In Vivo and In Vitro Antimalarial Activity of 4-Nerolidylcatechol

Luiz Francisco Rocha e Silva,1,2,3 Ana Cristina da Silva Pinto,4,5 Adrian Martin Pohlit,4 Etienne Louis Jacques Quignard,4 Pedro Paulo Ribeiro Vieira,1 Wanderli Pedro Tadei,6 Francisco Célio Maia Chaves,7 Jean Francisco Samonek,7 Carlos Alberto Jatoba Lima,7 Mônica Regina Farias Costa,1 Maria das Graças Costa Alecrim1 and Valter Ferreira de Andrade-Neto8*

1Laboratório da Gerência de Malária, Fundação de Medicina Tropical do Amazonas - FMTAM, 69040-000, Manaus, AM, Brazil
2Pós-graduação em Medicina Tropical, Universidade Estadual do Amazonas - UEA, 69050-030, Manaus, AM, Brazil
3Centro Universitário do Norte - UNINORTE, 69010-060, Manaus, AM, Brazil
4Laboratório de Princípios Ativos da Amazônia - LAPAAM, Coordenação de Pesquisas em Produtos Naturais - CPPN, Instituto Nacional de Pesquisas da Amazônia - INPA, 69060-001, Manaus, AM, Brazil
5Programa de Pós-graduação em Biotecnologia, Universidade Federal do Amazonas - UFAM, 69077-000, Campus Universitário, Manaus, AM, Brazil
6Laboratório de Dengue e Malária, Coordenação de Pesquisas em Ciências da Saúde - CPCS, Instituto Nacional de Pesquisas da Amazônia - INPA, 69060-003, Manaus, AM, Brazil
7Embrapa Amazonia Ocidental, 69010-970, Manaus, AM, Brazil
8Laboratório de Biologia da Malária e Toxoplasmose - LABMAT, Departamento de Microbiologia e Parasitologia, Universidade Federal do Rio Grande do Norte, UFRN, 59072-970, Natal, RN, Brazil

4-Nerolidylcatechol (4-NC) isolated from Piper peltatum L. (Piperaceae) was evaluated for in vitro antiplasmodial activity against Plasmodium falciparum (cultures of both standard CQR (K1) and CQS (3D7) strains and two Amazonian field isolates) and for in vivo antimalarial activity using the Plasmodium berghei-murine model. 4-NC exhibits significant in vitro and moderate in vivo antiplasmodial activity. 4-NC administered orally and subcutaneously at doses of 200, 400 and 600 mg/kg/day suppressed the growth of P. berghei by up to 63% after 4% daily treatments (days 1–4). Also, 4-NC exhibited important in vitro antiplasmodial activity against both standard and field P. falciparum strains in which 50% inhibition of parasite growth (IC50) was produced at concentrations of 0.05–2.11 μg/mL and depended upon the parasite strain. Interestingly, healthy (non-infected) mice that received 4-NC orally presented (denatured) blood plasma which exhibited significant in vitro activity against P. falciparum. This is evidence that mouse metabolism allows 4-NC or active metabolites to enter the blood. Further chemical and pharmacological studies are necessary to confirm the potential of 4-NC as a new antimalarial prototype. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: Plasmodium falciparum; Plasmodium berghei; antimalarial drug; 4-nerolidylcatechol; Piper peltatum; Piperaceae.

INTRODUCTION

Malaria is the most important parasitic disease in the world. It represents a major problem for public health services in tropical and sub-tropical regions of the planet. Half of the world’s population is at risk of contracting malaria and an estimated 243 million cases led to nearly 863,000 deaths in 2008 (WHO, 2009). Besides the distant perspective of the development of an effective vaccine, the rise and spread of populations of parasites which are resistant to traditionally employed antimalarial drugs represents a great challenge to control policies in countries where malaria is endemic (Menegon et al., 2008). On other hand, the use of ineffective antimalariais is considered to be partly responsible for the difficulties in reducing malaria morbidity and mortality. Given the current therapeutic scenario, identification of new lead substances exhibiting antimalarial activity is imperative, especially for the treatment of multi-drug resistant Plasmodium strains.

Root infusions of Piper peltatum L. and Piper umbellatum L. [syn. Pothisomorphe peltata (L.) Miq. and Pothosemorpe umbellata (L.) Miq.] are used in traditional medicine in the Amazon region and other parts of South America for the treatment of malaria (Mil liken, 1997). Both species are similar in terms of habitat and occur in humid, partially shaded areas where they receive abundant indirect and some direct sunlight, such as at forest edges, trails, roadsides and typically where anthropogenic alteration of forest has occurred. Both plants are morphologically very similar, the most ostensible difference being the position of attachment of the petiole to the leaf, as well as leaf shapes. In Brazil, these species are also known by the same indigenous names ‘caapeba’ and ‘pariparoba’ (Yuncker, 1973) and chemical studies have demonstrated that both plants produce the secondary metabolite 4-nerolidylcatechol (Kijjoo et al., 1980; Mongelli et al., 1999).

Early studies on the antimalarial activity of P. peltatum and P. umbellatum extracts produced...
contradictory results in assays using the rodent malaria parasite *Plasmodium berghei in vivo*. In what is apparently the first published study on the antimalarial activity of these *Piper* spp., Amorim et al. (1986) evaluated ethanol extracts of *P. umbellatum* leaves and *P. peltatum* whole plants in the 4 day suppressive test at doses of 500, 100 and 20 mg/kg (orally administered) in *P. berghei*-infected Swiss rats and found that *P. peltatum* extract was inactive and *P. umbellatum* extract presented moderate activity (parasitemia reduced by 66%, 55% and 28%, respectively). In a follow-up study, Amorim et al. (1988) administered ethanol extracts of leaves of *P. umbellatum* and of *P. peltatum* (250 and 1250 mg/kg orally; 100 and 500 mg/kg subcutaneously) to *P. berghei*-infected rats. *P. umbellatum* exhibited antimalarial activity (significant inhibition of parasite growth) while *P. peltatum* extract, in contrast, was inactive in rats treated both orally (500 mg/kg) and subcutaneously (highest dose: 500 mg/kg). Later, Adami (1995) tested hexane and methanol extracts of leaves of *P. peltatum* and of *P. umbellatum* in vivo with oral and subcutaneous administration in *P. berghei*-infected mice and concluded that the leaf extracts were inactive against blood stages of *P. berghei*. Later, this same group of researchers firmly rejected intraperitoneal injection in *P. berghei*-infected Swiss mice as a means of detecting antimalarial activity in *Piper peltatum* and *P. umbellatum* extracts (Ferreira-da-Cruz et al., 2000).

In the study discussed above, Adami (1995) also performed *in vitro* tests using *P. falciparum* strains and found that methanol extracts of the leaves of *Piper peltatum* and *P. umbellatum* exhibited greater parasite inhibition than hexane extracts of the leaves. Further support for *in vitro* antimalarial activity of *Piper peltatum* and *P. umbellatum* extracts was provided by Aïnèhouna et al. (2004) who demonstrated that methanol extracts of the leaves of *P. umbellatum* were active (IC₅₀ = 3.74 μg/mL) against chloroquine and pyrimethamine-resistant *P. falciparum* strains. In summary, these earlier studies showed that polar extracts of *P. peltatum* and *P. umbellatum* inhibit *P. falciparum* by *in vitro* but that *in vivo* suppression of *P. berghei* by extracts of these plants was absent or only moderate.

Using an alternative method, Sala-Neto et al. (1992) detected antimalarial activity in *P. peltatum* extracts. Initially, these authors confirmed that water extracts of different parts of *P. peltatum* were inactive towards blood stages of *P. berghei* in infected rats using the standard procedure. Then, *P. peltatum* water extracts of leaves, roots and stems were administered to healthy (non-parasitized) adult rats using a gastric tube in six doses of 6 mL each for 2 days. 30 min after administration of the last dose, the rats were bled, their blood centrifuged and the resulting blood plasma was added to *in vitro* cultures of *P. falciparum*. After 48 h, *in vitro* growth of *P. falciparum* was evaluated using the method of incorporation of [¹⁴C]-hypoxanthine. Blood plasma from healthy rats which had ingested meloquine (100 mg/kg) and water were used as positive and negative controls, respectively. Interestingly, blood plasma from healthy rats which had ingested water extracts of different parts of *P. peltatum* inhibited *P. falciparum* by 49% versus negative controls.

The work of these authors provides evidence that rat metabolism of *P. peltatum* extracts produces metabolites in blood plasma within 30 min after oral ingestion. Furthermore, these metabolites (presumably present in the plasma of *P. berghei*-infected rats which ingest *P. peltatum* extracts) do not exhibit appreciable inhibition of the growth of *P. berghei in vivo* but do inhibit *P. falciparum in vitro*. Thus, while the *in vivo* murine-*Plasmodium berghei* and *in vitro* *P. falciparum* culture models are firmly established in antimalarial drug discovery (Mons and Sinden, 1990) as powerful tools for predicting the protective value of new drugs (Peters and Robinson, 2000) differences in pharmacokinetics are frequently observed in *in vivo* and *in vitro* models where different *Plasmodium* species are used (Mons and Sinden, 1990). Presumably, pharmacokinetic differences between the *in vivo* *P. berghei* and *in vitro* *P. falciparum* models would explain differences in the apparent activity of blood plasma metabolites described by Sala-Neto et al. (1992) and the consistently different results of early *in vitro versus in vivo* studies on *P. peltatum* and *P. umbellata* extracts.

In a preliminary study, a major secondary metabolite found in *Piper peltatum* and *P. umbellatum* root extracts, 4-norolidylecatechol (4-NC), exhibited moderate *in vitro* inhibition (IC₅₀ = 9 μg/mL) of *P. falciparum* multidrug-resistant parasites (W2 - Indochina) (Pinto, 2002). Recently, *in vitro* antimalarial activity (IC₅₀ = 0.21 μg/mL) of 4-NC was confirmed against chloroquine-resistant K-1 strain (Andrade-Neto et al., 2007) providing compelling evidence that this antimalarial principle is responsible, at least in part, for the observed *in vitro* growth inhibition of *P. falciparum* exhibited by extracts of *P. peltatum* and *P. umbellatum* discussed above.

Given that 4-NC is present in *P. peltatum* and *P. umbellatum* polar extracts and that this substance actively inhibits *P. falciparum in vitro* (Andrade-Neto et al., 2007; Pinto et al., 2010), in the present study the *in vivo* antimalarial activity of 4-NC in *Plasmodium berghei*-infected mice and the effect that administration of 4-NC to healthy mice had on the antimalarial activity of the blood plasma of these rodents towards *P. falciparum in vitro* (sensu Sala-Neto et al., 1992) was studied. The *in vitro* antimalarial activity of 4-NC in stable cultures of field isolates of *P. falciparum* from Amazonas State, Brazil was also investigated as a means to evaluate the spectrum of activity of this substance and the susceptibility of parasites of clinical relevance to this novel class of natural product-based antimalarials (Pinto et al., 2009).

**MATERIALS AND METHODS**

**Plant material, extraction and chemical constituent isolation.** Plants (Fig. 1) were collected in the State of Amazonas between 2001 and 2006 and a voucher sample (INPA no. 210168) was deposited at the INPA Herbarium. Dried, ground *P. peltatum* roots (150 g) were extracted in an ultrasound bath in a mixture of CHCl₃: EtOH (1:1). Evaporation of solvents and freeze-drying yielded a dry extract (19.5 g) which was chromatographed on silica gel (0.50 = 0.0 cm; h = 38 cm) using CHCl₃: EtOH (9:1) as eluent. The final yield of 4-NC (Fig. 2) was 8.6 g (44.1% based on mass of dry extract, 5.7% based on mass of dried, ground roots).
Continuous culture of *Plasmodium falciparum*. Strains used in this study were the antimalarial drug-susceptible 3D7 clone of the NF54 isolate (unknown origin) and the chloroquine-resistant, pyrimethamine-resistant and cycloguanil-resistant K1 strain (Thailand). Strains were acquired from MR4 (Malaria Research and Reference Reagent Resource Center, Manassas, VA, USA). Additionally, two *P. falciparum* field isolates (M1 and M2; representative genotypes from the Brazilian Amazon region) were obtained from symptomatic malaria patients who presented themselves at the Tropical Medicine Foundation, in the city of Manaus, Brazilian Amazon, in search of diagnosis and treatment. Whole blood samples were processed as described elsewhere (Vieira *et al.*, 2004) and infected erythrocytes were directly added to complete RPMI medium and put in plastic flasks for *in vitro* culture (without cryopreservation) under low oxygen atmosphere. Patients presenting clinical symptoms related to non-severe malaria were invited to be enrolled in this study after confirmation of *P. falciparum* mono-infection by thick smear diagnosis and signing of an informed consent form (FMTAM Ethics in Research Commission - CEP no. 1838). Parasites were maintained in continuous culture using the method of Trager and Jensen (1976) at 5% hematocrit using type A + human erythrocytes in RPMI 1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine (Gibco), 25 mM HEPES (Sigma-Aldrich), 40 μg/mL gentamycin, 10% A + human plasma (donated by blood banks), 25 mM NaHCO₃. Cultures were maintained under an environment of 5% O₂, 5% CO₂ and 90% N₂ and incubated at 37°C. When cultures attained a parasitemia of 4–5% they were synchronized with 5% sorbitol (Lambros and Vanderberg, 1979).

**Test for in vitro inhibition of *P. falciparum* parasites.** This test was performed using the method of Rieckmann *et al.* (1978) with modifications which were described in Andrade-Neto *et al.* (2007). 4-NC was dissolved in DMSO at a stock concentration of 1.0 mg/mL. Seven dilutions were performed in culture medium (RPMI 1640) and represented final test concentrations of 50–3.2 × 10⁻³ μg/mL (in five dilutions). Each diluted sample was tested in duplicate in 96-well test plates containing a suspension of parasitized red blood cells at a hematocrit of 3% and initial parasitemia of 1% of synchronized young trophozoites (ring forms). The final concentration of DMSO in control wells was <1%. The final volume of each well was 200 μL. Reference antimalarial compounds chloroquine and quinine were used as positive controls at concentrations recommended by WHO (2001). The test plate was incubated for 48 h at 37°C under the same low oxygen gas mixture used for parasite culture. After the incubation period, thin smears were prepared from the contents of each well and evaluated using a microscope. The half-maximal inhibitory (IC₅₀) responses compared with the drug-free controls were estimated by interpolation using Microcal Origin® software.

**Animals and ethical approval.** Adult Webster Swiss albino mice (28 ± 2 g weight) were used for the antimalarial and toxicity tests and received water and food ad libitum. *In vivo* tests were performed using Guidelines for Ethical Conduct in The Care and Use of Animals of Federal University of Rio Grande do Norte (CEUA 043/2010).

**Test for in vivo suppression of *Plasmodium berghei*.** *In vivo* antimalarial activity was evaluated using *Plasmodium berghei* NK65 strain (drug-sensitive) from the Parasitology Department of the Universidade Federal do Rio Grande do Norte located in the city of Natal, State of Rio Grande do Norte, Brazil. This strain was maintained by successive passages of blood forms from mouse to mouse. The test protocol is based on the 4-day suppressive test as described by Peters (1965). Female Webster Swiss mice weighing 28 ± 2 g were used in this study. Animals were infected intraperitoneally...
with 0.2 mL of infected blood suspension containing $1 \times 10^5$ parasitized erythrocytes and randomly divided into groups of five individuals. Test groups were treated orally, subcutaneously (doses 200, 400, and 600 mg 4-NC/kg/day) and intraperitoneally (dose 200 mg 4-NC/kg/day). Positive control groups received a dose of 5 mg chloroquine/kg/day orally, subcutaneously or intraperitoneally, and negative control groups received 0.2 mL of Tween 2% Tween-20 or saline. The animals were treated for 4 days starting 24 h after inoculation with *P. berghei*. On days 5 and 7 after parasite infection, blood smears were prepared from all mice, fixed with methanol, stained with Giemsa, then microscopically examined (1000× magnification). Parasitemia was determined in coded blood smears by randomly counting 2000–4000 erythrocytes in the case of low parasitemias (≤10%); or up to 1000 erythrocytes in the case of higher parasitemias. Overall mortality was monitored daily in all groups during a period of 4 weeks following inoculation. The difference between the average parasitemia of control groups (100%) and test groups was calculated as a percentage of parasite growth suppression (PGS) according to the equation: $\text{PGS} = 100 \times (A - B)/A$, where $A$ is the average parasitemia of the negative control group and $B$ corresponds to the parasitemia of the test group.

**In vitro activity of mouse plasma against Plasmodium falciparum.** Three groups of five healthy mice were treated orally with 200 mg of 4-NC/kg (in 0.2 mL 2% Tween-20), 5 mg chloroquine per kg (in 0.2 mL 2% Tween-20) and 0.2 mL of 2% Tween-20, respectively. A 100 μL sample of blood was removed from each anaesthetized animal (under light diethyl ether anesthesia) from the eye orbital plexus with a Pasteur pipette 60 and 120 min after treatment began. Blood was centrifuged and after separation, the plasma was removed and deactivated for 30 min at 56°C. This plasma from animals which had previously ingested 4-NC was tested in vitro in cultures of *P. falciparum* in a procedure analogous to that described above. In this procedure, undiluted plasma and 1:10, 1:20 and 1:40 dilutions of plasma were tested. Undiluted plasma (data not shown) provided results which were similar to those for the 1:10 dilution. Plasma from animals which had received vehicle or chloroquine was also tested (negative and positive controls, respectively). The inhibition potential in the test wells containing plasma from animals treated with 4-NC was calculated by comparison of their parasitemias with those of control wells containing blood plasma from animals which had ingested only vehicle.

**Statistical analysis.** Average parasitemia was compared using ANOVA and Student’s $t$-tests. The Kruskal–Wallis test for survival analysis. Differences between IC_{50} values were calculated using the Mann-Whitney $U$ test performed with Biostat 1.0 MCT-CNPq. A value of $p \leq 0.05$ was considered to be statistically significant.

### RESULTS

**In vivo antimalarial activity**

4-NC was tested using the 4-day Peters’ suppression test against a drug-susceptible strain of *P. berghei*. All doses tested exhibited significant *in vivo* chemosuppression of parasitaemia in both routes of administration of sample (Table 1). Orally, at a daily dose of 200 mg/kg of body weight, 4-NC presented low suppression on day 5 (14.5%) and more expressive suppression on day 7 (54%). At a dose of 400 mg/kg/day, orally administered 4-NC exhibited 34.4% and 48.8% parasitaemia suppression on days 5 and 7, respectively. Significant chemosuppressive effects were observed on days 5 and 7 (63.1% and 59.7%) by oral doses of 600 mg 4-NC/kg/day. In contrast, 4-NC administered subcutaneously provided effective chemosuppression of parasites only on day 7 (40.6% up to 61.3%). Mortality was not significantly reduced by treatment of infected mice with doses of 200 and 400 4-NC mg/kg/day (oral and subcutaneous routes) compared with untreated infected mice (Table 1). However, the mouse survival time was significantly increased in groups treated with 4-NC 600 mg/kg/day by the oral and subcutaneous treatment routes used ($p \leq 0.05$).

Intraperitoneal administration of 200 mg 4-NC/kg/day for 4 consecutive days to *Plasmodium berghei*-infected mice led to the deaths of mice on day 3 after infection (60% mortality). Surviving mice presented day 5 and day 7 parasite suppressions, respectively, of

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>% Parasite inhibition</th>
<th>Average survival time±SD (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oral Day 5 Day 7</td>
<td>Subcutaneous Day 5 Day 7</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>14.5 54</td>
<td>0 41</td>
</tr>
<tr>
<td>400</td>
<td>34.4 48.8</td>
<td>0 40.6</td>
</tr>
<tr>
<td>600</td>
<td>63.1 59.7</td>
<td>0 61.3</td>
</tr>
</tbody>
</table>

SD, standard deviation.

*The significance of mortality by the Kruskal–Wallis test was $p \leq 0.05$.

Negativ control groups received 0.2 mL of Tween-20 2% or saline.

Positive control used received 5 mg chloroquine/kg/day (parasite inhibition: 91–97%). Average mouse survival was 32±3.0 days ($\rho = 0.007$).
24.4% and 63.3%. The antimalarial drug chloroquine was used in non-curative doses to mice but still caused a significant reduction in parasitaemia (91%–97%) and mortality in all experiments.

**In vitro antimalarial activity**

4-NC exhibited significant *in vitro* inhibitory activity towards both field isolates of *Plasmodium falciparum* from the Brazilian Amazon. *In vitro* inhibition data for 4-NC against standard and isolated *P. falciparum* from the Amazon region are presented in Table 2. IC$_{50}$ values varied from 0.05 to 2.11 μg/mL depending upon the *P. falciparum* strain used. 4-NC exhibited the greatest inhibition of locally obtained M1 strain and the lowest inhibition of the chloroquine-sensitive 3D7 strain. The IC$_{50}$ value of 4-NC in the multi-drug resistant K1 strain was consistent with previous results (Andrade-Neto et al., 2007).

The *in vitro* antimalarial activity of the blood plasma of healthy mice which ingested 4-NC 1–2 h prior to sampling was significant against standard strains and isolated field cultures of *P. falciparum* (Table 3). In preliminary tests, blood plasma removed 60 min after oral administration of 4-NC (200 mg/kg) and diluted by a factor of 1:10 suppressed the growth of blood forms of *P. falciparum* by 58.6–86.3%. At a dilution of 1:40, no suppression of parasite growth was observed by plasma removed 60 min after oral administration in any of the parasite strains assayed. There was a reduction in the suppression of parasite growth caused by mouse plasma removed 120 min after ingestion (compared with that removed after 60 min). At a dilution of 1:10, the plasma removed after 120 min presented growth suppression of 18.6–58.5%. Plasma removed after 120 min at a dilution of 1:40 exhibited no suppressive activity in standard and field isolated strains of *P. falciparum*. The blood plasma of healthy mice which received chloroquine had low *in vitro* activity towards the *P. falciparum* strains studied.

**DISCUSSION**

As presented in the introduction, previous studies on *Piper peltatum* and *P. umbellatum* extracts revealed consistent *in vitro* activity towards *Plasmodium falciparum*. Thus, screenings of water, water-alcohol, hexane and ethanol extracts for *in vitro* antimalarial activity in blood stages of *P. falciparum* revealed extracts exhibiting IC$_{50}$ as low as 3.7 μg/mL (Atindehou et al., 2004). There is ample evidence in the literature on *P. peltatum* and *P. umbellatum* (Ropke et al., 2003, 2005; Noriega et al., 2005) which is reinforced by our own experience in the laboratory (with *P. peltatum*) that extracts prepared with solvents of differing polarity contain significant and variable amounts of the secondary metabolite 4-NC (Pinto et al., 2010) which previous studies have shown to be an *in vitro* inhibitor of chloroquine-resistant K1 *P. falciparum* (Andrade-Neto et al., 2007).

There are often significant genetic differences between standardized laboratory strains and freshly isolated field strains of *P. falciparum* due to genetic polymorphism and resistance related to epidemiological specificities of parasite populations in circulation in the Western Brazilian Basin. The present study evaluated the *in vitro* susceptibility of recently isolated, continuously cultured parasites from the region near Manaus to 4-NC. The IC$_{50}$ of 4-NC towards the K1 strain is reproducible and in agreement with data obtained previously (Andrade-Neto et al., 2007). *In vitro* IC$_{50}$ values for 4-NC are evidence that this compound actively inhibits field isolates of *P. falciparum*. Considering the current state of affairs in antimalarial treatment and the urgency for finding new antimalarial lead compounds, evaluation of potential antimalarial drug candidates in field isolated parasite is very important since resistance of *P. falciparum* to almost all currently used antimalarials has been observed although the geographical distributions and rates of spread of drug resistance have varied considerably in different regions of the world (Vestergaard and Ringwald, 2007).

Amorim et al. (1986, 1988) demonstrated that ethanol extracts of *Piper peltatum* and *P. umbellatum* have only slight *in vivo* suppressive activity on blood forms of the rodent malaria parasite *P. berghei* (reduction of

---

**Table 2. In vitro median inhibition concentrations (IC$_{50}$ ± SD) of 4-NC against standard and Amazonian isolates of *Plasmodium falciparum***

<table>
<thead>
<tr>
<th>Strain/Field isolate</th>
<th>IC$_{50}$ ± SD (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>0.60 ± 0.41</td>
</tr>
<tr>
<td>3D7</td>
<td>2.11 ± 1.15</td>
</tr>
<tr>
<td>M1</td>
<td>0.05 ± 0.06</td>
</tr>
<tr>
<td>M2</td>
<td>0.83 ± 0.58</td>
</tr>
</tbody>
</table>

For K1 and 3D7 strains, respectively, IC$_{50}$ of chloroquine was 0.2 ± 0.1 and 0.05 ± 0.02 μg/mL; IC$_{50}$ of quinine was 0.04 ± 0.02 and = 0.06 ± 0.02 μg/mL.

**Table 3. In vitro inhibition by mouse blood plasma (withdrawn 60 and 120 min after oral ingestion of a single dose of 200 mg 4-NC/kg) on blood forms of *Plasmodium falciparum***

<table>
<thead>
<tr>
<th>Plasma dilution</th>
<th>% Inhibition by plasma 60 min</th>
<th>% Inhibition by plasma 120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain/Field isolate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K1</td>
<td>3D7</td>
</tr>
<tr>
<td>1:10</td>
<td>86.3</td>
<td>73.3</td>
</tr>
<tr>
<td>1:20</td>
<td>16.3</td>
<td>0</td>
</tr>
<tr>
<td>1:40</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

parasitemia by 66% at relatively high concentration). Other studies, such as those of Adami (1995) and Ferreira-da-Cruz et al. (2000), reported no activity for orally and subcutaneously administered ethanol and hexane extracts from these same plants. All of these studies are consistent with the notion that these extracts have little or no significant in vivo activity towards P. berghei and that differences in the results obtained can best be explained by variation in the composition of these extracts as suggested by Ferreira-da-Cruz et al. (2000) or perhaps by pharmacokinetic factors as presented in the introductory discussion of the work of the Sala-Neto, et al. (1992).

In the present study, the in vivo suppressive action of 4-NC on Plasmodium berghei parasitemia was evaluated. In this test, parasitemias were evaluated on days 5 and 7 after oral administration on days 1-4. On day 5, suppression was negligible while on day 7 it was more expressive. The observed in vivo suppression of rodent parasite growth by orally administered 4-NC is similar to that reported previously and discussed above for extracts of Piper peltatum and P. umbellatum. The in vivo activity of 4-NC only on day 7 by the subcutaneous route may be the result of slow uptake and a delayed effect of this drug; being as effective as the oral treatment on day 7. A hypothesis is that first-pass metabolism in the liver may be important and when 4-NC is administered subcutaneously this does not occur or results in inadequate blood plasma concentrations, or both. For example, in the pharmacokinetics of artemisinin and derivatives variability in blood plasma drug concentrations, high oral clearance, low to moderate apparent volume of distribution and short half-lives are observed (Nagelschmitz et al., 2008; White et al., 2008).

4-NC injected intraperitoneally in P. berghei-infected mice proved to have significant acute toxic effects. This may be the result of immunosuppressive (Pereira et al., 1999; Andrade-Neto et al., 2004) or immunomodulatory (Nergard et al., 2005) activities which have been observed for plant extracts in vivo or isolated compounds. It has also been shown that results can vary greatly depending on the route of drug administration (Ferreira-da-Cruz et al., 2000). However, no acute toxic effects were mentioned in previous reports involving oral, intraperitoneal or subcutaneous injection of Piper peltatum and P. umbellatum extracts. Little toxicological data have been published for Piper peltatum and/or P. umbellatum extracts or 4-NC. In previous work, 4-NC was shown to exhibit cytotoxic potential in several tumor cell lines (Pinto et al., 2009). Also, extracts and fractions of P. umbellatum reportedly have in vivo anticancer activity suggestive of the participation of different compounds with distinct mechanisms of action (Sacoman et al., 2008). Ropke et al. (2005) demonstrated that 4-NC inhibited the activity of the metalloproteinases MMP-2 and MMP-9 and suggested the use of this plant for attenuation of solar UVB light-induced skin carcinogenesis. In another study, extracts of Piper umbellata and 4-NC were shown not to exhibit mutagenic effects on mouse bone marrow cells. Furthermore, a protective effect was observed against genotoxicity induced by cyclophosphamide which suggests potential clinical applications (Valadares et al., 2007).

While in vitro evaluation of antimalarial activity presents the advantage of safety and in general uses the human malaria parasite P. falciparum, this method provides little information regarding pharmacokinetic factors such as absorption, distribution and biotransformation. In vitro tests do not include host factors and the correlation between results of in vitro and in vivo tests is not well understood (Vestergaard and Ringwald, 2007). Methods of evaluation of the antimalarial activity of plant derivatives which rely on the in vivo treatment of rodents infected by P. berghei provide insights into the pharmacokinetics and immunological factors, but use non-human malaria parasites (Peters, 1965).

Together with the above standard approaches, a synthetic or combined approach was used for the evaluation of antimalarial activity which is able to provide some pharmacokinetic information and has the additional advantage of using the human malaria parasite P. falciparum. To our knowledge this method was introduced by Sala-Neto et al. (1992), attracted little attention, and was never followed up with a more complete investigation of the potential of this method despite the original promise shown for investigating the antimalarial activity of plant extracts. Sala-Neto et al. (1992) demonstrated that blood plasma of healthy rodents which had ingested P. peltatum extracts inhibited P. falciparum growth in vitro by 49% at a dilution of 1:25.

In vitro susceptibility of standard strains and field isolates to 4-NC was demonstrated while low, variable in vivo activity was observed for this substance in the NK65 strain of P. berghei. Very interestingly, plasma from healthy, non-infected rodents which had ingested 4-NC presented significant in vitro inhibitory activity in all of the P. falciparum strains studied which varied from 83.3% (standard strain K1) to 58.6% (field isolate M1) at dilutions of 1:10 (plasma removed 1 h after treatment of animals). At this same dilution, plasma removed from animals 2 h after initial ingestion of 4-NC inhibited up to 58.5% of the in vitro growth of P. falciparum. Interestingly, the blood plasma of healthy animals which received chloroquine exhibited low in vitro activity against P. falciparum strains. This may be related to the fact that a sub-dose of chloroquine is used in the bioassays and in the single dose administered to healthy animals whose plasma was assayed. The distribution of this drug may be relatively slow. Chloroquine exhibits complex pharmacokinetics such that plasma levels of the drug soon after administration are determined by the rate of distribution and not by the rate of elimination. Because of extensive association with tissues an attack dose is necessary to obtain effective blood plasma concentrations and a sub-dose and a single treatment for the blood plasma assay were used. Another very likely reason for the low activity of blood plasma from healthy rodents which had received chloroquine is that the field and standard parasite strains used with the unique exception of 3D7 all exhibit resistance to chloroquine.

Taken together, our experimental observations are evidence that 4-NC: (1) administered intraperitoneally does not significantly inhibit P. berghei in vivo and is acutely toxic to infected mice, (2) administered orally and subcutaneously significantly inhibits P. berghei in vivo (3) significantly inhibits P. falciparum in vitro and (4) administered orally can give rise to sufficient concentration of active metabolite in mouse blood plasma to significantly inhibit P. falciparum growth.
in vitro. These data are consistent with an important role for 4-NC and perhaps a metabolite of 4-NC as antimalarial principle and/or pro-drug in *Piper peltatum* and *P. umbellatum* extracts similar, perhaps, to the pro-drug proguanil which is activated in *vivo* to its main antimalarial metabolite cycloguanil (Birkett et al., 1994; Yeo et al., 1994; Funck-Brentano et al., 1997). Additionally, the plasma concentration–time profiles of 4-NC and elimination half-life profile will need to be studied in future work.

In conclusion, although 4-NC isolated from *Piper peltatum* is less active or at best comparable to known antimalarials such as quinine or chloroquine in *vivo* and less active than chloroquine in *vivo*, the data reported here provide some rational evidence to support the use of extracts of this plant for the treatment of malaria in traditional medicine of Brazilian Amazon region and others parts of South America. Traditionally, infusions of extracts of this plant for the treatment of malaria in *P. umbellatum* and antimalarial metabolite cycloguanil (Birkett et al., 1990). Hence, it is possible that cycloguanil may be a useful drug proguanil which is activated in future work. The plasma concentration–time profiles of 4-NC (Pinto et al., 2010) lending more support to the traditional medicinal assertions of the usefulness of *Piper peltatum* and *P. umbellatum*. Investigations are now planned to determine the chemical identity of antimalarial principles, presumably 4-NC and/or its metabolites, present in rodent blood after ingestion of 4-NC, using hyphenated liquid chromatography and mass spectrometric techniques. Considering the consistent results obtained in this preliminary study, 4-NC (and derivatives) may represent an important class of new antimalarial compounds for further drug development (Pinto et al., 2009).

**Acknowledgements**

We thank FAPEAM and CNPq for fellowships to the authors (LFRS, ACSP, AMP, WPT, PPRV and VFAN); staff from LAPAAM and INPA Herbarium for sharing information about the medicinal use and identification for the plant material collected in the Amazon region. This project was sponsored by grants PPG-7 563892/2005-6 and 557106/2005-2 PNPQ 520354/1999-0 and 550260/2001-3), CNPq 561588-2005-3, CNPq 555669-2009-2 (Brazilian Malaria Network), Bioamazonia-Basa-Fepad Contract and FAPEAM (PIPT 6/2003, CBA-UFAM 1577/2005 and PINFRA 1928/2005).

**Conflict of Interest**

The authors have no conflicts of interest concerning the work reported in this paper.

**REFERENCES**


Ferreira-da-Cruz MF, Adami YL, Espinola-Mendes EC. 2000. The intraperitoneal *Plasmodium berghei*-Pastore infection of Swiss mice is not a system that is able to detect the antiplasmodial activity in the *Pothomorphe* plant extracts that are used as antimalarials in Brazilian endemic areas. *Exp Parasitol* 94: 243–247.


Peters W. 1965. Drug resistance in *Plasmodium berghei*—Pastore infection of Swiss mice is not a system that is able to detect the antiplasmodial activity in the *Pothomorphe* plant extracts that are used as antimalarials in Brazilian endemic areas. *Exp Parasitol* 94: 243–247.


