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Research Article

Antimalarial Activity of Compounds and Mixed Fractions of *Cecropia pachystachya*

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ABSTRACT Extracts from *Cecropia pachystachya* (Cecropiaceae), a medicinal plant used in Brazil, were tested for their antimalarial activity against *Plasmodium falciparum* and/or *Plasmodium berghei* in mice. The ethanol extracts of wood, root, and leaves reduce parasitemia of malaria-infected mice from 35–66% in relation to nontreated control mice. The plant roots extracts, with stronger activity, were further analyzed and provided subfractions also active in vivo from which two compounds were isolated and chemically characterized as β-sitosterol and tormentic acid. Although both compounds were active in mice malaria, only the tormentic acid inhibited *P. falciparum* chloroquine-resistant parasites (W2) growth in cultures, reducing parasitemia dose-responsively (IC_{50} = 11–15 μg/ml). The antimalarial activity of the plant extracts kept refrigerated 7 years after the initial isolation, when tested in parallel with new extracts freshly isolated from plants collected at the same geographical site, was similar, confirming the superiority of the plant roots and the stability of the active extracts. In these experiments, inhibition of parasite growth was measured by hypoxanthine incorporation and by immunoenzymatic assay (ELISA) with monoclonal antibodies against the *P. falciparum* histidine-rich protein (HRP2), expressed by the erythrocytic forms. The plant roots showed the lowest IC_{50} value and displayed the lowest toxicity, thus having the best therapeutic index. *C. pachystachya* species is therefore a good candidate for phytotherapeutic use against malaria. Further studies are ongoing to isolate new active compounds through bioguided fractionation analysis, in an effort to develop a new drug prototype against *P. falciparum* erythrocytic parasites. Drug Dev Res 71:82–91, 2010.

Key words: *Cecropia pachystachya*; tormentic acid; antimalarial activity

INTRODUCTION

Historically, natural products have provided an endless source of medicines. Despite the diversification of drug discovery technology and low funding for natural product-based drug discovery, natural products from plants and other biological sources remain an undiminished source of new pharmaceuticals. It is often noted that 20% of all prescribed drugs originate from plants [Farnsworth and Morris, 1976; Raskin and Ripoll, 2004; Schmidt et al., 2007], suggesting that

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plant-derived drugs constitute a significant segment of natural product-based pharmaceuticals.

Studies have demonstrated the ability of plant secondary metabolites to potentiate the activity of anticancer drugs and/or other phytochemicals [Hemalswarya and Doble, 2006]. Some plant secondary metabolites can overcome multiple drug resistance in tumors [Hemalswarya and Doble, 2006] or in pathogenic bacteria when used in combination with other natural products or antibiotics. In particular, a number of plant extracts and natural products work synergistically with existing antibiotics, restoring antibiotic activity against resistant strains of bacteria such as *Staphylococcus aureus* and *Escherichia coli* [Marquez et al., 2005; De Lima et al., 2006].

Terpenoids have greatly contributed to human health and as antimalarials, among them artemisinin from *Artemisia annua*, currently the best antimalarial [Abdin et al., 2003; Schmidt et al., 2007]. Current technologies for new chemical entities discovered in plants heavily favor constitutive compounds that are present at higher concentrations. Bioguided assays of extracts and assays conducted after the application of chromatographic or partitioning methodologies may improve the chances of detecting minor pharmacologically active components.

The *Cecropia pachystachya* Trec. (Cecropiaceae) tree is 5–15 feet tall, with trunks and segments where ants (*Azteca*) live in metabiosis. This plant originates from tropical zones in the Americas [Oliveira et al., 2003]. In Brazil, it is commonly known as "embaúba" or "imbaúba," deriving from the word "ambaíba," which means "tree with a hole" or "tree that doesn't serve for building," in the old Tupi language.

In traditional medicine, *C. pachystachya* is popularly used as a diuretic, tonic, antihemorrhagic, astringent, antiasthmatic, antitussive, and antiblenorrhagic extract. It serves to treat whooping cough, respiratory infections, cardiorenal and cardiopulmonary disorders, as well as tachycardia, bronchitis, high blood pressure, tuberculosis, wounds, and fever [Consolini et al., 2006]. Several other Brazilian plants used in the Amazon region where malaria is still endemic have been studied [Carvalho et al., 1991] and shown important antimalarial activities, including *Cecropia* sp. [Krettli, 2009], a plant species occurring widely in Brazil. Most importantly, species of the genus *Cecropia* are popularly used to treat fever, a symptom typical of acute malaria (when the infected red blood cells rupture, releasing the parasite, which invades new erythrocytes).

In previous studies we observed that *Cecropia glaziovii* extracts had antimalarial activity in vitro and in vivo models (unpublished results); the plant was chemically characterized by Prof. Dr. Antonio J. Lapa's group (Escola Paulista de Medicina, UNIFESP, SP), who kindly provided the plant extracts and fractions. In the present work, we describe the chemical and pharmacological characteristics of the species *C. pachystachya*, reported in to possess several biological activities: cardiotonic and sedative [Consolini et al., 2006], cardiovascular [Consolini and Migliori, 2005], antioxidant [Velázquez et al., 2003], and angiotensive [Lacaille-Dubois et al., 2001].

EXPERIMENTAL

Experimental Procedures, Plant Material, and Fractionation

Solvents were purchased from Morais de Castro (Recife-PE) and used after distillation. Thin-layer chromatographic plates (TLC) (aluminum-backed silica gel 60 F_{254} pre-coated 20×20 cm, 0.2-mm layer thickness) were purchased from Merck (Darmstadt, Germany). Chromatographic columns (CC) were performed with silica gel 60 (70–230 mesh) from Merck and Sephadex LH-20 from Pharmacia (Uppsala, Sweden). The TLC plates were developed by irradiation with a UV lamp (λ 254 nm), by immersion in I_2 vapor, followed by staining with anisaldehyde acid solution (50% acetic acid) or Liebermann–Burchard solution or ceric sulfate acid solution (10% sulfuric acid) and then oven-heated to 80°C for 15 min.

Plant Materials

Wood, leaves, and roots of *C. pachystachya* were collected in the city of São Miguel, State of Alagoas, by botanist Dr. Rosangela Lemos in March 2002. A voucher was deposited at the Herbarium of IMA–Alagoas State Environmental Institute, Brazil, under number MAC 14,305. New specimens were collected in March 2009 at the site where the plant was originally collected.

Preparation of Extracts

The plant was divided into five parts: root wood (3.7 kg), root bark (4.05 kg), wood (7 kg), wood bark (5 kg), and leaves (4.7 kg). The parts were oven-dried at 40°C under forced ventilation, ground in a feed mill (Nogueira Itapira, SP, 2-mm mesh), and extracted three consecutive times with 90% ethanol for 72 h in a stainless steel extractor at 27.1°C.

After concentration in a rotary evaporator under low pressure (1 mm Hg), the following ethanol extracts were obtained: root wood (170 g, 4.6%), root bark (125.22 g, 3.0%), wood (200 g, 3.0%), wood bark (94.35 g, 1.9%), and leaves (248.86 g, 5.3%). The residual moisture was removed by freeze drying or by drying in a desiccator. The ethanol extracts were fractionated (Fig. 1) and the fractions subjected to in vivo antimalarial tests, using *Plasmodium berghei* in mice and cultures of the human malaria parasite

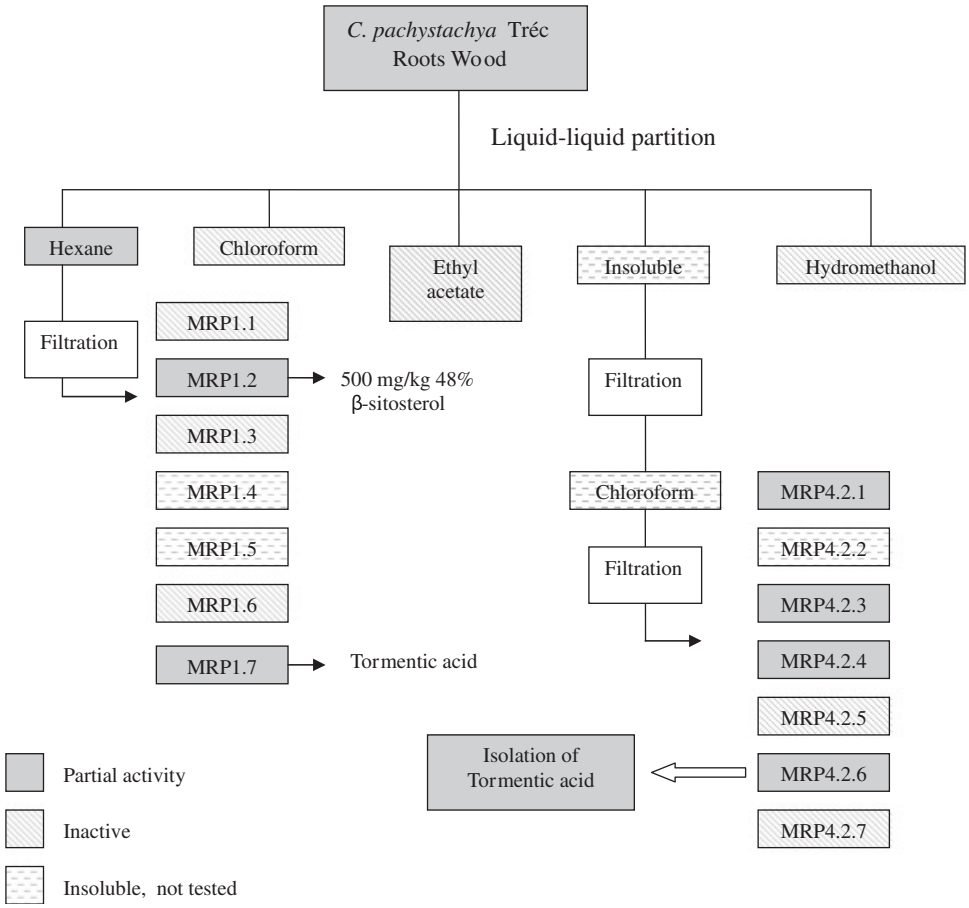

Fig. 1. Phytochemical studies and bioguided characterization of *Cecropia pachystachya* tested in vivo against malaria parasites. Fractions MRP1.2; MPR4.2.1; MRP4.2.2 yielded β-sitosterol.

Plasmodium falciparum (Table 1), as described by Andrade-Neto et al. [2004a] and discussed in depth in a recent review [Krettli et al., 2009].

Fractionation of the Root Extract

After suspension in a methanol-water (2:3) mixture, the ethanol extract of the root wood (160 g) was subjected to liquid–liquid partition, using hexane (4 × 350 ml), chloroform (4 × 350 ml), and ethyl acetate (4 × 350 ml). This procedure yielded five fractions: hexane (MRP1) (9.22 g, 5.8%), chloroform (MRP2) (0.91 g, 0.6%), ethyl acetate (MRP3) (12.47 g, 7.8%), hydromethanol (MRP-5) (36.61 g, 23.0%), and insoluble (MRP-4) (97.15 g, 60.8%). These fractions were also subjected to in vivo antimalarial tests (Table 2, Fig. 1).

The fractionation using a chromatographic column of MRP1 (8.50 g) in silica gel (70–230 mesh) yielded seven subfractions (Fig. 1), which were tested for in vivo antimalarial activity, as listed in Table 3.

The active subfraction MRP1.2 (2.65 g, 30.2%) was subjected to column chromatography using silica gel treated with 10% potassium hydroxide ethanol solution and then activated. One hundred fractions of 10 ml each were collected using hexane, chloroform, methanol, and different mixtures as eluants. After the TLC analysis (15% hexane-chloroform stained with anisaldehyde acid solution), fractions 14–36 eluted with chloroform (1.50 g, 56.6%) yielded the compound called CPMR-1 (1 g, 38%), which

TABLE 1. In Vivo Antimalarial Activity of Ethanol Extracts of *C. pachystachya* Tested in Swiss Albino Mice Infected With *P. berghei**

Plant part	Dose (mg/kg)	Reduction of parasitemia	Results
Wood bark	250	11%	Inactive
	500	0%	
Leaves	250	24%	Borderline[a]
	500	37%	
Wood	250	35%	Active
	500	54%	
Root wood	250	66%	Active
	500	48%	

*A dosage of 15–25 mg/kg (90–95%) of chloroquine was used as a positive control.
[a]Extracts resulting in ≤20% reduction of parasitemia were considered inactive.

was crystallized in ethanol, resulting in an odorless white solid (mp 135°C, ETOH), identified as the steroid β-sitosterol (Fig. 2).

The subfraction MRP1.7 (3.92 g, 42.5%) was subjected to gel chromatography with Sephadex LH-20, using methanol as a single system solvent. Ninety fractions of 10 ml each were collected. Fractions 50–60 (400 mg, 10%) were combined after analysis by TLC (chloroform-methanol 1:1, stained with anisaldehyde acid solution) and, after crystallization in ETOH, yielded the compound CPMR-6 (a white odorless amorphous solid, mp 252°C, ETOH), which was identified as the triterpene tormentic acid by comparison with spectral data available in the literature [Taniguchi et al., 2002].

TABLE 2. Antimalarial Activity of Ethanol Extract Fractions From *C. pachystachya* Roots Tested in a Dosage of 500 mg/kg*

Fraction	Origin of the extract	Reduction of parasitemia	Results
MRP1	Hexane	42%	Active
MRP2	Chloroform	17%	Inactive
MRP3	Ethyl acetate	11%	Inactive
MRP5	Hydroalcoholic	0%	Inactive

*Fractions that reduced parasitemia by ≤20% were considered inactive. Doses of 1–25 mg/kg of chloroquine reduced parasitemia by about 90–95% in the test.

TABLE 3. In Vivo Antimalarial Activity of MRP1 Subfractions From Ethanol Extracts of *C. pachystachya* Roots Tested in a Dosage of 500 mg/kg

Name	Subfractions	Reduction of parasitemia		Conclusion[a]
		Day 5	Day 7	
MRP1.1	Hex/$CHCl_3$ 2.5%	9%	1%	Inactive
MRP1.2	Hex/$CHCl_3$ 10%	48%	49%	Active
MRP1.3	Hex/$CHCl_3$ 1:1	0%	12%	Inactive
MRP1.4	$CHCl_3$	0%	0%	Inactive
MRP1.5	$CHCl_3$/MeOH 5%	62%	55%	Active
MRP1.6	$CHCl_3$/MeOH 10%	0%	0%	Inactive
MRP1.7	$CHCl_3$/MeOH 1:1	55%	32%	Active

[a]Fractions that reduced parasitemia by ≤20% were considered inactive; fractions reducing parasitemia by 21–30% were partially active; and ≥31% considered active.

Identification of Tormentic Acid

Compound CPMR-6 is a white crystal, mp 252°C (ethanol), HRMS $C_{30}H_{48}O_5$ with an experimental m/z of 511.3394 [$M+Na^+$] and calculated m/z of 511.3382. The IR spectrum showed hydroxyl group signals at 3447 cm^{-1} and carbonyl signals at 1689 cm^{-1}. The 1 H NMR spectra showed signals at δ 5.28 (J = 3.8 Hz) related to an olefinic hydrogen, and signals at δ 3.95 (m) and δ 3.45 (d) (J = 9.5 Hz) assigned to carbinolic hydrogens.

The ^{13}C NMR spectra and DEPT showed 8 quaternary, 7 methynic, and 8 methylene carbons and 7 methyl groups, as well as a signal for a carbonyl group at δ 181.36 ppm, followed by signals at δ 138.08 (quaternary carbon) and δ 128.35 (methynic carbon). The signals at δ 79.11 and δ 66.17 for methynic carbons were associated with the hydrogens at δ 3.95 and δ 3.45, respectively. The spectral analyses allowed for the identification of CPMR-6 as tormentic acid. Tormentic acid is a six-member pentacyclic triterpenoid belonging to the ursane family (Fig. 2), that has been isolated from plants of other families (Table 4), e.g., Cecropiaceae, Combretaceae, Labiatae, Lamiaceae, Rosaceae, and Tiliaceae. Tormentic acid has been described as having the following activities: antileishmanial [Santos et al., 2004], anti-inflammatory [Jung et al., 2005; Murakami et al., 2002; Wang et al., 2004], antitumor [Gao et al., 2003], fungicidal [Hirai et al., 2000], antinociceptive [Jung et al., 2005], and cytotoxic [Taniguchi el al., 2002]. It inhibits DNA polymerase [Chen et al., 2003]. Its antimalarial activity is described herein for the first time.

Antimalarial Tests Against *P. falciparum*

The extracts and purified plant compounds were tested alone and in combination for antimalarial activity

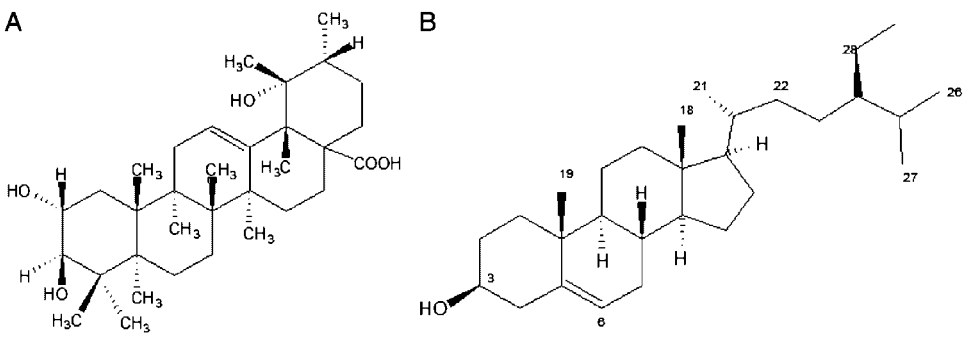

Fig. 2. Tormentic acid (**A**) and β-sitosterol (**B**) compounds.

TABLE 4. Families and Species of Plants Containing Triterpene Tormentic Acid, Some With Reported Biological Activities in the Literature

Family/species	Part of the plant	Biological activity	Reference
Cecropiaceae			
Myrianthus serratus	Stem wood	Not determined	Lontsi et al., 1998
Combretaceae			
Terminalia argentea	Leaves/Trunk bark	Not determined	Garcez et al., 2003
Labiatae			
Perilla frutescens	Leaves	Anti-inflammatory	Wang et al., 2004
Moraceae			
Pourouma guianensis	Leaves	Anti-*Leishmania*	Santos et al., 2004
Rosaceae			
Cotoneaster simonsii	Stem wood	Not determined	Palme et al., 1996
Eriobotrya japonica	Leaves	Cytotoxic	Taniguchi et al., 2002
Margyricarpus setosus	Stem wood	Anti-HIV	DeTommasi et al., 1998
Rubus sieboldii	Fresh leaves	Anti-inflammatory	Murakami et al., 2002

against *P. falciparum*, a human malaria parasite that was maintained in continuous cultures, as described by Trager and Jensen [1976]. The inhibition of parasitic growth was evaluated using different methodologies [Krettli et al., 2009], as described below.

Continuous Cultures of *P. falciparum* and Antimalarial Tests

P. falciparum W2 clone, which is chloroquine-resistant and mefloquine-sensitive [Andrade-Neto et al., 2004a,b], was kept in a continuous culture at 37°C on human erythrocytes using the candle jar method. The antimalarial effect of the extracts was measured: (1) by the traditional test, counting parasitemia in coded blood smears under the microscope, as described by Carvalho et al. [1991]; and (2) by the hypoxathine [^{3}H]-incorporation assay [Desjardins et al., 1979] with slight modifications [Zalis et al., 1998]. In both tests, the parasites were kept in complete culture medium (RPMI 1640 containing 10% human sera, 2% glutamine, and 7.5% $NaHCO_3$) on culture dishes whose culture medium was changed daily. All experiments were performed in duplicate; the extracts were tested in triplicate at each dose. For the [^{3}H]-hypoxanthine incorporation assay, the cultures were kept for 4 days or longer in medium without hypoxanthine; the ring-stage parasites were concentrated in sorbitol-synchronized blood [Lambros and Vanderberg, 1979]. A suspension of red blood cells with 2–3% parasitemia was distributed in a 96-well microtiter plate (180 µl/well) for the traditional test and with 1.0% for the hypoxanthine test, with 1–2% hematocrit. The methods used to evaluate parasite growth were traditional microscopy, hypoxanthine incorporation, and the ELISA immunoenzymatic anti-HRP2 test, as summarized below.

Traditional Microscopy Test

The extracts and fractions were tested at various concentrations, as specified in the results, using the stock solution of each extract (10 mg/ml) previously diluted in DMSO 2% (v/v). Controls without extracts or with chloroquine, a reference antimalarial, were run in parallel in each test. For the traditional method, the culture medium with or without extract was replaced after 24 and 48 h of incubation. After 72 h in culture, blood smears were prepared, coded, stained with Giemsa, and examined (× 1,000). The percentage reduction of parasitemia was calculated in relation to control cultures (average of six) grown in complete medium without extract.

Hypoxanthine Test

For the hypoxanthine test, extracts were diluted in an aqueous solution of DMSO 2% (v/v), then in medium without hypoxanthine, followed by incubation with the parasites for 24 h, after which 20 µl/well of medium containing [^{3}H]-hypoxanthine (0.5 µCi/well) were added to each well, followed by incubation for 18 h at 37°C [Andrade-Neto et al., 2004a]. The plates were frozen and thawed and the cells harvested [Tomtec 96-Harvester (Tomtec, Handem, CT)] on glass-fiber filters (Wallac Ou, Turku, Finland), which were placed in sample bags (Wallac) and immersed in scintillation fluid (Optiphase super mix, Wallac). Radioactivity was counted in a 1450 Microbeta reader (Wallac). The inhibition of parasite growth was evaluated from the [^{3}H]-hypoxanthine incorporation levels, which were plotted to generate dose/response curves. The half-maximal inhibitory response (IC_{50}) compared with the drug-free controls was estimated by using curve-fitting software (Microcal Origin Software 8.0).

ELISA-HRP2

The HRP2 test was performed as previously described [Noedl et al., 2002]. Briefly, cultures of *P. falciparum* (1.5% hematocrit; 0.05% parasitemia) were placed in 96-well plates with the test extracts and control antimalarial at different concentrations, and

were incubated for 72 h under the same culture conditions as described above. After 24-h incubation, the content of six wells (controls in medium and no extract) were harvested and frozen in microtubes, as to allow subtracting the average value obtained from these wells from the other wells to exclude the background value (production of HRP2 during the first 24 h of incubation). After a total of 72 h incubation, the plates were frozen and thawed twice for total erythrocyte lyses and 100 µl/well of the material was placed in another plate for the ELISA test. This plate was pre-coated overnight at 4°C with 1 µg/ml of the primary antibody anti-HRP2 (MPFM-55A ICLLAB®), after which the content was discarded, replaced by the blocking solution (PBS/BSA 2% 200 µl/well), incubated for 2 h, and the content discarded. The hemolyzed cultures were transferred to the ELISA pre-coated plate, incubated (1 h, room temperature), discarded, incubated for 1 h with 0.05 µg/ml of the secondary antibody (MPFG55P-ICLLAB; 100 µl/well), then incubated with 100 µl /well of TMB chromogen (15 min at room temperature) in the dark. The reaction was stopped with 50 µl/L of 1 M sulfuric acid and the absorbance read at 450 nm in a spectrophotometer (SpectraMax340PC^{384}, Molecular Devices).

In Vivo Antimalarial Tests Against *P. berghei*

The suppressive test in mice infected with malaria [Peters, 1965] was used with a few modifications [Carvalho et al., 1991]. The decision to test the samples directly in mice rather than first in vitro was due to the high insolubility of most of them, which required using DMSO (at a concentration unsuitable for in vitro tests), as well as gum arabic. Briefly, adult Swiss outbred mice weighing 20 ± 2 g were inoculated ip with 1×10^5 red blood cells infected with *P. berghei* NK65 strain, a chloroquine-sensitive parasite. The infected animals were divided randomly into groups of 3–5 per cage, and treated daily p.o for 4 consecutive days with the dose specified in the Results section. The extract samples dissolved in DMSO 0.2% (v/v) were diluted with saline to the desired final volume, administering 200 µl to each mouse. Two control groups were used, one treated with chloroquine (≤ 15 mg/kg) and one not treated or treated with saline. On days 5 and 7 after parasite inoculation, blood smears were prepared from the mouse tail, methanol-fixed, stained with Giemsa, and examined microscopically by counting parasitemia in 1,000–6,000 erythrocytes. Overall mortality was monitored daily until day 30 after infection. Inhibition of parasite growth in the drug-treated groups was calculated in relation to the untreated control group. All extracts and combinations were tested in parallel in two or three independent experiments.

Cytotoxicity Tests With a Human Hepatoma Cell Line

The hepatoma cells Hep G2A16 were stored at 37°C, 5% CO_2 in 25-cm^2 sterile culture flasks (Nunc®) with RPMI culture medium (Sigma) supplemented with 5% FBS, penicillin (10 U/ml), and streptomycin (100 g/ml), with changes of medium twice a week. The cells were maintained in weekly passages (at 1:3 dilutions in sterile culture flasks) and grown to confluence [Varotti et al., 2008]. They were used for experiments after being trypsinized (0.05% trypsin/0.5 mM EDTA) and plated on Lab-Tek slides [Calvo-Calle et al., 1994]. When confluent, the monolayers were trypsinized, washed, counted, diluted in complete medium, distributed in 96-well microtiter plates (40×10^3 cells/well), then incubated for another 18 h at 37°C. The extracts and control drugs were diluted to a final concentration of 0.02% Tween-20 or DMSO in culture medium to yield six extract concentrations obtained in serial dilutions starting at 1,000 µg/ml. After a period of 24 h incubation at 37°C, 20 µl of MTT solution (5 mg/ml in RPMI 1640 without phenol red) were added to each well, followed by another 3 h incubation at 37°C. The supernatant was then removed, and 200 µl of acidified isopropanol were added to each well. The culture plates were read in a spectrophotometer with a 570-nm filter and a background of 630 nm. The minimum lethal dose was determined as described [Madureira et al., 2002] with slight modifications. Each test was performed in duplicate; the doses that killed 50% of the cells (MLD 50%) were considered toxic. The selective index of antimalarial activity was then calculated based on the in vitro activity against *P. falciparum* and the MLD 50% as described [Sá et al., 2009].

RESULTS AND DISCUSSION

Terpenoids, also known as isoprenoids, are perhaps the most diverse family of natural products synthesized by plants, serving a range of important physiological and social functions. More than 40,000 different terpenoids have been isolated from plants, animals, and microbial species [Rohdich et al., 2005; Withers and Keasling, 2007]. Plant terpenoids are classified as primary metabolites necessary for cell function and maintenance, or secondary metabolites, that are not involved in development and growth. Secondary metabolic terpenoids are often commercially attractive because of their uses as flavor and color enhancers, agricultural chemicals and medicines [Roberts, 2007]. Very few of these have been investigated from a functional standpoint (Table 4). To some extent, this is a legacy of the once widely held

belief that all natural products are metabolic wastes. However, starting in the 1970s, some terpenes were demonstrated to be toxins, repellants, or attractants to other organisms, raising the hypothesis of their ecological roles in antagonistic or mutualistic interactions among organisms [Langenheim, 1994].

Aiming to find new antimalarial drugs based on ethnopharmacology, we selected the species *C. pachystachya* to conduct chemical studies and evaluate its activity against blood malaria parasites, first in mice infected with *P. berghei*. Two of the five tested extracts and fractions of *C. pachystachya* were active: the wood and wood roots, causing 35–54% and 48–66% inhibition of parasitemia, respectively, on days 5 and 7 after parasite inoculation in comparison with untreated control mice (Table 1).

The fractions from the root ethanol extract obtained after partitioning by liquid-liquid chromatography were then evaluated as antimalarials (Table 2). Only the hexane fraction was active, reducing parasitemia by 42% (500 mg/kg). It was then subjected to a chromatographic process that resulted in seven subfractions (Fig. 1), also tested in vivo (Table 3). The subfractions MRP1.2 (Hex/$CHCl_3$ 10%), MRP1.5 ($CHCl_3$/MeOH 5%), and MRP1.7 ($CHCl_3$/MeOH 1:1) actively suppressed parasitemia by 48%, 62%, and 55%, respectively, at day 5 of inoculation.

After the chromatographic process, subfractions MRP1.2 and MRP1.7 led to the isolation, respectively, of β-sitosterol and tormentic acid (Fig. 2A,B), which were tested in vivo for antimalarial activity against *P. berghei*. Both subfractions showed significant activity at a dose of 15 mg/kg, reducing parasitemia by up to 58% on day 8 after parasite inoculation (Table 5). Chloroquine tested in parallel inhibited 100% and 76% at doses of 15 and 10 mg/kg, respectively.

Tormentic acid tested in serial dilutions by the method of hypoxanthine incorporation in two independent experiments, in parallel with chloroquine, allowed for the determination of the IC_{50} values (Fig. 3). The activities measured by the IC_{50} values were further confirmed in the traditional test, when tormentic acid and β-sitosterol were tested in parallel; β-sitosterol showed only borderline activity (Table 6). Next, tormentic acid was tested alone (Fig. 4A) and in association with β-sitosterol, in a molar ratio of 1:1 (Fig. 4B). The activity of the mixture was reduced by half (IC_{50} = 23.13 μg/ml) when compared with that of tormentic acid alone (IC_{50} = 15.1 μg/ml). The low activity of β-sitosterol alone or combined with tormentic acid was surprising, since both compounds showed similar activity in vivo, a result that requires further clarification.

In another series of experiments, summarized in Table 7, the plant extracts kept frozen for several years were studied in parallel with freshly prepared plant extracts, against *P. falciparum* blood cultured parasites. In this case, in addition to the hypoxanthine test, an immunoenzymatic ELISA-HRP2 was also performed in parallel, as described by Noedl et al. [2002]. The production of histidine-rich protein II (HRP2) by *P. falciparum* parasites is believed to be closely associated with the parasite proliferation, and allows evaluation of the inhibition of parasite growth as a measure of drug susceptibility.

TABLE 5. Antimalarial Activity of Tormentic Acid and β-Sitosterol Tested Against *P. berghei* in Mice, Measured by Percentage Inhibition of Parasitemia in Relation to Untreated Infected Mice on Day 8 After Malaria Infection*

Dose (mg/kg)	Tormentic acid	β-Sitosterol	Chloroquine
15	58%	51%	100%
10	35%	20%	76%

*Parasitemia reduction ≥20% indicates antimalarial activity; see footnote of Table 3.

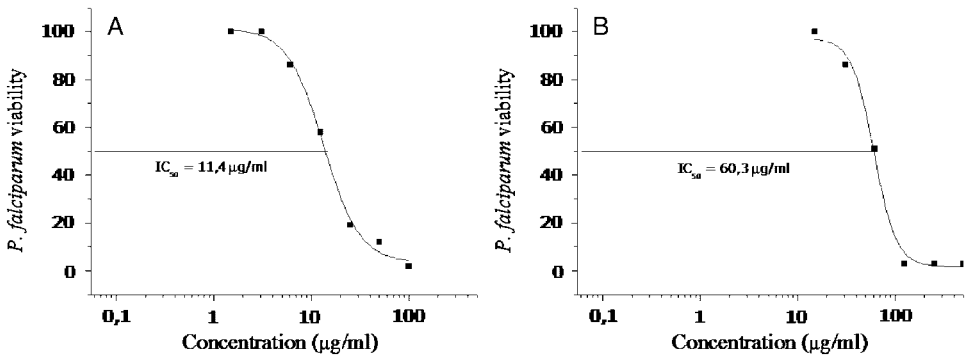

Fig. 3. Dose-response effect of tormentic acid (**A**) and chloroquine (**B**), tested in parallel against the chloroquine-resistant *P. falciparum* strain W2.

TABLE 6. Antimalarial Activities of Tormentic Acid and β-Sitosterol Extracted From *C. pachystachya* Root Wood Fractions, Tested Against *P. falciparum* Strain W2, Evaluated in Parallel With Antimalarial Chloroquine (Doses in μg/ml) by the Traditional Method

	Parasitemia reduction (%)		
Dose (μg/ml)	Tormentic acid	β-Sitosterol[a]	Chloroquine[b]
100	100	71	100
50	100	17	100
25	92	42	100
12.5	81	23	100
6.12	56	46	81
3.06	21	0	58
1.50	0	8	0

[a]The activity of β-sitosterol was considered borderline with no dose response being observed.
[b]Doses of chloroquine in ng/ml tested in parallel with the compounds.

The chemotherapy test to quantify HRP2 protein is presumably easier to establish and perform, and is considered as reliable as the hypoxanthine incorporation assay [Noedl et al., 2002]. In fact, we found the ELISA- HRP2 more practical than either method. The test, which was run in parallel with the hypoxanthine, confirmed the stronger activity of the plant roots wood, although the IC_{50} values in ELISA- HRP2 were slightly lower then those found in the hypoxanthine test. Based on the activity-to-toxicity ratio, it was also observed that, the root wood extracts displayed the highest selective antimalarial activity (>529.1) (Table 7).

Other studies of Brazilian medicinal plants with collaborators in Brazil have enabled us to select candidates for antimalarial drug development: (1) lignans isolated from *Holostylis reniformis* [Andrade-Neto et al.,

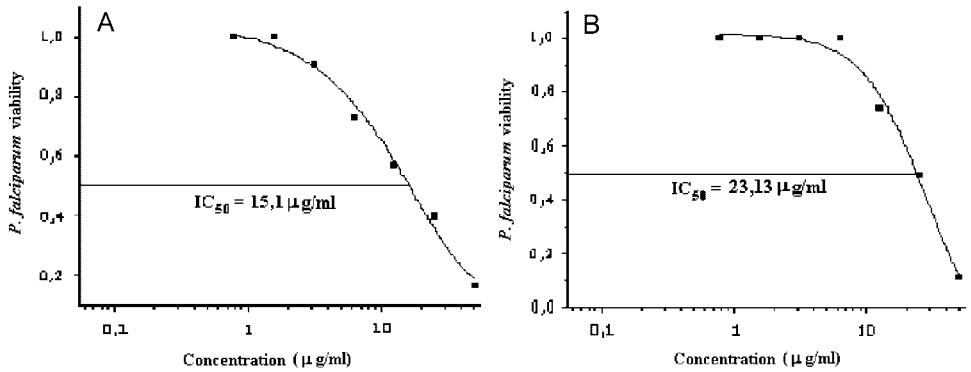

Fig. 4. Dose-response effect of tormentic acid (**A**) and of the mixture tormentic acid and β-sitosterol (**B**).

TABLE 7. Antimalarial Activity of *C. pachystachya* Ethanol Extracts Kept Under Refrigeration (AA) for 7 years, or Freshly Obtained (AN), Measured Against *P. falciparum* (Strain W2) in ELISA-HRP2 or by Hypoxanthine Incorporation, Determined From the Minimal Inhibitory Dose (IC_{50})*

Extract Code	Plant parts used	IC_{50} (μg/ml) ELISA-HRP2	IC_{50} (μg/ml) hypoxanthine	Cytotoxicity (ED_{50}) μg/ml	Selectivity index
AA 1	Wood bark	11.7/5.6	10.8	507	58.1
AA 3	Root bark	8.7	24	348	16.5
AA 5	Root wood	1.9	7.5/2.6	>1,000	>529.1
AA 6	Wood	7.9/4.9	14/24	>1,000	>155.2
AN 10	Root bark	8.5/6.4/4.2	10.6/11.7	414	64.9
AN 12	Wood	4.7/3.9	5.2/6.2	ND	ND
AN 13	Wood bark	24.6	>50	>1,000	>40.5
CQ[a]	(Chloroquine)	0.094	0.062	311	3987

ND, not determined; HRP2, histidine-rich protein expressed by the parasite; AA, old frozen samples (obtained in March 2002); AN = new samples (obtained in March 2009).
*The cytotoxic dose against HepG2 hepatoma cells allowed for the evaluation of the compounds selectivity index. Some tests were performed two or three times, as shown for AA1, AA6, AN10, and AN12.
[a]Chloroquine used as a control antimalarial.

2007]; (2) phenazines derived from β-lapachone and lapachol (a prenyl naphthoquinone isolated from plants of the Bignoniaceae family and structurally related to atovaquone, which is an antimalarial drug used against *P. falciparum*) [Andrade-Neto et al., 2004a; Carvalho et al., 1988]; (3) polyacetylene and flavonoids from *Bidens pilosa* root extract [Brandão et al., 1988; Andrade-Neto et al., 2004b; Oliveira et al., 2004]; and (4) betulinic acid from *Ziziphus joazeiro* Mart. (Rhamnaceae) and its derivatives, which were active against *P. falciparum* chloroquine-resistant parasites and in malaria-infected mice [Sá et al., 2009].

In short, *C. pachystachya*, a plant used against several pathologies, is a potential phytotherapeutic against malaria, since extracts from different parts of the plant were active in vivo against malaria parasites, thus confirming its medicinal properties. Two compounds were isolated from the active extracts, but only tormentic acid extracted from the plant root extracts was active in vivo against a rodent malaria and in cultures of the human malaria parasite *P. falciparum*. This plant therefore represents a potential source of a new prototype compound, confirming ethnopharmacology as an important approach in the search for new antimalarial drugs.

CONCLUSIONS

Several extracts of the medicinal plant *Cecropia pachystachya* showed consistent activity against malaria, in vivo in infected mice and in vitro against the human parasite *P. falciparum*. Tormentic acid isolated from the hexane extract of the roots was active against the W2 strain of *P. falciparum* and in mice with malaria, whereas β-sitosterol tested in parallel showed only a borderline activity. This was surprising because tormentic acid and β-sitosterol were active in mice at concentrations of 15 mg/kg. The use of different methodologies with *P. falciparum* cultures for the antimalarial tests confirmed the stronger activity of the *Cecropia* roots, whose extracts inhibited the parasite's growth. In preliminary studies, we also confirmed the activity of frozen and fresh plant extracts, tested in parallel in the hypoxanthine and ELISA-HRP2 tests, the latter performed with species-specific monoclonal antibodies. The plant's root extracts also displayed low toxicity and hence a good selectivity index, making it a candidate for antimalarial phytotherapy in support of disease control, as well as for further isolation of active products with a view to designing prototypes for new antimalarials.

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