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In Vivo and In Vitro Antiplasmodial Activities of Some Plants Traditionally Used in Guatemala against Malaria

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We present an evaluation of the antiplasmodial and cytotoxic effects of four plants commonly used in Guatemalan folk medicine against malaria. Methanol extracts of *Simarouba glauca* D. C., *Sansevieria guineensis* Willd, *Croton guatemalensis* Lotsy, and *Neurolaena lobata* (L.)R.Br. significantly reduced parasitemias in *Plasmodium berghei*-infected mice. Dichloromethane fractions were screened for their cytotoxicities on *Artemia salina* (brine shrimp) larvae, and 50% inhibitory concentrations were determined for *Plasmodium falciparum* in in vitro cultures. Both chloroquine-susceptible and -resistant strains of *P. falciparum* were significantly inhibited by these extracts. Of all dichloromethane extracts, only the *S. glauca* cortex extract was considered to be toxic to nauplii of *A. salina* in the brine shrimp test.

Malaria is the most important parasitic disease in the world. Control of the main causative agents of malaria, *Plasmodium falciparum* and *Plasmodium vivax*, by use of the classical drugs chloroquine and primaquine has been frustrated by the resistance of the malarial parasites to these drugs (5, 9, 12, 14). Hence, new antimalarial drugs with novel actions are being sought. Medicinal plant research has become more important, especially after the studies of the Chinese antimalarial drug artemisinin, isolated from *Artemissia annua* (19, 26, 30). More recently, a number of studies have been undertaken to evaluate the inhibitory effects of various plant extracts on *P. falciparum* (3, 7, 15, 17, 23, 31) and *Plasmodium berghei* (31) in culture. The in vivo antimalarial properties of several plant extracts have been studied in mice infected with *P. berghei* (6, 28, 34), *Plasmodium vinckei* (17, 23), and *Plasmodium yoelii* (1, 17). Following this trend, this study presents the results obtained from the evaluation of the in vivo and in vitro antiplasmodial activities of four plants most commonly used in Guatemalan folk medicine against malaria: *Simarouba glauca* D. C. (family *Simaroubaceae*), *Sansevieria guineensis* Willd (family *Agavaceae*), *Croton guatemalensis* Lotsy (family *Euphorbiaceae*), and *Neurolaena lobata* (L.)R.Br. (family *Asteraceae*). *S. glauca*, *C. guatemalensis*, and *N. lobata* are indigenous to Guatemala, and *S. guineensis* originates in South Africa (24).

The possible cytotoxic activities of these plants were determined in the brine shrimp (*Artemia salina*) assay. This test was developed by Michael et al. (21) and adapted by others (20, 33). It is a convenient preliminary toxicity test, since the brine shrimp is highly sensitive to a variety of chemical substances. Toxicity to brine shrimp coincides with cytotoxicity to mammalian cells in many cases. However, there is no correlation in the degree of toxicity between the two systems (20, 33).

Quassinoids, a class of chemicals commonly found in members of the family *Simaroubaceae*, are toxic to the brine shrimp (33), strongly antiplasmodial (7), and toxic to mice (4). For this type of compound, toxicity in the brine shrimp test (BST) is often used as a tool for biologically guided fractionation of extracts (33).

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MATERIALS AND METHODS

Plant extracts. The plants used in our study were collected at different places in Guatemala, where they are being used by the local population to prepare febrifuge and antimalarial herbal teas (24), which are taken for 1 week or longer (8). The different specimens were authenticated by Juan José Castillo, botanist from the Herbarium of the Faculty of Agronomy, Universidad de San Carlos de Guatemala. One or two parts of each plant were processed for our studies (Table 1). The leaves of *C. guatemalensis* and *N. lobata* were air dried at room temperature under shade, while the rest of the plant material was dried at 45°C. Considering that people in Guatemala usually use water to prepare their herbal remedies, aqueous infusions of each plant were prepared at a 10% (wt/vol) concentration. The extracts that were obtained were then freeze-dried. Methanol extracts were also prepared by maceration of the plant material with solvent at room temperature for 24 h. The latter extraction was repeated three times. We fractionated the methanol extracts of the plants which had been tested in mice with a mixture of water and dichloromethane (1:1; vol/vol). The organic phase was concentrated by means of a rotary evaporator and was then freeze-dried for use in antiplasmodial and cytotoxicity tests.

Parasites. To test for the antimalarial activities of the aqueous and methanol extracts, we used the mouse-infective, chloroquine-sensitive strain *P. berghei* (donated by E. Rowton, Department of Entomology, Walter Reed Army Institute of Research, Washington, D.C.).

The multidrug-resistant strain *P. falciparum* K1 (donated by D. Warhurst, London School of Hygiene and Tropical Medicine, London, United Kingdom) and the chloroquine-susceptible strain NF54 (donated by W. Eling, Institute for Cytology and Histochemistry, Nijmegen, The Netherlands) were used to test the antiplasmodial activities of the dichloromethane plant extracts in vitro.

Antiplasmodial activities of methanol and aqueous extracts against *P. berghei*. Swiss mice (body weight, 20 to 25 g; bred at Universidad de San Carlos) were inoculated intraperitoneally at 10^5 *P. berghei*-infected erythrocytes per mouse on day 0, resulting in a peak parasitemia on day 7 postinfection in nontreated control mice, as described previously by Eling et al. (10). A standard 7-day in vivo test was used (29). Oral treatment was started on day 0 with freeze-dried aqueous or methanol extracts, dissolved in drinking water, at a dose of 750 mg/kg of body weight and was then continued daily for 7 days. Groups of three to four mice each were used to assay the different plant extracts. Three control groups were also included; the mice in the control groups were treated with chloroquine (20 mg/kg), artemisinin (50 mg/kg), or water. In all cases, a volume of 0.2 ml was administered through a gastric tube. Every assay was repeated two or three times. The aqueous and methanolic extracts were tested in two independent experiments. The effect of each treatment was determined by microscopic examination

TABLE 1. Names and origins of the evaluated plants

Botanical and vernacular name[a]	Part evaluated	Collection location in Guatemala
C. guatemalensis (copalchí)	Leaves and cortex	Escuintla
N. lobata (tres puntas)	Leaves	Sierra de las Minas
S. guineensis (curarina)	Leaves and root	Guatemala City
S. glauca (jocote de mico)	Cortex	Amatitlán

[a] The different specimens were authenticated by agronomy engineer Juan José Castillo, from the Herbarium of the Faculty of Agronomy, Universidad de San Carlos de Guatemala.

of thin blood smears stained with Wright's stain. The blood smears were made on day 7 postinfection.

Cytotoxic activities of dichloromethane extracts. Dichloromethane extracts of all plants were screened for toxicity with larvae (nauplii) of *A. salina* (brine shrimp) as described by Solís et al. (33). The dichloromethane fractions were dissolved in seawater. Water-insoluble extracts were primarily dissolved in dimethyl sulfoxide (DMSO; final concentration, 0.5%). The test was performed in triplicate in microwell plates, with extract concentrations of 1,000, 250, 125, 62.5, and 31.25 μg/ml. *Artemia* eggs were incubated for 48 h in artificial seawater. The nauplii were collected and brought into contact with the test substances. After 24 h of incubation at room temperature in the light, the number of surviving nauplii in each well was determined with a stereomicroscope. As controls, *A. salina* nauplii were submitted to 95% ethanol (100% lethality) and seawater containing 1% DMSO (100% survival). The 50% lethal concentrations (LC_{50}s) of the dichloromethane extracts were determined by Finney's probit analysis. The mean ± standard deviation of the mean LC_{50} was calculated from two independent experiments. Extract dilutions that did not show toxicity at 1 mg/ml were considered nontoxic (2, 20, 21, 33).

Culture system and drug solutions. Sorbitol-synchronized (18) *P. falciparum* K1 and NF54 were cultured in suspension with 2% human type O-positive erythrocytes under a gas atmosphere of 2% O_2, 5% CO_2, and 93% N_2. The culture medium consisted of modified RPMI 1640 (11) supplemented with 10% human type AB-positive serum, 35 μM hypoxanthine, and 50 μg of gentamicin per ml. Stock solutions of drugs were prepared by dissolving freeze-dried dichloromethane plant extracts in methanol at a concentration of 250 mg/ml. The stocks were then stored at −20°C and diluted further in culture medium prior to each experiment.

Evaluation of drug efficacy and determination of IC_{50}s. Synchronized *P. falciparum* parasites were cultured in 6-ml volumes in 25-cm^2 tissue culture flasks (Greiner GMBH, Frickenhausen, Germany) in the presence of various concentrations of plant extract. As controls, parasites were cultured without any drug (100% growth) and with 2 μM chloroquine (100% inhibition). Since all plant extracts were dissolved in methanol, the influence of this solvent on parasite growth was determined in serial dilutions at concentrations ranging from 0.003 to 1.6% (vol/vol). All plant extracts were tested in three independent experiments. The effect of each extract was evaluated by measurement of parasite DNA increase, as described by Smeijsters et al. (32). In brief, synchronized ring-form cultures (2% erythrocytes, of which 1% was infected) were exposed to various concentrations of plant extract for 48 h. Parasites from duplicate 0.85-ml culture samples were liberated by the addition of 0.85 ml of saponin (0.08% in phosphate-buffered saline). After centrifugation (15,800 × *g*, 3 min) the supernatant was discarded and the parasites were lysed by the addition of 50 μl of guanidinium-sodium acetate (pH 5.5). Subsequently, 2 ml of 0.33 μg of Hoechst 33258 per ml–2 M NaCl–50 mM Tris-HCl (pH 7.8) was added. The interfering protein was removed by extraction with 50 μl of chloroform-isoamyl alcohol and brief centrifugation. The parasite DNA was quantified by measuring the Hoechst 33258 fluorescence, and the fluorescence was corrected for background fluorescence by subtraction of the fluorescence value for the chloroquine-treated cultures. The concentration of each plant extract at which the fluorescence equals 50% of the net value for the nontreated control cultures (IC_{50}) was determined graphically by use of the computer programs SlideWrite, version 6.1 (Advanced Graphics Software Inc., Carlsbad, Calif.), and Number Cruncher Statistical System (NCSS, Kaysville, Utah).

RESULTS

Antimalarial effects of aqueous and methanol extracts on *P. berghei*. After 7 days of treatment with aqueous extracts, mean parasitemias in the *P. berghei*-infected mice ranged from 4.4% ± 3.6% to 13.3% ± 4.7%, whereas the mean parasitemia in the control group was 18.1% ± 4.0% (Table 2). Of the aqueous extracts, only the *C. guatemalensis* cortex extract induced significant inhibition of parasitemia compared to that for the control group, as did artemisinin (overall analysis of variance significance, $P = 0.0002$ by Scheffe's procedure at $\alpha = 0.05$). Mean parasitemias in the methanol extract-treated groups ranged from 6.5% ± 3.5% to 9.6% ± 4.7%. In the control group the mean parasitemia was 26.0% ± 7.5% (Table 2). All methanol plant extracts inhibited parasitemias in *P. berghei*-infected mice significantly (overall analysis of variances significance, $P < 0.0001$ by Scheffe's procedure at $\alpha = 0.05$).

TABLE 2. parasitemia after 7 days of treatment with 750 mg/kg per os

Treatment	Mean ± SD parasitemia (%)	
	Aqueous extracts	Methanol extracts
Water (control)	18.1 ± 4.0	26.0 ± 7.5
Artemisinin	3.5 ± 3.6	ND[a]
Chloroquine	ND	0.0
C. guatemalensis leaves	11.2 ± 5.4	9.6 ± 4.7
C. guatemalensis cortex	4.4 ± 3.6	9.3 ± 4.5
S. glauca cortex	9.5 ± 7.1	8.1 ± 5.4
N. lobata leaves	9.8 ± 6.3	6.5 ± 4.0
S. guineensis leaves	13.3 ± 4.7	8.4 ± 4.8
S. guineensis roots	10.0 ± 6.7	6.5 ± 3.5

[a] ND, not done.

Cytotoxic activities of the dichloromethane extracts. On the basis of the more reproducible inhibition with the methanol extracts in mice, we carried out a liquid-liquid extraction of the methanol fractions with dichloromethane. The dichloromethane fractions were screened for cytotoxicity in the BST and for inhibition of *P. falciparum* in in vitro cultures. DMSO at a concentration of 1% (vol/vol), which was used as the solvent for the freeze-dried dichloromethane plant extracts, had no effect on the nauplii of *A. salina*. Ethanol (95%) killed all nauplii upon exposure (data not shown).

Three of six dichloromethane extracts, *N. lobata*, *C. guatemalensis* (leaves), and *C. guatemalensis* (cortex), were nontoxic in the BST up to a concentration of 1,000 μg/ml (Table 3). The leaves of *S. guineensis* showed moderate toxic activity (LC_{50}, 775.3 ± 127.9 μg/ml), while the root of the same plant induced a higher mortality (LC_{50}, 414.0 ± 12.4 μg/ml). The extract of *S. glauca* cortex showed considerable toxicity (LC_{50}, 51.2 ± 13.8 μg/ml).

Antiplasmodial actions of the plant extracts in vitro. Methanol, which was used as the solvent for the freeze-dried dichloromethane plant extracts in the in vitro experiments, had no inhibitory effect on *P. falciparum* at concentrations below 0.8% (vol/vol) (data not shown). The highest concentration of methanol used with the various concentration ranges of the extracts was 0.5% (vol/vol).

With the exception of the *S. glauca* cortex extract, the IC_{50}s of the tested extracts ranged from 15.8 ± 2.6 to 39.5 ± 3.8 μg/ml; the *S. glauca* cortex extract gave much lower IC_{50}s: 0.195 ± 0.04 and 0.184 ± 0.024 μg/ml for *P. falciparum* NF54

TABLE 3. LC_{50}s of the dichloromethane extracts against *A. salina*

Plant extract	LC_{50} (μg/ml)[a]
N. lobata leaves	>1,000
C. guatemalensis leaves	>1,000
C. guatemalensis cortex	>1,000
S. guineensis leaves	775.3 ± 127.9
S. guineensis roots	414.0 ± 12.4
S. glauca cortex	51.2 ± 13.8

[a] Values are means ± standard deviations of the means.

TABLE 4. IC_{50}s of dichloromethane plant extracts for *P. falciparum* NF54 and K1 in culture calculated from three independent experiments

Plant extract	Mean ± SD IC_{50} (μg/ml)	
	NF54	K1
S. glauca cortex	0.195 ± 0.04	0.184 ± 0.024
S. guineensis roots	17.0 ± 2.0	15.8 ± 2.6
S. guineensis leaves	38.5 ± 3.0	39.5 ± 3.8
N. lobata leaves	8.6 ± 1.0	10.6 ± 1.3
C. guatemalensis cortex	27.8 ± 2.2	23.1 ± 2.6
C. guatemalensis leaves	19.8 ± 0.9	22.1 ± 2.1

and K1 strains, respectively (Table 4). There was no significant difference in susceptibility to the tested plant extracts between the multidrug-resistant *P. falciparum* K1 strain and the drug-susceptible NF54 strain (overall analysis of variance significance, $F = 0.01$ and $P = 0.9$).

DISCUSSION

The plants used in our study were collected at different places in Guatemala, where they are being used by the local population to prepare herbal febrifuge and antimalarial remedies. To our knowledge, this is the first report of the biologic activity of *C. guatemalensis* and *S. guineensis* extracts.

We investigated the antimalarial potencies of aqueous and methanol extracts in *P. berghei*-infected mice. Treatment with the aqueous *C. guatemalensis* cortex extract significantly inhibited parasitemia compared to no treatment. Although the suppression of parasitemias in the mice treated with the other aqueous extracts were not significant, due to within-group divergence in peak parasitemias on day 7, they are indicative of antiplasmodial potential. Similarly, François et al. (13) found that their crude aqueous extract of *N. lobata* leaves had no in vitro activity against *P. falciparum* NF54, although they were able to demonstrate the presence of several lipophilic plasmodicidal compounds.

The results obtained with the methanol extracts were more homogeneous, and all treated groups showed a significant reduction in parasitemias compared to those for the control group. On the basis of the more reproducible inhibition with the methanol extracts, we started the biologically guided fractionation by carrying out a liquid-liquid extraction of the methanol fractions with dichloromethane. The dichloromethane fractions were screened for their cytotoxicities against *A. salina* (brine shrimp) larvae and for their abilities to inhibit *P. falciparum* cultures. In vitro, all dichloromethane plant extracts were active against both *P. falciparum* strains, with IC_{50}s well below 50 μg/ml. The *P. falciparum* K1 strain is highly resistant to chloroquine (IC_{50}, 459 ± 74 nM), whereas the NF54 strain is susceptible to chloroquine (IC_{50}, 15.1 ± 3.1 nM) (32). These results are comparable to what others have found with extracts of other, in some cases related, plants (3, 7, 13, 25, 26).

For quassinoids, bitter compounds commonly found in members of the family *Simaroubaceae*, toxicity in the BST is often used as a tool for biologically guided fractionation of extracts (33). Quassinoids were found in *S. glauca* seeds (4, 22). The compounds glaucarubol, glaucarubin, and glaucarubinone obtained from these seeds have been shown to have activity against *P. falciparum* in vitro (IC_{50}s, 0.410, 0.055, and 0.004 μg/ml, respectively). These compounds have also shown cytotoxic activity in vitro against 9 KB cells from human epidermoid carcinoma of the mouth (50% effective doses of 5.5 μg/ml for glaucarubol, 5.1 μg/ml for glaucarubin, and 0.04 μg/ml for glaucarubinone) (25). However, the cortex, which is the plant part used against malaria in Guatemala (8), had not been investigated yet. In our studies, the dichloromethane extract of the *S. glauca* cortex was the most toxic in the BST (LC_{50}, 51.2 μg/ml) and the most potent inhibitor of parasite growth of *P. falciparum* (IC_{50}, 0.195 μg/ml). The *Sansevieria* extracts were moderately toxic to brine shrimp (LC_{50}s, 775 and 414 μg/ml), and the differences in toxicity between leaves and roots in the BST were reflected in the differences in antiplasmodial action in culture (IC_{50}s, 38.5 and 17.0 μg/ml, respectively). It is unknown whether the inhibition by *Simarouba* and *Sansevieria* extracts is caused by specific antiplasmodial action or general cytotoxicity. For *N. lobata* it has been published that the aerial parts contain sesquiterpene lactones (27) and flavonoids (16). On the basis of the cytotoxicity index for mammalian cells, it was assumed that the antiplasmodial effect was not due to the cytotoxicities exhibited by these compounds (13). Our dichloromethane extract of *N. lobata* did not show any toxicity against the brine shrimp. It is interesting that the IC_{50} of the dichloromethane extract of *N. lobata* reported by François et al. (13) is identical to the value that we obtained, even though we prepared the extracts differently.

Our two *C. guatemalensis* extracts were nontoxic to brine shrimp, but their antipasmodial activities (IC_{50}s, 27.8 and 19.8 μg/ml) were similar to those of the *S. guineensis* extracts. In contrast to the nontoxic properties of both of our *C. guatemalensis* extracts, Meyer et al. (20) found rather high toxicities of ethanolic *Croton tiglium* seed extracts (IC_{50}, 30 μg/ml) in the BST. In our mouse experiments, we saw no overt signs of toxicity within the 7-day period that the animals were treated orally.

The differences in the IC_{50}s of our extracts in vitro were not reflected in the effectiveness of the extracts in the mouse model. Possibly, the effectiveness of the extracts is influenced by differences in gastrointestinal uptake, the half-life in plasma, or the metabolism of the active compounds.

In conclusion, we have demonstrated the antimalarial and antiplasmodial effects of methanol and dichloromethane extracts from four commonly used Guatemalan medicinal plants. Efforts will be undertaken to continue the biologically guided fractionation in order to isolate and identify the active compounds, as well as to understand the mechanism of inhibition.

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