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Antiplasmodial Activity of Aryltetralone Lignans from Holostylis reniformis

Valter F. de Andrade-Neto,1,2† Tito da Silva,3 Lucia M. Xavier Lopes,3* Virgílio E. do Rosário,4 Fernando de Pilla Varotti,1 and Antoniana U. Krettli1

Laboratório de Malaria, Centro de Pesquisas René Rachou/FIOCRUZ, 30190-002, Belo Horizonte, MG, Brazil; Departamento de Parasitologia, Universidade Federal de Minas Gerais, UFMG, Av. Augusto de Lima, 1715, 30190-002, Belo Horizonte, MG, Brazil; Instituto de Química, Universidade Estadual Paulista, UNESP, C. P. 355, 14801-970, Araçoiaba, SP, Brazil; and Instituto de Higiene e Medicina Tropical, Centro de Malaria e Doenças Tropicais, Universidade Nova de Lisboa, UNL, 1349-008, Lisboa, Portugal

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Extracts from Holostylis reniformis were tested in vivo against Plasmodium berghei and in vitro against a chloroquine-resistant strain of Plasmodium falciparum. The hexane extract of the roots was the most active, causing 67% reduction of parasitemia in vivo. From this extract, six lignans, including a new (7'R,8,8'S)-3',4',4'-methylenedioxy-4,5-dimethoxy-2,7'-cyclolignan-7-one, were isolated and tested in vitro against P. falciparum. The three most active lignans showed 50% inhibitor concentrations of \( \leq 0.32 \) \( \mu \)M. An evaluation of minimum lethal dose (30%) values showed low toxicity for these lignans in a hepatic cell line (Hep G2A16). Therefore, these compounds are potential candidates for the development of antimalarial drugs.

Malaria is the most important parasitic disease in the world, responsible for 500 million new cases and 2 to 3 million deaths every year (http://www.who.int/malaria/epidemicsandemergencies.html). The number of clinical attacks due to Plasmodium falciparum seems to be 50% higher than WHO estimates (24). This situation, together with the progressive spread of chloroquine-resistant strains of P. falciparum and, more recently, Plasmodium vivax, has caused an intensive search for novel blood schizonticides to replace chloroquine, a cheap, safe, and, formerly effective therapeutic antimalarial drug (9, 20, 21). Many natural products of various structural types have shown antimalarial activity (22, 26, 27). The toxicities of compounds isolated from several species are active against P. falciparum in vitro. The extracts were bioassayed in vitro for their antimalarial activities and toxicities. The structures of lignans 1 to 5 had been determined by spectroscopic methods and chemical transformations (5, 6). Lignan 6 is reported here for the first time.

MATERIALS AND METHODS

Plant material. The plant material was collected in Ituiutaba, MG, Brazil, in February 1998 and identified as H. reniformis Duch. by Condorect Aranha and Lindolpho Cappellari Jr. A voucher specimen (ESA88282) was deposited at the herbarium of the Escola Superior de Agricultura “Luiz de Queiroz”, Piracicaba, SP, Brazil. The material was separated according to the plant parts, dried (\(-45^\circ C\)), and ground (4-6). Extracts and isolation of the chemical constituents. The plant material was extracted exhaustively at room temperature with hexane, acetone, and ethanol, successively, and the extracts were individually concentrated (4-6). The hexane extract (6.17 g) from the roots was fractionated by column chromatography (60.0 by 4.8 cm; silica gel 60 H; 151.0 g; hexane-ethyl acetate gradient, 95:5 to 100% ethyl acetate) to give 28 fractions (100 ml), as previously described (6). Several of these fractions were subjected to semipreparative HPLC (MeOH-H2O, 3:2). Fraction 10 was comprised of lignans 1, 5, and 6 (11:3:2) and gave lignans 1 (67.6 mg), 5 (18.4 mg), and 6 (12.3 mg). Fraction 11 gave lignans 1 (25.7 mg) and 5 (22.1 mg). Fraction 12, comprised of lignans 2, 3, and 4 (3:8), and subjected to semipreparative HPLC (MeOH-H2O, 3:2) to give lignans 2 (255.7 mg), 3 (275.8 mg), and 4 (28.1 mg).

Instrumentation. One-dimensional (\( ^1H \), \( ^13C \), and distortionless enhancement by polarization transfer [DEPT]) and two-dimensional (\( ^1H-^1H \) gradient-selected correlated spectroscopy [gCOSY]; gradient-selected heteronuclear multiple-quantum coherence, inverse detected \( ^1H-^1C \) one-bound correlation experiment [gHMQC]; gradient-selected heteronuclear multiple-bond correlation, inverse detected \( ^1H-^1C \) long-range correlation experiment [gHMBC]; and gradient-selected nuclear Overhauser enhancement spectroscopy [gNOESY]) nuclear magnetic resonance (NMR) experiments were recorded on a Varian INOVA 500 spectrometer (11.7 T) at 500 MHz (\( ^1H \)) and 125 MHz (\( ^13C \)), with the residual solvent CDCl3 used as an internal standard for \( ^1H \) (6.23 and 126 MHz (\( ^13C \)), with the residual solvent CDCl3 used as an internal standard for \( ^1H \) (8.72 and 77.0)). Mass spectra (electrospray ionization-mass spectrometry [ESI-MS]) were obtained on a Finnigan Platform II, and flow injection into the electrospray source was used for ESI-MS. Infrared (IR) spectra were obtained on a Nicolet-730 FT-IR spectrometer using KBr discs. UV absorptions were measured on a Hewlett-Packard 8452A diode array spectrophotometer. Optical rotations were measured on a Polamat A (Carl Zeiss, Jena, Switzerland). Circular dichroism

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* Corresponding author. Mailing address: Instituto de Química, Universidade Estadual Paulista, UNESP, R. Francisco Degni s/n, C. P. 355, 14801-970, Araçoiaba, SP, Brazil. Phone: (55) 16-3301-6663. Fax: (55) 16-3301-6662. E-mail: lopes@iq.unesp.br.
† Present address: Departamento de Microbiologia e Parasitologia, Universidade Federal do Rio Grande do Norte, UFRN, 59072-970, Natal, RN, Brazil.
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(five mice per group) received 10^5 infected red blood cells (day zero), followed by NK-65, originally received from New York University Medical School. Each mouse test was performed as previously described (3) in mice infected with

\[ \text{Microquimica MQAPF-301 melting point apparatus and were uncorrected. Chromatograms were acquired at 254 nm. Melting points were recorded on a} \]

\[ \text{for analytical analysis and 250 by 20 mm for semipreparative analysis), and} \]

\[ \text{H}_{2}O_{851}/H_{9251}/H_{20852}-\text{aristotetralone; lignan 6] was obtained as a yellow solid, m.p. 136.2 to 138.0°C; epi} \]

\[ \text{repeated three times. When this reduction was} \]

\[ \text{parasitemia in relation to untreated mice, and a compound was considered active} \]

\[ \text{smears were taken on days 5 and 7 after parasite inoculation, and mortality was} \]

\[ \text{not treated or were treated with Tween 20 (2% final concentration) immediately before use and then diluted so that} \]

\[ \text{daily treatment, via gavage, for 4 consecutive days. The extracts were suspended in} \]

\[ \text{Swiss albino mice (body weight, 20} \]

\[ \text{Antimalarial tests in vivo. The antimalarial tests were performed with adult} \]

\[ \text{Quinidine Sulfate (Q) and 10-[2-(2,4-dichlorophenyl)benzylideneamino]-2,2'-bipyridyl (25). The antiparasitic effects of extracts, purified compounds (lignans 1 to 6), and fractions were measured by the percent inhibition of parasite growth in relation to the control (parasites cultivated in drug-free medium), as previously described (3). Briefly, the drugs tested were diluted with Tween 20 at a final concentration of 0.02% in culture medium (RPMI 1640). These stock solutions were further diluted in complete medium (RPMI 1640 plus 10% human serum) to give each of the concentrations used (0.02 to 20 \mu M for purified compounds and fractions and 0.2 to 50 \mu M/ml for extracts). The cultures, with trophozoites in sorbitol-synchronized blood (14) at 1 to 2% parasitemia and 2.5% hematocrit, were incubated at 37°C in an enriched CO2 environment for 24 h (17). The compounds were mixed with William’s E culture medium in 96-well microtiter plates and incubated at 37°C for 48 h. The culture medium was replaced with 200 \mu M fresh medium with or without the drugs. At the end of the incubation periods, 20 \mu l of MTT solution (5 mg of thiazolyl blue salt in RPMI 1640) without phenol red was added to each well, and the plates were incubated for three more hours. The supernatant was then removed, and 200 \mu l of acidified isopropanol was
**TABLE 1. NMR spectroscopic data (500 MHz, CDCl$_3$) for lignans 5 and 6**

<table>
<thead>
<tr>
<th>Position</th>
<th>Lignan 5 (S)</th>
<th>Lignan 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\delta_C$</td>
<td>$\delta_H$ ($J$ in Hz)</td>
</tr>
</tbody>
</table>
| 1        | 127.0       | 125.6     | 3, 6, $7'$
| 2        | 141.0       | 138.4     | 6
| 3        | 109.5       | 108.2     | 7'$
|          | 6.35 s      | 6.35 s    | OCH$_3$-4, 7'
| 4        | 152.2       | 153.8     | 3, 6, OCH$_3$-4
| 5        | 147.2       | 148.3     | 3, 6, OCH$_3$-5
| 6        | 105.8       | 108.1     | OCH$_3$-5
| 7        | 199.5       | 199.8     | 6, 9
| 8        | 43.0        | 42.6      | 9, 7', 9'
|          | 2.71 dq (4.5, 7.0) | 2.70 dq (3.5, 7.0) | 9, 8', 2'
| 9        | 11.7        | 11.9      | 8, 9'
| 1'       | 136.0       | 137.7     | 5'
| 2'       | 111.9       | 111.8     | 4.67 d (1.5)
| 3'       | 149.2       | 147.9     | OCH$_3$-O$_2$, 2', 5'
| 4'       | 147.9       | 146.3     | 2', 6'; OCH$_3$-O
| 5'       | 111.1       | 109.0     | 6.67 d (8.0)
|          | 6.72 d (8.0) | 6.44 dd (8.0, 1.5) | 2', 7'
| 6'       | 121.1       | 122.0     | 8, 7', 8'
|          | 6.49 dd (8.0, 1.5) | 3.85 d (5.5) | 3, 6', 9'
| 7'       | 50.6        | 50.5      | 3, 2', 8', 9'
|          | 3.86 d (5.5) | 2.35 dd (5.5, 3.5, 6.5) | 9, 7', 9'
| 8'       | 42.0        | 42.5      | 9, 8', 2', 6', 7'
|          | 2.35 ddq (5.5, 4.5, 6.5) | 0.92 d (6.5) | 9, 7', 8'
| 9'       | 15.9        | 15.9      | OCH$_3$-4, 4'
|          | 0.91 d (6.5) | 0.92 d (6.5) | 3, 2', 6'
| OCH$_3$-4 | 56.0      | 56.0      | 3.72 s
| OCH$_3$-4' | 56.0      | 56.0      | 3.87 s
| OCH$_3$-O | 101.6      | 101.0     | 5.87 s

$^a$ The $^{13}$C NMR data were assigned with the assistance of DEPT, gHMOC (optimized for 140 Hz), and gHMBC experiments.

$^b$ gHMBC correlations (optimized for 7 Hz) are from the proton(s) stated to the indicated carbon.

The crude hexane, acetone, and ethanol extracts of the roots, stems, and leaves of *H. reniformis* partially reduced the malaria parasitemia and mortality of mice infected with *P. berghei*. The hexane extracts were the most active, especially the root and leaf extracts, which caused 67% and 48% reduction of parasitemia, respectively, at doses of 500 mg/kg (day 5; $P \leq 0.05$). Lower doses tested were inactive. The extracts were also screened in vitro against *P. falciparum* parasites (isolate BHZ 26/86; chloroquine resistant). The apolar extracts (hexane and acetone) exhibited the best antiplasmodial activities, and they exhibited the lowest IC$_{50}$ values ($\leq$0.70 µg/ml), whereas the positive control (chloroquine) showed an IC$_{50}$ of 0.09 µg/ml.

All of the isolated lignans were tested for antimalarial activity in vitro; their IC$_{50}$ and IC$_{90}$ values, as well as the values for the standard antimalarial chloroquine, obtained in three sets of experiments are shown in Table 2. Lignans 1 to 3 exhibited IC$_{50}$ values of $\leq 0.32$ µM ($\leq 0.12$ µg/ml). The lowest IC$_{50}$ value obtained was for lignan 3 (0.20 µM), whereas the lowest IC$_{90}$ value was for lignan 4 (2.61 µM), which showed that these lignans are active and that they are the major active principles in the extracts. Lignan 5 exhibited low activity, with the highest IC$_{50}$ (8.00 µM) and IC$_{90}$ (19.7 µM) values, whereas lignan 6 did not exhibit any activity under the same experimenter.

**TABLE 2. IC$_{50}$ and IC$_{90}$ of lignans, alone or in mixtures, tested against *P. falciparum* isolate BHZ26/86**

<table>
<thead>
<tr>
<th>Lignan(s)</th>
<th>Compound</th>
<th>IC$_{50}$ (µM)$^a$</th>
<th>IC$_{90}$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(7'R,8'S,8'R)-4,5-Dimethoxy-3',4'-methyleneoxy-2,7'-cycloglignan-7-one</td>
<td>0.26 ± 0.08</td>
<td>3.35 ± 0.12</td>
</tr>
<tr>
<td>2</td>
<td>(7'R,8'S,8'R)-3',4',4'-Tetramethoxy-2,7'-cycloglignan-7-one</td>
<td>0.32 ± 0.11</td>
<td>4.60 ± 0.30</td>
</tr>
<tr>
<td>3</td>
<td>(7'R,8'S,8'R)-3',4',5'-Tetramethoxy-2,7'-cycloglignan-7-one</td>
<td>0.20 ± 0.09</td>
<td>3.00 ± 0.15</td>
</tr>
<tr>
<td>4</td>
<td>(7'R,8'S,8'S)-3',4',4'-Tetramethoxy-2,7'-cycloglignan-7-one</td>
<td>0.63 ± 0.20</td>
<td>2.61 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>(7'R,8'S,8'S)-3',4'-Dimethoxy-4,5-methyleneoxy-2,7'-cycloglignan-7-one</td>
<td>8.00 ± 0.65</td>
<td>19.70 ± 0.42</td>
</tr>
<tr>
<td>6</td>
<td>(7'R,8'S,8'S)-4,5-Dimethoxy-3',4'-methyleneoxy-2,7'-cycloglignan-7-one</td>
<td>&gt;140.00</td>
<td>&gt;140.00</td>
</tr>
<tr>
<td>3 + 4</td>
<td>Combination (3:8)</td>
<td>2.80 ± 0.34</td>
<td>9.13 ± 0.30</td>
</tr>
<tr>
<td>2 + 3 + 4</td>
<td>Combination (3:1:2)</td>
<td>6.00 ± 0.50</td>
<td>18.20 ± 0.40</td>
</tr>
<tr>
<td>1 + 5 + 6</td>
<td>Combination (11:3:2)</td>
<td>1.90 ± 0.09</td>
<td>8.40 ± 0.15</td>
</tr>
<tr>
<td>Chloroquine$^b$</td>
<td></td>
<td>0.19 ± 0.02</td>
<td>0.70 ± 0.13</td>
</tr>
</tbody>
</table>

$^a$ Values are means ± standard deviations in triplicate.

$^b$ Antimalarial reference drug.
Experimental conditions at the maximal dose tested (140.0 µM = 50 µg/ml). Mixtures of these lignans, which were not previously subjected to semipreparative HPLC (3 and 4; 2, 3, and 4; and 1, 5, and 6), also showed some activity and exhibited significant IC$_{50}$ (from 1.9 to 6.0 µM) and IC$_{90}$ (from 8.4 to 18.2 µM) values (Table 2).

The cytotoxicities of the active lignans evaluated in vitro were considered low, since the mean minimum lethal dose that killed 30% of the cells (450 µg/ml) was at least 5,000 times higher than the mean IC$_{50}$ value obtained for them.

Compound 6 has not yet been described in the literature. It was isolated from the active fraction 1, 5, and 6 (11:3:2) by semipreparative HPLC. The $^1$H and $^{13}$C NMR, UV, IR, and ESI-MS data for lignan 6 were similar to those reported for lignan 5 (5).

**DISCUSSION**

Lignoids with different structural types (up to 60) have been previously isolated from the family Aristolochiaceae (4–6, 8, 16, 19). The biosynthesis, functions, and pharmacological and physiological effects of lignans have been studied, and these compounds have been shown to possess a wide range of biological activities (15, 18, 23). Lignans have been used as lead compounds for the development of new drugs, mainly due to their low cytotoxicity and their antiangiogenic, antiviral, anti-inflammatory, antifungal, antihypertensive, and antihyperinc activity (2). Here, we show that they also have an antiplasmodial activity, as well as rather low cytotoxicity, as tested for one cell line so far.

Compound 6 was suggested to be an aryltetralone lignan, since it showed quasi-molecular ions at m/z 355 [M+H]$^+$, which were consistent with the molecular formula C$_{22}$H$_{22}$O$_8$, and its IR, $^1$H, and $^{13}$C NMR spectra were very similar to those of lignan 5 (5). A detailed analysis of $^1$H and $^{13}$C NMR, $^1$H-$^1$H COSY, DEPT, gHMOC, and gHMBC experiments enabled the precise assignment of all hydrogens and carbons in the basic structure of lignan 6 (Table 1). $^1$H-$^1$H COSY and $^1$H selective-irradiation NMR experiments with lignan 6 allowed us to establish the same conformations and relative configuration for the B ring as in lignan 5 (Fig. 2) (5). Therefore, the main difference between lignans 6 and 5 is due to the interchange of substituents on the A and C rings. This deduction was further confirmed by NOESY experiments (Table 1 and Fig. 2). Moreover, the similarity between the CD curves of these lignans allowed us to determine the same absolute configuration for stereocenters on the B ring (5, 6). Thus, the absolute configuration 7'R,8S,8'S was determined for lignan 6.

Based on an analysis of the structure-activity relationships for these lignans, we could infer that the activity was affected by the configurations of the stereocenters on the B ring and by the substituents (methylenedioxy or dimethoxy groups) on the A and C rings. The best activity was achieved for lignan with dimethoxy substituents on the A ring and with the substituents on the B ring (CH$_3$-9 and CH$_3$-9' and veratryl) in a trans-trans orientation (lignan 3).

Aryltetralone lignans from *H. reniformis* showed antiplasmodial activities and low toxicity on hepatic cells, and the three most active lignans (1 to 3) showed IC$_{50}$ values of ≤0.32 µM. Although mixtures of the lignans were at least 10 times less active than lignan 3 and the standard chloroquine, their IC$_{50}$ values were still low, i.e., in the micromolar range. However, a lower antimalarial activity than one would expect for fractions comprising mixtures of these lignans was observed. Whether this reflects an antagonist effect is unclear, and further work must be undertaken to elucidate this finding. These lignans are worthy of further investigation, including chemical transformations, to optimize the activity and to study structure-activity relationships of this class of antimalarial compounds. As toxicity is a very important parameter for a suitable lead candidate in the development of antimalarial drugs, it also has to be further investigated for the active lignans using other cell lines, as well as animal models.

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**REFERENCES**

control, diagnosis, treatment, and a proposed agenda for research and development. 


