI 10.1055/s-2006-931569 · Published online May 29, 2006
ISSN 0032-0943

Analytical methods

Analytical HPLC experiments were performed on an Agilent 1100 series HPLC instrument (Agilent Technologies Japan, Ltd.; Tokyo, Japan) equipped with a column of PEGASIL (250×4.6 mm i.d., Senshu Co. Ltd.; Tokyo, Japan). The solvent system used was a linear gradient of acetonitrile from 30% to 60% during 30 minutes in 10 mM phosphoric acid. Flow rate was 1 mL/min. Injection volume was 10 µL for MeOH solution of all the samples. Concentrations of the samples were 10 mg/mL for extracts and 1 mg/mL for the isolated compounds.

Plant material

The outer and inner barks of *O. integerrima* Merr. were collected from Ubon Ratchathani in Thailand. The plants were identified by Associate Professor Wongsatit Chuakul, Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University. Voucher specimens are kept at the Herbarium of Faculty of Pharmacy, Mahidol University.

Extraction and isolation

The outer barks of *O. integerrima* (485 g) were extracted with 80% EtOH at room temperature for 3 days, and the extract was evaporated under vacuum to yield 150 g of the crude EtOH extract. The EtOH extract was chromatographed on silica gel (500 g) and eluted with CHCl₃/MeOH (10:1, 1:1, 0:1; 2 L each) to give three fractions (Fr. 1–3). Fr. 1 (50 g, CHCl₃/MeOH, 10:1) was further fractionated by silica gel column chromatography (1000 g) using CHCl₃/MeOH containing 0.05% TFA [50:1 (3 L), 20:1 (2 L), 10:1 (2 L), 5:1 (4 L), 1:1 (2 L)] to give eight fractions (Fr. 1/1–1/8). Fr. 1/4 (0.5 g of 16.5 g, CHCl₃/MeOH, 5:1) was separated by a reverse phase HPLC (40% CH₃CN containing 0.05% TFA, flow rate, 8.0 mL/min; UV detector, 254 nm) to give the active fraction (Fr. 1/4–3, 266.8 mg, *t_R* = 19.2 min). Fr. 1/4–3 (133.4 mg) was further purified by the same reverse phase HPLC (solvent A, 40% MeOH containing 0.05% TFA; B, MeOH; A/B, 7:1 → 6:2 for 40 minutes; flow rate 8.0 mL/min; UV detector; 254 nm) to give **1** (97.9 mg, *t_R* = 38.0 min) and **2** (7.3 mg, *t_R* = 41.8 min).

Compound 1: brown oil; [α]_D²⁵: +6.2° (c 1.00, MeOH); HR-FAB-MS: *m/z* = 511.1399 [M + H]⁺, (calcd. for C₃₀H₂₃O₈: 511.1393); LR-FAB-MS: *m/z* (rel. int.) = 511 (60), 255 (45); ¹H-NMR (acetone-*d*₆, 300 MHz, 23°C): δ = 6.29 (2H, d, *J* = 2.0 Hz, H-8/H-8''), 6.59 (2H, dd, *J* = 8.5, 2.0 Hz, H-6/H-6''), 6.88 (4H, d, *J* = 8.5 Hz, H-3'/H-3''' and H-5'/H-5'''), 7.14 (4H, d, *J* = 8.5 Hz, H-2'/H-2''' and H-6'/H-6'''), 7.78 (2H, d, *J* = 8.5 Hz, H-5/H-5''); ¹³C-NMR (acetone-*d*₆, 75 MHz, 23°C): δ = 49.8 (C-3/C-3'), 82.5 (C-2/C-2'), 103.4 (C-8/C-8''), 111.5 (C-6/C-6''), 114.4 (C-4a/C-4a''), 116.2 (C-3'/C-3''' and C-5'/C-5'''), 129.1 (C-1'/C-1'''), 130.0 (C-5/C-5''), 130.6 (C-2'/C-2''' and C-6'/C-6'''), 159.0 (C-4'/C-4'''), 164.2 (C-8a/C-8a''), 165.4 (C-7/C-7''), 190.7 (C-4/C-4'').

Compound 2: brown oil; [α]_D²⁵: +83.2° (c 0.25, MeOH); HR-FAB-MS: *m/z* = 533.1209 [M + H]⁺, (calcd. for C₃₀H₂₂O₈Na: 533.1212); ¹H-NMR (acetone-*d*₆, 300 MHz, 23°C): δ = 2.73 (2H, d, *J* = 12.0 Hz, H-3/H-3''), 5.98 (2H, d, *J* = 12.0 Hz, H-2/H-2''), 6.33 (2H, d, *J* = 1.5 Hz, H-8/H-8''), 6.58 (2H, dd, *J* = 8.5, 1.5 Hz, H-6/H-6''), 6.86 (4H, d, *J* = 8.0 Hz, H-3'/H-3''' and H-5'/H-5'''), 7.02 (4H, d, *J* = 8.0 Hz, H-2'/H-2''' and H-6'/H-6'''), 7.75 (2H, d, *J* = 8.5 Hz, H-5/H-5''); ¹³C-NMR (acetone-*d*₆, 75 MHz, 23°C): δ = 51.8 (C-3/C-3''), 84.7 (C-2/C-2''), 103.4 (C-8/C-8''), 111.3 (C-6/C-6''), 115.2 (C-

4a/C-4a''), 116.2 (C-3'/C-3''' and C-5'/C-5'''), 129.1 (C-1'/C-1'''), 129.8 (C-5/C-5''), 130.1 (C-2'/C-2''' and C-6'/C-6'''), 159.0 (C-4'/C-4'''), 164.2 (C-8a/C-8a''), 165.3 (C-7/C-7''), 191.6 (C-4/C-4'').

Sikokianins B and C: Both were isolated from *Wikstroemia indica* as reported previously [11].

Antimalarial activity and cytotoxicity

The assays were performed as described previously [13]. Antimalarial assays were conducted using the multidrug-resistant (K1) and drug-sensitive strains (FCR3) of *Plasmodium falciparum* (gift from Prof. K. Kita, University of Tokyo, Tokyo, Japan). Chloroquine (Sigma-Aldrich Japan K.K.; Tokyo, Japan) and artemisinin (Sigma-Aldrich Japan K.K.; Tokyo, Japan) were used as positive controls. Cytotoxicity was assayed against the human diploid embryonic cell line MRC-5 as described previously [13].

Results and Discussion

The 80% EtOH extract of the outer barks of *O. integerrima* showed significant antimalarial activity (IC₅₀ value: 6.5 µg/mL against the multidrug-resistant strain of *P. falciparum* and 4.5 µg/mL against the sensitive one, respectively). However, the extract from the inner barks of *O. integerrima* was not active (Table 1). According to bioassay-guided purification, a biflavanone **1** was isolated as the major active compound (IC₅₀ value: 80 ng/mL). The stereoisomer of **1**, compound **2** (Fig. 1), was also found in the extract as the other active ingredient, but **2** showed significantly weaker activity than **1** (Table 1).

The structure of **1** was analyzed by 1D and 2D NMR, MS and optical rotation. The HR-FAB-MS of **1** gave [M + H]⁺ at *m/z* = 511.1393 and LR-FAB-MS gave the fragment ion at *m/z* = 255 in addition to [M + H]⁺. The presence of seven phenyl protons and twelve phenyl carbons was confirmed with a 1D-NMR spectrum, and a keto group (δ = 190.7) was also detected in the ¹³C-NMR spectrum. In addition, **1** was also analyzed by HMQC and HMBC experiments, and the obtained data for **1** were very similar to the reported data for the *meso* form of 3,3''-di(7,4'-dihydroxyflavanone-3-yl) which had been obtained only as the biotransformation product [8], [9]. Although the signals of H-2, H-2'', H-3 and H-3'' almost could not be observed due to the fixed conformation at 23°C, these appeared as doublets (δ_H = 4.86, 2H, d, *J* = 12.0 Hz, H-2/H-2'' and 3.55, 2H, d, *J* = 12.0 Hz, H-3/H-3'') on NMR analysis at 80°C in DMSO-*d*₆ in a similar manner as reported previously [9], suggesting that H-2, H-2'', H-3 and H-3'' were each located at axial sites. Compound **1** also gave a very small optical rotation, and from these observations **1** was identified to be the *meso* form of 3,3''-di(7,4'-dihydroxyflavanone-3-yl) (Fig. 1). Compound **2** gave similar spectra upon ¹H-NMR, ¹³C-NMR and MS analyses as the other biotransformation product, the diastereomer of **1** [9]. Although this biotransformation product of **1** has been reported as the racemate, which showed no optical rotation [9], **2** showed significant dextrorotation (+83.2° in MeOH at 25°C). These findings suggested that **2** might be a diastereomer of **1**, but the absolute stereostructure of **2** could not be determined in the present study. Compound **1** and the racemate of **2** had been obtained only as biotransformation products from chalcones with peroxidases of cultured plant cells [8], [9], [10].

Table 1 Antimalarial and cytotoxic activity of the extracts, fractions and compounds from *O. integrerrima* Merr.

Extract/Fraction/Compound	K1 [$\mu\text{g/mL}$]	FCR3 [$\mu\text{g/mL}$]	MRC-5 [$\mu\text{g/mL}$]	Selectivity ^c
80% EtOH extract (outer barks)	6.5	4.5	4.5	0.7
80% EtOH extract (inner barks)	> 12.5	N.T. ^b	N.T. ^b	
Fraction 1	5.0	4.5	4.5	0.9
Fraction 2	40.0	40.0	40.0	1.0
Fraction 3	50.0	45.0	45.0	0.9
1	0.08	0.26	30.0	375
2	5.2	4.5	27.0	5.2
Sikokianin B	0.54	0.54	22.5	41.7
Sikokianin C	0.56	0.34	11.2	20.0
Artemisinin ^a	0.007	0.005	N.T. ^b	
Chloroquine ^a	0.11	0.009	N.T. ^b	

^a Positive control.^b N.T. = not tested.^c IC₅₀ on MRC-5/IC₅₀ on K1.

The present paper is the first report on the isolation of compounds **1** and **2** directly from natural plant extracts. When the 80% extracts from both the inner and outer barks of *O. integrerrima* were compared by HPLC, compounds **1** and **2** could not be detected in the extract from the inner barks, therefore explaining why the extracts of the inner barks were not active (Fig. 2).

Compounds **1** and **2** were evaluated *in vitro* for antimalarial activity (Table 1). The antimalarial activity of compound **1** against the multidrug-resistant strain (K1) was three times stronger than against the sensitive strain (FCR3), and the selectivity (IC₅₀ value against MRC-5/IC₅₀ value against K1) was 375. However, the antimalarial activity of compound **2** was 65 and 17 times weaker than those of **1** for the K1 and FCR3 strains, respectively, although similar cytotoxic activities against MRC-5 were observed for both compounds **1** and **2**.

The antimalarial biflavanones, sikokianins B and C, have been isolated from *Wikstroemia indica* [11], and sikokianins B and C both showed ten times weaker activity than compound **1**, assuming that the stereochemistry of the C3/C3'' coupling bond and methoxy substitution might affect the degree of activity. The monomer of compound **1** is liquiritigenin which has no antiplasmodial activity, while a chalcone (could be related to **1/2**), name-

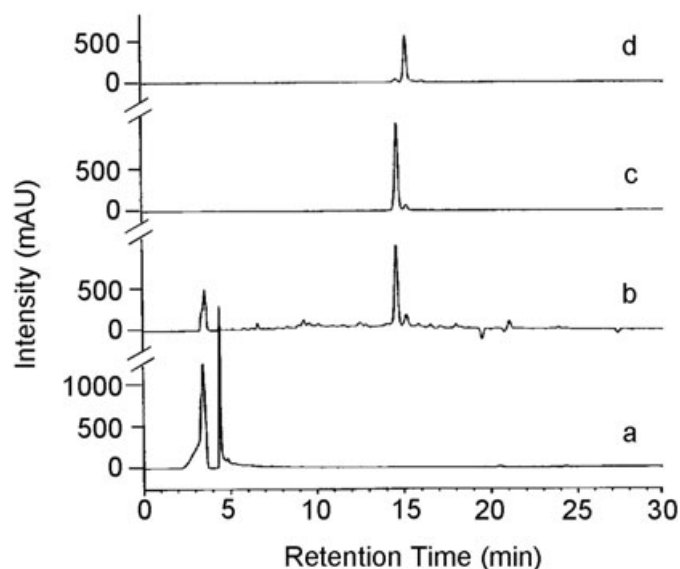


Fig. 2 HPLC profiles at 254 nm of (a) the extract of inner bark of *Ochna integrerrima* Merr.; (b) the extract of outer bark of *Ochna integrerrima* Merr.; (c) compound **1**; (d) compound **2**.

ly isoliquiritigenin, has weaker *in vitro* antimalarial activity than the present biflavanone (**1**) [12]. Finally, biflavanone **1** could be a promising compound for investigation of *in vivo* antimalarial activity in animal models.

Acknowledgements

This work was supported, in part, by funds from the UNICEF/UNDP/World Bank/WHO special Program for Research and Training in Tropical Disease (grants ID A10124), the 21st century COE Program, Ministry of Education, Culture, Sports, Science and Technology (MEXT), and JSPS/NRCT Core University Program for Natural Products in the category of pharmaceutical sciences. The authors appreciate Ms. A. Nakagawa and Ms. C. Sakabe for mass measurements and Ms. N. Sato for NMR measurements.

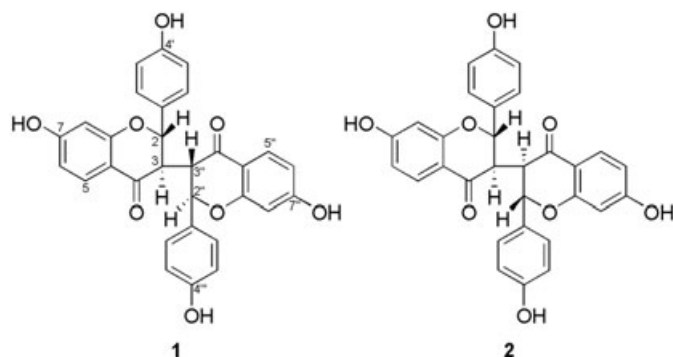


Fig. 1 Antimalarial biflavanones from *Ochna integrerrima* Merr.

References

- ¹ Greenwood B, Mutabingwa T. Malaria in 2002. *Nature* 2002; 415: 670–2
- ² Sachs J, Malaney P. The economic and social burden of malaria. *Nature* 2002; 415: 680–5
- ³ Smitinand T, Larsen K. *Flora of Thailand*; Vol. 2 Bangkok: Tistr Press, 1970: p 24–6
- ⁴ Perry LM. *Medicinal plants of East and Southeast Asia*. Massachusetts: MIT Press, 1980: p 289–90
- ⁵ Likhitwitayawuid K, Rungserichai R, Ruangrunsi N, Phadungcharoen T. Flavonoids from *Ochna integerrima*. *Phytochemistry* 2001; 56: 353–7
- ⁶ Kaewamatawong R, Likhitwitayawuid K, Ruangrunsi N, Takayama H, Kitajima M, Aimi N. Novel biflavonoids from the stem bark of *Ochna integerrima*. *J Nat Prod* 2002; 65: 1027–9
- ⁷ Likhitwitayawuid K, Kaewamatawong R, Ruangrunsi N. Mono- and biflavonoids of *Ochna integerrima*. *Biochem Syst Ecol* 2005; 33: 527–36
- ⁸ Botta B, Vinciguerra V, DeRosa MC, Scurria R, Carbonetti A, Ferrari F et al. Studies in cell suspension cultures of *Cassia didymobotrya*. Part III. The biotransformation of chalcones to flavones and biflavanones. *Heterocycles* 1989; 29: 2175–83
- ⁹ Vitali A, Botta B, Delle Monache G, Zappitelli S, Ricciardi P, Melino S et al. Purification and partial characterization of a peroxidase from plant cell cultures of *Cassia didymobotrya* and biotransformation studies. *Biochem J* 1998; 331: 513–9
- ¹⁰ Botta B, Ricciardi P, Vitali A, Vinciguerra V, Garcia C, Delle Monache G. Peroxidase from cell cultures of *Cassia didymobotrya*: a review and comparison with horseradish peroxidase. *Heterocycles* 1999; 50: 757–66
- ¹¹ Nunome S, Ishiyama A, Kobayashi M, Otoguro K, Kiyohara H, Yamada H et al. *In vitro* antimalarial activity of biflavonoids from *Wikstroemia indica*. *Planta Med* 2004; 70: 76–8
- ¹² Beldjoudi N, Mambu L, Labaied M, Grellier P, Ramanitrahasimbola D, Rasoaivo P et al. Flavonoids from *Dalbergia louvelii* and their anti-plasmodial activity. *J Nat Prod* 2003; 66: 1447–50
- ¹³ Otoguro K, Kohana A, Manabe C, Ishiyama A, Ui H, Shiomi K et al. Potent antimalarial activities of polyether antibiotic, X-206. *J Antibiot* 2001; 54: 658–63