Antiplasmodial Activity of Aryltetralone Lignans from Holostylis reniformis

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Abstract

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 parasitaemia *in vivo*. From this extract six lignans, including a new (7'R,8S,8'S)-3',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 IC₅₀ values less than or equal to 0.32 µM. An evaluation of minimum lethal dose (30%) values showed low toxicity for these lignans on a hepatic cell line (Hep G2A16). Therefore, these compounds are potential candidates for the development of antimalarial drugs.

Keywords: Aristolochiaceae; *Holostylis reniformis*; Malaria; Antiplasmodial activity; Cytotoxicity; Lignans; Aryltetralone lignans.
Introduction

Malaria is the most important parasitic disease in the world, responsible for 500 million new cases and 2-3 million deaths every year (27, 28). 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, *P. vivax*, have urged an intensive search for novel blood schizonticides to replace chloroquine, a cheap, safe and, formerly more effective therapeutic antimalarial drug (9, 20, 21). Many natural products with various structural types have shown antiparasitic potency in the laboratory and represent interesting lead structures for the development of new drugs (12). The molecular diversity and efficacy of antiparasitic plants, extracts and herbal preparations have been intensively discussed in recent reviews (22, 26, 29).

More than 60 Brazilian plant species used in traditional medicine to treat malaria and/or fever have been screened by our group, and several species are active against *P. falciparum* in culture and *P. berghei* in mice (1, 13). In the present study, we investigated the antimalarial activity and toxicity of compounds isolated from *Holostylis reniformis* Duch. (Aristolochiaceae), which is used in traditional Brazilian medicine as an antirheumatic, stomachic, and depurative (10). *H. reniformis* is a rich source of aryltetralone lignans (4-6). Several lignans (1-6, Fig. 1) isolated from extracts of this species by chromatography (column, TLC, and HPLC) were then bioassayed *in vitro* for their antiplasmodial activity and toxicity. The structures of lignans 1-5 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 *Holostylis reniformis* Duch. by Dr. Condorcet Aranha and Dr. Lindolpho Cappellari Júnior. A voucher specimen (ESA88282) was deposited at the herbarium of the Escola Superior de Agricultura “Luiz de Queiroz” (ESALQ), Piracicaba, SP, Brazil. The material was separated according to the plant parts, dried (~45 °C), and ground (4-6).

Extraction 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 × 4.8 cm, silica gel 60 H, 151.0 g, hexane-EtOAc gradient: 95:5 to 100% EtOAc) to give 28 fractions (100 mL), as previously described (6). Several of these fractions were subjected to semi-prep. HPLC (MeOH-H₂O 3:2). Fraction 10 was comprised of 1+5+6 (11:3:2) and gave 1 (67.6 mg), 5 (18.4 mg), and 6 (12.3 mg). Fraction 11 gave 1 (25.7 mg) and 5 (22.1 mg).

Fraction 12, comprised of 2+3+4 (3:1:2), was combined to fraction 13, comprised of 3+4 (3:8), and subjected to semi-prep. HPLC (MeOH-H₂O 3:2) to give 2 (255.7 mg), 3 (275.8 mg), and 4 (28.1 mg).

Instrumentation. 1D- (¹H, ¹³C, and DEPT) and 2D- (¹H-¹H gCOSY, gHMQC, gHMBC, and gNOESY) NMR experiments were recorded on a Varian INOVA 500 spectrometer (11.7 T) at 500 MHz (¹H) and 126 MHz (¹³C), with the residual solvent (CHCl₃) used as an internal standard for ¹H (δ 7.23) and CDCl₃ for ¹³C (δ 77.0). Mass spectra (ESI-MS) were obtained on a Fisons Platform II, and flow injection into the electrospray source was used for ESI-MS. IR spectra were obtained on a
Nicole-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. Circular dichroism spectra were recorded on a JASCO J-720 spectrometer. HPLC analyses were carried out using a Shimadzu liquid chromatograph 10Avp equipped with a UV-vis detector. Columns were RP18 (Shimadzu, C18, 3.9 × 150 mm for analytical analysis and 250 × 20 mm for semi-preparative analysis), and chromatograms were acquired at 254 nm. Melting points were recorded on a Microquimica MQAPF-301 melting point apparatus and were uncorrected.

(7'R,8S,8'S)-4,5-Dimethoxy-3',4'-methylenedioxy-2,7'-cyclolignan-7-one [(−)-8'-epi-aristotetralone, 6] was obtained as a yellow solid, m.p. 136.2-138.0 °C; [α]25D −57.0 (c 1.18, CHCl3); UV (MeOH) λmax nm (log ε) 239 (3.6), 278 (3.4), 323 (3.2); IR (KBr) υmax 3469, 3022, 2959, 2924, 2862, 1667 cm−1; for 1H and 13C NMR see Table 1; ESI-MS (+35 eV) m/z 355 [M+H]+ (100); CD (MeOH, c 0.1) θ212 +34650, θ227 +1155, θ238 +12177, θ247 0, θ254 −5709, θ265 0, θ275 +5181, θ280 0, θ291 −17061, θ303 −11616, θ307 −11616, θ315 −11880; anal. C 71.2 %, H 6.2%, calcd for C21H22O5, C 71.2%, H 6.3%.

**Antimalarial tests in vivo.** The antimalarial tests were performed in Swiss albino adult mice (body weight 20 ± 2 g) and their use approved by the Ethical Committee for Using Animals (CEUA-P0094-01, Fundação Instituto Oswaldo Cruz, FIOCRUZ). The animals received water and food ad libitum. The antimalarial suppressive test was performed, as previously described (3), in mice infected with *P. berghei*, strain NK-65, originally received from New York University Medical School. Each mouse (five mice per group) received 10⁵ infected red blood cells (day zero), followed by daily
treatment, via gavage, during four consecutive days. The extracts were suspended in Tween-20 (2% final concentration) immediately before use, and then diluted so that doses of 100 up to 500 mg/Kg were delivered in 0.2 mL per animal. Three control groups were used in each test: one received chloroquine and the others were not treated or treated with Tween-20 (concentration < 0.2% final concentration). Blood smears were taken on days 5 and 7 after parasite inoculation and mortality was monitored for three weeks. The results are expressed as the percent reduction of parasitaemia in relation to non-treated mice, and a compound was considered active when this reduction was ≥ 30\% (3). Each experiment was performed in triplicate and repeated three times.

Parasite culture and in vitro antimalarial tests. The P. falciparum, a chloroquine-resistant isolate (BH\textsc{z} 26/86), used for the in vitro tests was from an imported case of malaria from the Amazon region (3). Parasites were maintained in continuous culture on human erythrocytes (blood group AB+ or A+ using RPMI medium supplemented with 10% of human serum), as previously described (25). The antiparasitic effects of extracts, purified compounds (1-6) and fractions, were measured by the percent growth inhibition of parasite 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 up to 20 \( \mu \text{M} \) for purified compounds and fractions, and 0.2 up to 50 \( \mu \text{g/ml} \) for extracts). The cultures, with trophozoites in sorbitol-synchronized blood (14) at 1-2\% parasitaemia and 2.5\% hematocrit, were then incubated with extracts, fractions or isolated compounds for a total of 72 h at 37 \(^\circ\text{C}\). A positive
control with chloroquine (reference antimalarial drug) and a control with medium and the Tween-20 solution were used in each experiment. The 50% and 90% inhibitory concentrations (IC\textsubscript{50} and IC\textsubscript{90}, respectively), compared to the drug-free control responses, were estimated by linear interpolation (11). Each experiment was performed in triplicate and repeated three times. The blood smears were read in a double-blind manner.

**Cytotoxicity test.** In vitro culture of Hep G2 A16 hepatic cells (8) was mixed with William's E. culture medium in 96-well microtiter plates, and incubated at 37 °C in an enriched CO\textsubscript{2} environment for 24 hours (17). The compounds were diluted with 0.02% final concentration of Tween-20 solution in culture medium to obtain six concentrations: 500, 250, 100, 50, 10, and 5 µg/ml. After incubation periods of 24 and 48 hours, the culture medium was replaced with 200 µl fresh medium with or without the drugs. At the end of the incubation periods, 20 µl of MTT solution (5mg of Thiazoly blue salt in RPMI 1640) without phenol red was added to each well and incubated for 3 more hours. The supernatant was then removed, and 200 µl of acidified isopropanol was added to the wells. The culture plates were read by spectrophotometer with a filter of 570 ηm and a background of 630 ηm. The minimum lethal dose that killed 30% of the cells (MLD 30%) was determined (17). The assays were performed in three independent experiments.

**Statistical analysis.** The average parasitaemias in vivo were compared using ANOVA and Student t tests. Differences between IC\textsubscript{50} values were evaluated by the Mann-Whitney U test performed with Biostat 1.0 MCT-CNPq. \( P \leq 0.05 \) was considered to be statistically significant.
RESULTS

The crude hexane, acetone, and ethanol extracts of the roots, stems, and leaves of *H. reniformis* partially reduced malaria parasitaemia 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 parasitaemia, respectively, at doses of 500 mg/Kg (day 5, *P* ≤ 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 activity, and they exhibited the lowest IC$_{50}$ values (~0.70 µg/ml), whereas the positive control (chloroquine) showed IC$_{50} = 0.09$ µg/ml.

All of the isolated lignans were tested for antiplasmodial 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 1-3 exhibited IC$_{50}$ values ≤ 0.32 µM (≤ 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 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 in the same experimental conditions at the maximal dose tested (140.0 µM = 50µg/mL). Mixtures of these lignans, which were not previously subjected to semi-preparative HPLC (3+4, 2+3+4, and 1+5+6), also showed some activity and exhibited significant IC$_{50}$ (from 1.9 up to 6.0 µM) and IC$_{90}$ (from 8.4 up to 18.2 µM) values (Table 2).
The cytotoxicity of the active lignans evaluated in vitro were considered low, since the mean MLD 30% (450 µg/ml) was at least five thousand times higher than the mean IC₅₀ value obtained for them.

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

**DISCUSSION**

Lignoids with different structural types (up to 60) have been previously isolated from the Aristolochiaceae family (4-7, 16, 19). The biosynthesis, function, 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 cytotoxic, and their antiangiogenic, antiviral, antileishmanial, antifungal, hypolipidemic, and antirheumatic activities (2). Here we show that they also have an antiplasmodial activity, as well as a 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 C₂₁H₂₂O₅, and its IR, ¹H and ¹³C NMR spectra were very similar to those of lignan 5 (5). A detailed analysis of ¹H and ¹³C NMR, ¹H-¹H COSY, DEPT, gHMOC, and gHMBC experiments enabled the precise assignment of all hydrogens and carbons in the basic structure of lignan 6 (Table 1). ¹H-¹H COSY and ¹H selective irradiation NMR experiments of 6 allowed us to establish the same conformations and relative
configuration for the B ring as in 5 (Fig. 2) (5). Therefore, the main difference between 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, 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 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 (3).

Aryltetralone lignans from *Holostylis reniformis* showed antimalarial activities and low toxicity on hepatic cells, the three most active lignans (1-3) showed IC$_{50}$ values $\leq$ 0.32 $\mu$M. Although mixtures of the lignans are at least 10 times less active than lignan 3 and the standard chloroquine, their IC$_{50}$ values are 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 has to 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 antiplasmodial compounds. As toxicity is a very important parameter for a suitable lead candidate to 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.
ACKNOWLEDGEMENTS

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REFERENCES


Captions for Figures and Tables

FIG. 1. Bioassayed lignans from *H. reniformis*.

FIG. 2. Selected nOe interactions and conformation for lignan 6.

TABLE 1. NMR spectroscopic data (500 MHz, CDCl$_3$) for 5 and 6

Footnote:

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

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

TABLE 2. Inhibitory concentrations (IC$_{50}$ and IC$_{90}$) of lignans, either alone or in mixtures, tested against *Plasmodium falciparum* BHz26/86 isolate

Footnote: $^a$ Values are mean ± standard deviation in triplicate;

$^b$ Antimalarial reference drug.
### TABLE 1. NMR spectroscopic data (500 MHz, CDCl₃) for 5 and 6

<table>
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<th>Position</th>
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<th>NOESY-2D</th>
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<tr>
<td></td>
<td>δ_Cᵃ</td>
<td>δ_H (J in Hz)</td>
<td>δ_Cᵃ</td>
<td>δ_H (J in Hz)</td>
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<tr>
<td>1</td>
<td>127.0</td>
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<td></td>
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<td>148.3</td>
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<td>105.8</td>
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<td>43.0</td>
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<td>2.70 dq (3.5, 7.0)</td>
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OCH$_3$-4'  56.0  3.80 s  
OCH$_2$O  101.6  5.87 s  101.0  5.87 s  

$^a$The $^{13}$C NMR data were assigned with the assistance of DEPT, gHMQC (optimized for 140 Hz), and gHMBC experiments;  
$b$gHMBC correlations (optimized for 7 Hz) are from proton(s) stated to the indicated carbon.
TABLE 2. Inhibitory concentrations (IC<sub>50</sub> and IC<sub>90</sub>) of lignans, either alone or in mixtures, tested against <i>Plasmodium falciparum</i> BH26/86 isolate

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Inhibitory concentrations (µM)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
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<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
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<tr>
<td>(1) (7'R,8S,8'R)-4,5-Dimethoxy-3',4'-methylenedioxy-2,7'-cyclolignan-7-one</td>
<td>0.26 ± 0.08</td>
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<tr>
<td>(2) (7'R,8S,8'R)-3',4,4',5-Tetramethoxy-2,7'-cyclolignan-7-one</td>
<td>0.32 ± 0.11</td>
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<tr>
<td>(3) (7'R,8R,8'S)-3',4,4',5-Tetramethoxy-2,7'-cyclolignan-7-one</td>
<td>0.20 ± 0.09</td>
</tr>
<tr>
<td>(4) (7'R,8S,8'S)-3',4,4',5-Tetramethoxy-2,7'-cyclolignan-7-one</td>
<td>0.63 ± 0.20</td>
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<tr>
<td>(5) (7'R,8S,8'S)-3',4'-Dimethoxy-4,5-methylenedioxy-2,7'-cyclolignan-7-one</td>
<td>8.00 ± 0.65</td>
</tr>
<tr>
<td>(6) (7'R,8S,8'S)-4,5-Dimethoxy-3',4'-methylenedioxy-2,7'-cyclolignan-7-one</td>
<td>&gt; 140.00</td>
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<tr>
<td>Combination of 3+4 (3:8)</td>
<td>2.80 ± 0.34</td>
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<td>Combination of 2+3+4 (3:1:2)</td>
<td>6.00 ± 0.50</td>
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<td>Combination of 1+5+6 (11:3:2)</td>
<td>1.90 ± 0.09</td>
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<tr>
<td>Chloroquine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.19 ± 0.02</td>
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</table>

<sup>a</sup> Values are mean ± standard deviation in triplicate;

<sup>b</sup> Antimalarial reference drug.
FIG. 1. Bioassayed lignans from *H. reniformis*. 
FIG. 2. Selected nOe interactions and conformation for lignan 6.