Antimalarial 4-Phenylcoumarins from the Stem Bark of *Hintonia latiflora*

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The EtOAc extract of the stem bark of *Hintonia latiflora* showed the suppression of total parasitemia and the chemosuppression of schizont numbers, when tested in vivo against *Plasmodium berghei* infection in mice. Bioassay-directed fractionation of the EtOAc extract, using the in vitro 16 h and the in vivo 4-day suppression tests on *P. berghei* schizont numbers, led to the isolation of the new compound 5-*O*-*β*-D-glucopyranosyl-7,4′-dimethoxy-3′-hydroxy-4-phenylcoumarin (**1**), along with the known 5-*O*-*β*-D-glucopyranosyl-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin (**2**). The structure of compound **1** was established on the basis of spectroscopic data interpretation. Compounds **1** and **2** suppressed the development of *P. berghei* schizonts in vitro with IC_{50} values of 24.7 and 25.9 μM, respectively. Compound **2** suppressed the development of schizonts at the dose of 40 mg/kg by 70.8% in the in vivo assay.

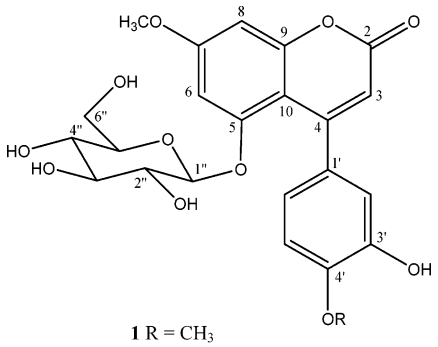
Malaria continues to be a major health problem throughout the tropical and subtropical regions of the world, particularly in sub-Saharan Africa.[1] The increasing rate at which the primary human malaria parasites *Plasmodium falciparum* and *P. vivax* are developing resistance to current antimalarial drugs has stimulated an intensive search for new, safe, and economically affordable antimalarial agents.[2]

Plasmodium development (gametocytes, trophozoites, merozoites, schizonts) in the vertebrate host occurs through a series of processes that present many potential drug targets. Schizont development is a key step in the parasite life cycle, during which intracellular merozoites are formed and released to invade erythrocytes. Newly invaded erythrocytes again produce schizonts, maintaining the blood cycle, or produce gametocytes to maintain transmission.[3] Several compounds have shown to be effective against different development stages of malaria parasites. Natural products for the treatment of malaria such as quinine and their derivatives, as well as artemisinin, are the most successful antimalarial drugs ever used to date. Quinine and the structurally related synthetic compounds chloroquine and mefloquine disrupt the process of hemoglobin degradation, resulting in the persistence of toxic heme molecules that would otherwise be polymerized into inert hemozoin.[4,5] Parasite resistance against many antimalarial drugs has arisen[6,7] and has led to renewed interest in evaluating natural products with the potential to treat malaria with reduced side effects.[8]

Results and Discussion

The stem bark of *Hintonia latiflora* Sessé & Moc. ex DC. Bullock (Rubiaceae), known as "Copalchi", has been used in Mexican traditional medicine as a febrifuge and as an ingredient of many remedies to treat patients with suspected malaria[9−11] and diabetes.[12−14] Previously, a hydrolyzed ethyl acetate extract from the stem bark of *H. latiflora* demonstrated in vitro antimalarial activity against *P. falciparum*, with this activity attributed to the coumarin content of the extract.[15] Phytochemical studies on the stem bark of this plant have determined the presence of 4-phenylcoumarins,[15−18] cucurbitacins,[16,19] and a phenylstyrene,[16] but the effect of these isolated compounds on the malaria parasite in vivo still requires investigation.

Using bioactivity-directed isolation procedures, two 4-phenylcoumarins, the new 5-*O*-*β*-D-glucopyranosyl-7,4′-dimethoxy-3′-hydroxy-4-phenylcoumarin (**1**) and the known 5-*O*-*β*-D-glucopyranosyl-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin (**2**), were isolated from the stem bark of *H. latiflora*. Compound **2** was previously isolated from *Coutarea hexandra*.[20] In this paper we provide an account of the isolation and structural elucidation of **1** and describe the in vitro and in vivo suppressive effects of compounds **1** and **2** against *P. berghei* schizont development.



1 R = CH_3

2 R = H

The administration of the EtOAc and MeOH extracts of *H. latiflora* stem bark to *P. berghei*-infected mice, at doses of 500 and 800 mg/kg/day, respectively, in an in vivo 4-day suppression test,[21] indicated that these extracts were active (Table 1). Of these, the EtOAc extract had the best potency in producing the chemosuppression of total parasitemia on day 4 post infection (p.i.) of 51.1 ± 9.1% ($p < 0.05$) and an 80.0 ± 7.8% ($p < 0.05$) chemosuppression of schizont numbers. On the other hand, the EtOAc extract did not show discernible cytotoxicity (ED_{50} >20 μg/mL) when tested against KB, P388, and SQC-1 UISO tumor cells, which indicated that the antimalarial activity of this extract is not due to toxic effects; ellipticine (ED_{50} = 1.31, <1, and 1.77 μg/mL, for KB, P388, and SQC-1 UISO cells, respectively) was used as the positive control. Bioassay-guided fractionation of this EtOAc extract, assessed by parasite suppression in 16 h *P. berghei*

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Table 1. In Vivo and In Vitro Antimalarial Activities of Extracts, Fractions, and Compounds **1** and **2** of *H. latiflora* Stem Bark against Schizonts of *P. berghei*

	in vivo		in vitro	
extract/fraction	dose (mg/kg/day)	chemosuppression % schizonts (4-day suppressive test)	dose (μg/mL)	chemosuppression % schizonts (16 h)
MeOH extract	800	51.1 ± 9.1	N/D	N/D
EtOAc extract	500	80.0 ± 7.8	N/D	N/D
F2	200	N/D	50	54.4 ± 30.0[a]
F3	200	N/D	50	16.3 ± 1.6[a]
F4	200	N/D	50	32.9 ± 18.2[a]
F5	200	43.2 ± 7.7	50	51.7 ± 39.2
F6	200	73.9 ± 19.4		35.8[b]
F7	200	37.4 ± 22.1	50	64.6 ± 26.2
F8	200	49.4 ± 11.2	50	41.8 ± 2.7
1	40	35.1 ± 0.04		24.7[c]
2	40	70.8 ± 0.1		25.9[c]
chloroquine	5	95.7 ± 8.1	10	100
control (−)		0		0

[a] Cytopathic effect on a schizont culture ($p < 0.05$). [b] IC_{50} value expressed in μg/mL. [c] IC_{50} values expressed in μM.

schizont cultures in vitro, resulted in seven active fractions (F2−F8) (Table 1). The administration of fractions F2, F3, and F4 produced cytopathic effects on erythrocytes and were thus excluded from further investigation. Fractions F5−F8 were tested for their ability to suppress the development of *P. berghei* schizonts in infected mice. All of the tested fractions markedly reduced schizont numbers compared to untreated controls (Table 1), with F6 being the most active, with a 73.9 ± 19.4% ($p < 0.05$) suppression of schizont development ($IC_{50} = 35.8$ μg/mL). The in vitro and in vivo effects of fraction F6 on schizonts with respect to untreated controls are shown in Figures S1 and S2 of the Supporting Information, respectively. Consequently, this active fraction was subjected to purification by a combination of silica gel column and preparative TLC chromatography, leading to a fraction constituted by a mixture of two coumarins that showed suppression of *P. berghei* schizont development in vitro ($IC_{50} = 11.8$ μg/mL). Preparative TLC purification of this fraction led to the isolation of compounds **1** and **2**.

The less polar compound **1** differed from **2** by the presence of an additional methoxyl group, as shown by the molecular ion peak at *m*/*z* 476.4433 observed in the HRFABMS. Compound **1** was spectroscopically very similar to **2**, as a result of having almost the same structural features.[20] The UV spectrum of **1** showed the characteristic maxima of a coumarin structure at 231, 252, and 325 nm.[22] Its ^{1}H NMR data exhibited signals corresponding to the aromatic protons H-6 and H-8 (δ 6.64 and 6.60, respectively) of a coumarin skeleton, with the olefinic proton singlet at δ 6.01 assigned to H-3, and two signals for methoxyl groups found at δ 3.89 and 3.86, as well as an anomeric proton signal at δ 4.75 (d, $J = 7.5$ Hz). The phenyl substituent at C-4 showed an aromatic AMX system at δ 6.90 (d, $J = 8.4$ Hz), 6.87 (d, $J = 1.6$ Hz), and 6.78 (dd, $J = 8.4$, 1.6 Hz). The ^{13}C NMR resonances of **1** were assigned from its HMQC, HMBC, and NOESY spectra (Table 2). The HMBC spectrum revealed cross-peaks between H-3 and C-1′ and C-10, supporting the localization of the phenyl group at C-4. In the NOESY spectrum of **1**, the aromatic protons H-6 (δ 6.64) and H-8 (δ 6.60) exhibited correlations with the methoxyl group at δ 3.89, which in turn showed a cross-peak with C-7 (δ 184.1). The remaining methoxyl signal at δ 3.86 was assigned to C-4′ because of a long-range correlation with the aromatic quaternary carbon at δ 148.8 and the NOE correlation with H-5′ (δ 6.90). This substitution pattern was supported by the NOE cross-peaks observed between the hydroxyl proton at C-3′ (δ 8.53) and H-2′ (δ 6.86) and between H-3 (δ 6.01) and H-6′ (δ 6.78). Also, the position of the sugar residue was determined unambiguously to be at C-5 due to the long-range correlation observed between C-5 (δ 156.1) of the aglycon and H-1 (δ 4.75) of the glucopyranosyl unit. Thus, this compound was proposed as 5-*O*-β-D-glucopyranosyl-7,4′-dimethoxy-3′-hydroxy-4-phenylcoumarin (**1**), which is a new compound.

Table 2. ^{1}H and ^{13}C NMR Spectroscopic Data for Compound **1** (400, 100 MHz, $CDCl_3$−DMSO-d_6)[a]

position	δ_C	δ_H
2	162.4	
3	113.2	6.01 (1H, s)
4	157.3	
5	156.1	
6	100.1	6.64 (1H, d, $J = 1.2$ Hz)
7	164.1	
8	95.9	6.60 (1H, d, $J = 1.2$ Hz)
9	156.8	
10	105.1	
1′	133.4	
2′	111.4	6.86 (1H, d, $J = 1.6$ Hz)
3′	146.2	
4′	148.8	
5′	104.6	6.90 (1H, d, $J = 8.4$ Hz)
6′	113.2	5.78 (1H, dd, $J = 8.4$, 1.6 Hz)
OMe-7	56.5	3.89 (3H, s)
OMe-4′	56.3	3.86 (3H, s)
OH-3′		8.53 (1H, s)
1″	101.1	4.75 (1H, d, $J = 7.5$ Hz)
2″	72.9	2.58 (1H, dd, $J = 8.5$, 7.5 Hz)
3″	76.1	3.14 (1H, m)
4″	69.3	3.03 (1H, m)
5″	77.0	3.24 (1H, m)
6″	60.6	3.67 (2H, m)

[a] Proton resonance integral, multiplicity, and coupling constant (J = Hz) are in parentheses.

Compound **2** was identified as 5-*O*-β-D-glucopyranosyl-7-methoxy-3′,4′-dihydroxy-4-phenylcoumarin, by comparison of its physical and spectroscopic data to those published in the literature.[20]

Compounds **1** and **2** were evaluated in vitro for chemosuppression in 16 h *P. berghei* schizont cultures. The results indicated that both compounds were active against this parasite blood stage in the in vitro assay, with IC_{50} values of 11.8 μg/mL (24.7 μM) and 11.9 μg/mL (25.9 μM), respectively. Evaluation of compounds **1** and **2** in the in vivo 4-day suppression test at a dose of 40 mg/kg showed a chemosuppression of total parasitemia of 19.7 ± 3.9% and 39.5 ± 0.8% ($p < 0.05$), respectively, and a 35.1 ± 0.04% and 70.8 ± 0.1% ($p < 0.05$) chemosuppression of schizont numbers, respectively. The in vivo effect of compound **2** on schizonts with respect to untreated control is shown in Figure S3 of the Supporting Information. The in vivo activity displayed by pure compound **2** was greater than that of the natural mixture of **1** and **2** (73.9 ± 19.4%) at a dose of 200 mg/kg, but much lower than that exhibited by chloroquine, which in this study displayed a 94.0 ± 1.6% ($p < 0.05$) chemosuppression of total parasitemia and a 97.0 ± 0.06%

($p < 0.05$) chemosuppression of schizont numbers at dose of 5 mg/kg. All animals treated with compounds **1** and **2**, and chloroquine, survived until 15 days and did not show evident signs of toxicity. Without treatment, all mice died between 6 and 7 days with a gradual loss in body weight. Thus, these results clearly show that compound **2** had a significant effect in reducing *P. berghei* schizonts in vivo in the animal model used in this study. This supports the use of *H. latiflora* as an antimalarial drug in Mexico.

Experimental Section

General Experimental Procedures. Melting points were determined on a Fisher-Johns melting point apparatus and were uncorrected. Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter. The IR spectra were obtained on a Bruker Vector 22 spectrometer. NMR spectra were recorded on a Varian Unity 400 spectrometer at 400 MHz for ^{1}H NMR, ^{1}H−^{1}H COSY, HMBC, HSQC, and ^{1}H−^{1}H NOESY and 100 MHz for ^{13}C NMR and ^{13}C DEPT using DMSO-d_6 and $CDCl_3$ as solvents. Chemical shifts are reported in ppm (δ) relative to TMS. HRFABMS in a matrix of glycerol were recorded on a JEOL JMX-AX 505 HA mass spectrometer. Column chromatography and TLC were carried out on silica gel 60 (0.063−0.2 mm, Merck).

Plant Material. The stem bark of *Hintonia latiflora* (Sessé & Mociño & DC.) Bullock, [syn. *Coutarea latiflora* (Sessé & Mociño & DC.)] was collected at Sierra de Huautla, Morelos, México, in July 2002. The plant material was identified by Abigail Aguilar-Contreras, IMSSM Herbarium, and deposited under voucher number 14,853.

Extraction and Isolation. *H. latiflora* bark was dried in the dark at room temperature and milled. The material (3.5 kg) was extracted sequentially at room temperature with *n*-hexane, EtOAc, MeOH, and H_2O (15 L each). Extracts were filtered and concentrated by distillation under reduced pressure and lyophilized to remove solvent traces to yield 19.6 g (0.56%) of the *n*-hexane, 33.0 g (0.94%) of the EtOAc, 663.4 g (18.95%) of the MeOH, and 295.6 g (8.44%) of the water extract, respectively. The most in vitro-active extract (EtOAc, 22 g) was fractionated on a chromatographic column of silica gel (900 g), eluting with *n*-hexane−EtOAc−MeOH mixtures of increasing polarity, to produce 207 fractions of 300 mL each, which were grouped into eight main fractions according their similarity by TLC analysis, as follows: F1, 1.50 g (95:5:0, 10.5 L), F2, 0.71 g (80:20:0, 9.0 L), F3, 0.419 g (70:30:0, 4.7 L), F4, 385 g (50:50:10, 4.25 L), F5, 0.30 g (50:50:20, 1.5 L), F6, 0.71 g (50:50:30, 2.7 L), F7, 1.22 g (50:50:40, 2.5 L), and F8, 0.33 g (50:50:50, 6.2 L).

Fraction F6 was chromatographed on a silica gel column, eluting with a gradient of CH_2Cl_2−MeOH (98:02 → 50:50) to yield 15 subfractions. Subfractions 8−12 (72 mg), eluted with CH_2Cl_2−MeOH (4:1), were purified by reversed-phase preparative TLC using a mixture of H_2O−CH_3CN−MeOH (3:2:1) to yield 12 mg of **1** and 40 mg of **2**.[17]

5-*O*-β-D-Glucopyranosyl-7,4′-dimethoxy-3′-hydroxy-4-phenylcoumarin (1): pale yellow solid; mp 162−165 °C; $[\alpha]^{25}_D$ −0.2 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ϵ) 231 (3.45), 252 (3.28), 325 (3.35) nm; IR (KBr) ν_{max} 3429, 2957, 2920, 1704, 1615, 1440, 1306, 1076, 882 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 2; FABMS *m*/*z* 476 $[M + H]^+$; HRFABMS *m*/*z*, 476.4433 $[M + H]^+$ (calcd for $C_{23}H_{24}O_{11}$, 476.4412).

In Vivo Antimalarial Assay. The evaluation of the activities of extracts, fractions, and pure compounds **1** and **2** against *P. berghei* (ANKA) parasites was carried out using the classical 4-day suppression test described by Peters and Robinson.[21] Briefly, experimental groups of five mice with initial 20% percent parasitemia were treated daily from day 0 to 3 post infection (p.i.), with an oral dose of 800 mg/kg of methanol extract, 500 mg/kg of ethyl acetate extract, 200 mg/kg of fractions F5, F6, F7, and F8, and 40 mg/kg of **1** and **2** per day. Control groups were treated with chloroquine (5 mg/kg) and 0.2 mL of 0.1% DMSO in H_2O, respectively. The percentages of chemosuppression of total parasitemia and schizont numbers for each extract, fraction, and pure compound were calculated as $[(A - B)/A] \times 100$. In this expression, *A* is the mean parasitemia in the untreated control group and *B* the parasitemia in each experimental group. Total parasitemia was an arithmetic sum of the intracellular parasites in different stages of development.

In Vitro Antimalarial Assay. Cultured schizonts of *P. berghei* were used to assess antimalarial activity of *H. latiflora* fractions and pure compounds. Cultures of *P. berghei* schizonts were prepared as described by Thathy and Menard.[23] Fractions F5, F6, F7, and F8, dissolved in PBS containing 0.1% and 5% DMSO in ethanol at a concentration of 50 μg/mL, were added. Parasites were incubated for 16 h at 37 °C as described. Chloroquine was used as positive control. Samples of each culture were taken to prepare smears and used to count numbers of schizonts in 2000 erythrocytes. The IC_{50} values of fraction F6 and compounds **1** and **2** were determined using concentrations of 1, 5, 50, 100, 250, and 500 μg/mL, according to Khalid et al.[24] and by extrapolation from the concentration response curve. The IC_{50} value represents the drug concentration producing a 50% reduction in number of *P. berghei* schizonts (compared to drug-free control cultures).

Statistical Analysis. Statistical differences between mean parasitemias of control and experimental culture and mice groups were assessed using the Student's *t*-test and ANOVA with the Tuckey test (*p*-values of 0.05 or less were considered significant).

Cytotoxic Activity. Cytotoxic evaluation was determined in KB (nasopharyngeal carcinoma), P388 (murine leukemia carcinoma), and SQC-1 UISO (uterine-cervix cancer) cell cultures according to Geran and Greenberg's screening protocols.[25]

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Supporting Information Available: In vitro and in vivo effects on schizonts by fraction F6 (Figures S1 and S2) and compound **2** (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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