TRABALHO DE CONCLUSÃO DE CURSO DE GRADUAÇÃO
Curso de Ciências Biológicas

Nicolli Bellotti de Souza

Juiz de Fora
2009
The antimalarial activity of *Zanthoxylum chiloperone* methanolic extract in vivo in *Plasmodium berghei* infected mice

**ABSTRACT**

Malaria is one of the most important diseases nowadays. Therefore, much effort has been done to obtain new drugs from different sources and plants have shown promising results in several reports. Some plant species belonging to the *Zanthoxylum* genus are widely used as antimalarials or antipyretics in traditional medicine. A decoction of *Z. chiloperone* root bark is used traditionally in Paraguay for its antimalaric, emmenagogue and antirheumatic properties. Besides, because of its also known antiparasitic properties, we decided to assess the bioactivity of methanolic extracts of *Zanthoxylum chiloperone* on *Plasmodium berghei*-infected mice. It was tested *in vivo* at the doses of 50mg/Kg, 100mg/Kg and 200mg/Kg using the 4-day suppressive test. The inhibition of parasite multiplication (ipm) was almost 40% for both 50mg/Kg and 200mg/Kg treated groups and almost 52% for 100mg/Kg. Besides, mice survived for a few days longer than the untreated group. Cytotoxicity in mice peritoneal macrophage was 7.27% for 50mg/Kg, 1.82% for 100mg/Kg and 0% for 200mg/Kg. These results may be explored for the study of new antimalarial drugs, since the inhibition for 100mg/Kg dose was higher than 50%.

*Keywords:* antimalarial activity; *Plasmodium berghei*; *Zanthoxylum chiloperone*
1. Introduction

Human malaria is caused by species of Plasmodium: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Malaria, besides being the main cause of high morbidity and economic loss in the world nowadays (Alecrim et al., 2007), attacking 41% of world population (Witkowski et al., 2009). It takes almost one million lives per year and afflicts as many as half a billion people in 109 countries in Africa, Asia and Latin America (WHO, 2009). The control of the disease is difficult due to the lack of an effective vaccine (Alecrim et al., 2007) and the emergence of the parasite resistance to drugs, specially *Plasmodium falciparum* and also *P. vivax* (Cao et al., 2007; Fidock et al., 2008; Anstey et al., 2009; Witkowski et al., 2009), and of their vectors to insecticides (Bourdy et al., 2008). Because of this, the research for new antimalarial drugs has been emphasized (Fidock et al., 2008) and plants consist in a very promising and low-cost strategy – mainly in the case of malaria, when the new drug discovery must be affordable in all ways because of many limitations (Njommang and Benoit-Vical, 2007; Pagnoni, 2009) – also being promoted by the World Health Organization (WHO), which recognizes that the centuries-old use of certain plants as therapeutic resources should be taken into account of their efficacy (Chhabra et al., 2007; Gertsch, 2009).

It is estimated that 80% of the world’s population depends on herbal remedies for treatment of diseases, mainly because the pharmaceutical medicines are not available and either because people can not afford it (Dolabela et al., 2008). The accumulated knowledge of the malaria endemic regions inhabitants about the curing capacity of plants (Alecrim et al., 2007) ends up providing natural products, as was the case of not only two of the most important currently available drugs to treat malaria, quinine and artemisinin (Dolabela et al., 2008; Pillay et al., 2008) but also 30 drugs
originated from plants from the 250 deline as essential by the WHO (Bourdy et al., 2008). In addition, natural products and derivates represent more than 30% of the current pharmaceutical market, consisting in a big source of drugs (Newman et al., 2003; Njomnang et al., 2007; Boyom et al., 2009). Therefore, it should be safe to say that ethnopharmacological approaches could provide new antimalarial agents (Phillipson and Wright, 1991).

In many tropical and sub-tropical countries, plants have traditionally been used to treat fever and other symptoms associated with malaria (Noster and Kraus, 1990; Bickii et al., 2000), the single most important condition treated with herbal remedies (Chhabra et al., 2007). Indeed, in malaria endemic areas, plant remedies are still widely used without assurance of their efficacy, what can be proved by clinical trials in order to validate the traditionally used plants. Knowledge of active compounds of a medicinal plant is important for development of standardized preparations for pre-clinical and clinical assays (Dolabela et al., 2008) so as to provide scientific understanding (Chhabra et al., 2007), what has encouraged the researchers to screen plant extracts as well as their naturally occurring compounds (Koumaglo et al., 1991; Gessler et al., 1994; Munõz et al., 2000).

*Zanthoxylum* is the largest genus in the family Rutaceae and comprises about 200 species of trees and shrubs, distributed worldwide, but predominantly found in tropical and temperate regions (Ross et al., 2004) and in Brazil it is found in all regions, including the Amazon forest (Silva et al., 2007).

*Zanthoxylum* species are reported to have many medicinal properties and phytochemical studies on the genus have shown it to be a rich source of coumarins, lignans, and alkaloids, with febrifuge, sudorific, and diuretic properties (Talapatra et al., 1973; Ross et al., 2004; Ross et al., 2005; Diehl et al., 2000). This genus embraces
species used for different medical purposes, and some attention has been given to the
toxic effects of its different species. The absence of clinical toxicity is reported by
several studies from which it is possible to confirm several potential therapeutical uses
of plants from the genus *Zanthoxylum* (Silva et al., 2007). Some plant species belonging
to the *Zanthoxylum* genus (*Z. usambarense*, *Z. tsihanimposa*, *Z. rhoifolium*, *Z. rubescens*
and *Z. chiloperone*) are widely used as antimalariais or antipyretics in traditional
medicine (Milliken, 1997; Radrianarivelosia et al., 2003; Kirira et al., 2006; Jullian et
al., 2006; Penali et al., 2007).

*Zanthoxylum chiloperone* Mart. ex Engl. is a dioic tree growing in South
America. A decoction of *Z. chiloperone* root bark is used traditionally in Paraguay for
its antimalaric, emmenagogue and antirheumatic properties (Miliken, 1997; Guy et al.,
2001). Previous studies reported that three canthin-6-one-related alkaloids, isolated
from the Paraguayan medicinal plant *Zanthoxylum chiloperone*, were active against 13
fungi (Thouvenel et al., 2003), effective in vivo against *Leishmania amazonensis*
(Ferreira et al., 2002) and also effective in vivo against *Trypanosoma cruzi* (Ferreira et
al., 2007). Due to its also known antiparasitic properties, we evaluated herein the
bioactivity of methanolic extracts of *Zanthoxylum chiloperone* on *Plasmodium berghei-
infected* mice. This model was recommended by the World Health Organization for
primary screening of potential blood schizonticidal agents (Carvalho et al., 1991).

2. Materials and methods

2.1. Plant materials

The leaves of *Zanthoxylum chiloperone* were collected and dried at room
temperature. Plant samples were collected in Concepción, Paraguay, in January 2000.
The plant was identified by Dr. F. R. G. Salimena in the Herbarium Leopoldo Krieger, of
the Department of Botany, Federal University of Juiz de Fora, where voucher specimen (CESJ – 41. 683) was deposited.

2.2. Methanol extracts preparation

Plant samples (100 g) were pulverized, and the respective plant’s powder was subjected to Soxhlet extraction of 48h using methanol. The methanolic extract was concentrated in a rotary evaporator and reconstituted into 100 mg/mL stock solution. The respective stock solution was stored at -18ºC until use. This part was performed a Faculty of Pharmacy, by Felipe Villela Gomes and Profa. Dra. Magda Narciso Leite.

2.3. Phytochemical screening

Standard screening tests of the extract were carried out for various constituents (Matos, 1989). The extract was screened for the presence of alkaloids, flavonoids, triterpenes/steroids, leucoantocianidines, anthraquinones, coumarins, saponins, tannins and volatile oils using standard procedures. This part was performed a Faculty of Pharmacy, by Felipe Villela Gomes and Profa. Dra. Magda Narciso Leite.

2.4. Animals

For the in vivo tests, Swiss albino adult mice, 6-8 weeks of age and weighting 20-22g, were infected with 1 x 10⁶ Plasmodium berghei infected red blood cells (NK65 strain) by the intraperitoneal route, randomly distributed in groups of five mice each. These animals were obtained from Centro de Biologia da Reprodução, in Federal University of Juiz de Fora (process in Ethic Committee number 063/2007-CEEA).
2.5. “In vivo” antimalarial activity evaluation

Using the 4-day suppressive test (Peters, 1965), 5 animals per group were inoculated intraperitoneally with 0.2ml of that solution (day 1) and divided into 5 groups. These groups were treated orally with 0.1ml of water or drugs, as following: Group 1 (G1) was untreated (control untreated group); Group 2 (G2) was treated with 200mg/Kg of chloroquine (control treated group); Group 3 (G3), with 50mg/Kg of the extract; Group 4 (G4), with 100mg/Kg of the extract; and Group 5 (G5), with 200mg/Kg of the extract. Groups 3, 4 and 5 were test groups. These treatments were performed for 4 days, starting on the day of infection. Blood smears were made on days 1, 3, 5, 7, 11 and 14 after treatment, Giemsa stained and examined microscopically to assess the percentage parasitaemia. At the day 14 after treatment all mice died, except the chloroquine treated group.

2.6. Inhibition of parasite multiplication (ipm) percentage

The antimalarial activity of the drugs was established on the basis of average parasitaemia of each group. The percent inhibition of parasite multiplication was calculated using treated group compared with untreated group, by means of the following formula (Carvalho et al., 1991):

\[
\frac{\text{percent average parasitaemia in the control group (pcg)} - \text{percent average parasitaemia in the test group}}{\text{pcg}} \times 100.
\]

2.7. Cytotoxicity evaluation

The cytotoxicity effect against mammalian cells expressed as cell viability was assayed on mice’s peritoneal macrophages. Swiss albino mice received thyoglicolate 4%. After 72h, the cells were isolated from peritoneal cavity mice using cold Hank solution. The 1 × 10^6 cells per well were cultivated on a 96-well plate with RPMI 1640
supplemented with 10% FBS and incubated at 37 °C in 5% CO₂ atmosphere for 24h, so that the macrophages could adhere. After this, cells not adhered were washed with phosphate buffered saline (PBS), then adhered cells were covered with RPMI 1640 supplemented with 10% FBS and the extract was added in the doses equivalent to those used in the in vivo test. Control wells without extract were included. Then, the plate was incubated at 37 °C in 5% CO₂ atmosphere for 48h. After this, the viability of the macrophages was determined with the colorimetric 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) method based on tetrazolium salt reduction by mitochondrial dehydrogenases. After 2h, the reaction was interrupted by the addition of isopropanol acid and absorbance at 570 nm was measured in an ELISA reader. Each assay was performed in triplicate.

3. Results

The evaluation of parasitemia is shown by means of average per group (Fig.1 and Table 1) and separately per mouse (Fig. 2).
Fig. 1 – Percent average of parasitaemia per group, after the end of treatment until the death of mice.

Table 1 – Average parasitemia per group, on days after treatment

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>Untreated group</th>
<th>Treated groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50 mg/Kg</td>
</tr>
<tr>
<td>Day 1</td>
<td>8.47</td>
<td>7.16</td>
</tr>
<tr>
<td>Day 3</td>
<td>11.98</td>
<td>13.99</td>
</tr>
<tr>
<td>Day 5</td>
<td>14.60</td>
<td>11.48</td>
</tr>
<tr>
<td>Day 7</td>
<td>17.80</td>
<td>8.46</td>
</tr>
<tr>
<td>Day 11</td>
<td>27.13</td>
<td>26.74</td>
</tr>
<tr>
<td>Day 14</td>
<td>45.63</td>
<td>42.16</td>
</tr>
</tbody>
</table>
The inhibition of parasite multiplication took place mostly on day 5 after treatment (Table 2), but only the dose of 100mg/Kg presented a relevant value, higher than 50% of inhibition. The control treated group (chloroquine 200mg/kg) presented an inhibition of parasite multiplication of 100% on all days.
Table 2 – Percentage of inhibition of parasite multiplication on days 1, 3 and 5 after treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg/Kg</td>
<td>15.47%</td>
<td>0%</td>
<td>39.99%</td>
</tr>
<tr>
<td>100 mg/Kg</td>
<td>0%</td>
<td>0%</td>
<td>51.65%</td>
</tr>
<tr>
<td>200 mg/Kg</td>
<td>0%</td>
<td>0%</td>
<td>39.31%</td>
</tr>
</tbody>
</table>

Mice treated with chloroquine 200mg/Kg survived for all days. Mice treated with *Z. chiloperone* 50mg/Kg, 100mg/Kg and 200mg/Kg survived a few days longer than the untreated group (Fig. 3). The survival can also be seen separately in Figure 4.

![Figure 3](image-url)

Fig 3 – Survival of mice from day 1 of infection to death. G1 – untreated group; G2 – chloroquine 200mg/Kg; G3 – *Z. chiloperone* 50mg/Kg; G4 – *Z. chiloperone* 100mg/Kg; G5 – *Z. chiloperone* 200mg/Kg.
Fig 4 – Survival of mice separated by group from day 1 of infection to death. G1 – untreated group; G2 – chloroquine 200mg/Kg; G3 – *Z. chiloperone* 50mg/Kg; G4 – *Z. chiloperone* 100mg/Kg; G5 – *Z. chiloperone* 200mg/Kg.

Concerning cytotoxicity, *Z. chiloperone* methanolic extract was not considered cytotoxic for mouse peritoneal macrophage. Data shown in Table 3.
Table 3 - Cytotoxicity of the methanolic extract for mouse peritoneal macrophage

<table>
<thead>
<tr>
<th>Dose</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg/Kg</td>
<td>7.27%</td>
</tr>
<tr>
<td>100 mg/Kg</td>
<td>1.82%</td>
</tr>
<tr>
<td>200 mg/Kg</td>
<td>NC*</td>
</tr>
</tbody>
</table>

* Not Cytotoxic

4. Discussion

Our main result was that 100mg/Kg dose of *Z. chiloperone* displays a good antimalarial activity, inhibiting more than 50% of the parasite multiplication. The present work is in accordance with a large variety of other studies (Jonville et al., 2008; Céline et al., 2008; Boyom et al., 2009; Esmaeili et al., 2009; Bero et al., 2009; Rukunga et al., 2009; Okokon and Nwafor et al., 2009), supporting the relevance of using natural compounds in attempt to provide antimalarials.

Some plant species belonging to the *Zanthoxylum* genus (*Z. usambarense*, *Z. tsihanimposa*, *Z. rhoifolium*, *Z. rubescens* and *Z. chiloperone*) are widely used as antimalarials or antipyretics in traditional medicine (Miliken, 1997; Randrianarivelosia et al., 2003; Kirira et al., 2006; Jullian et al., 2006; Penali et al., 2007;). *Z. rubescens* was tested against *Plasmodium falciparum* exhibiting low activity (Penali et al., 2007), in contrast to *Z. chiloperone* trypanocidal *in vivo* (Ferreira et al., 2007), leishmanicidal *in vitro* (Ferreira et al., 2002) and antifungal *in vitro* (Soriano-Agatón et al., 2005). In order to confirm activity based on the traditional usage, using ethnopharmacological approach, we selected *Z. chiloperone* species to be tested against *Plasmodium berghei* in mice. Models can help the comprehension of questions that are too complex, diverse and expensive to be approached by other means. They can offer research a more realistic expectation, which is extremely important to the optimization
of intervention programs (McKenzie, 2000). Moreover, in vivo tests provide the evaluation of metabolic transformations by the host and the late action of the metabolic compound after digestion. They are reproducible, give helpful information on structure-activity relationships (Loiseau and Xuong, 1996), and are easy and simple to be maintained (Carvalho and Krettli, 1991).

The inhibition of parasite multiplication takes place mostly on day five after treatment. It was near 40% for the 50mg/Kg and 200mg/Kg doses, and higher than 50% for 100mg/Kg dose. This late activity suggests a matter of drug bioavailability. Actually, herbal products are composed by combinations of biologically active compounds with chemicals and inherent pharmacological activities. Thus, they may suffer some transformations during metabolism altering the consequent action (Venkataramanan et al., 2006).

Bioavailability is defined by the fraction of administered drug that reaches systemic circulation and many physiological/pharmacological parameters are involved, besides the ones concerning the compound itself (Agoram et al., 2001). Its related factors have received marked attention (Martin, 2005) since potential drug candidates are in urgent need of discovery due to the rise of world-wide malaria drug resistance, mainly to Plasmodium falciparum. Taking it in account, studies on the relationship between chemical and biological properties have been conducted in order to reach optimization, as the case of halofantrine (Khoo et al., 1998; Khoo et al., 2000) and dihydroartemisinin (Kongpatanakula et al., 2007).

Besides, it is an important issue concerning the development of bioactive molecules as therapeutic agents, since it depends on many factors not only of the host – as variability of metabolizing enzymes, membrane permeation, water complexation, passive or active processes – but also of the drug properties as well, which includes
structure features, lipophilicity, molecular weight, ease of formulation and chemical stability (Veber et al., 2002). Structure-activity may also play a role, as demonstrated by the evaluation of canthin-6-one analogues extracted from *Zanthoxylum chiloperone*, whose changes in the structure incited changes in the antifungal ability (Soriano-Agáton et al., 2005). Indeed, concepts related to drugs can optimize pharmacokinetic and pharmaceutical properties, as defended by Vistoli et al. (2008) who demonstrated that they are not “frozen statues but dancing ballerinas”.

The result for the dose of 200mg/Kg (high dose versus low efficacy) brought us to an intriguing question, which has already been demonstrated by others (Adesomoju et al., 2007), whose report shows that the suppression due to drug of cytokines is correlated with exacerbation of early phase of malaria infection in mice. This may be the case and we have looked forward to clarifying it. It is important to highlight that, herein, it cannot be due to toxicity, since the methanolic extract demonstrated none at any doses tested (Table 1). Confirming the absence of clinical toxicity, reported by others (Silva et al., 2007), *Z. chiloperone* methanolic extract was not cytotoxic for mouse peritoneal macrophages.

The higher ipm for the intermediate dose leads us to the supposition that the lower dose was not enough to provide a considerably higher ipm. It may be due to matters related to the compound, such as the ones already discussed. Another supposition is about the fact that the inhibition of 100mg/Kg dose (51,65%) was higher than 200mg/Kg dose (39,31%). This phenomenon may be a matter of hormesis, which is defined in a general way as benefit at low doses and harm at high ones (Calabrese and Blain, 2005). Indeed, it is already clear that xenobiotics can modify endogenous substrates, altering the metabolic equilibrium, as the example of phenobarital (Kitano et al., 1998), and the suspicious presence of other variables must be taken into
consideration, since biphasic responses may derive from very diverse mechanisms (Murado and Vazquez, 2007).

Comparing to untreated group, the survival time of all doses of *Z. chiloperone* treated mice was longer. Although the parasitaemia of untreated group was lower than in some *Z. chiloperone* treated groups, as seen on day 7 and 11 after treatment, these mice survived longer than untreated mice. This may confirm the antimalarial activity of methanolic extract of *Z. chiloperone*.

This work revealed antiplasmodial activity *in vivo* of the *Z. chiloperone* methanolic extract, whose data is very scarce. It also brought up the intriguing question about high dose *versus* low efficacy. Furthermore, it represents an addition on *Z. chiloperone* range of bioactivity, as it was trypanocidal *in vivo* (Ferreira et al., 2007), leishmanicidal *in vitro* (Ferreira et al., 2002) and antifungal *in vitro* (Soriano-Agatón, 2005). Thus, these results may be objects of further researches for new antimalarial agents, since the inhibition of parasite multiplication for 100mg/Kg group was almost 52%, besides the results - almost 40% - for the other doses. Last but not least, essential to remember is the fact that natural products represent a potential source of antimalarials, as mentioned before.

Concluding, this report represents a contribution to a better understanding of the matters related to the attempts of controlling malaria. Therefore, more efforts on these shall be held.

**Aknowledgements**

We thank Federal University of Juiz de Fora for financial support to student’s fellowship and we are grateful to Dr. Erika Martins Braga (Department of Parasitology, UFMG) and Dr. Kézia Scopel for kindly providing *Plasmodium berghei* NK65 strain.
References


Jonville, M.C., Kodja, H., Humeau, L., Fournel, J., De Mol, P., Cao, M., Angenot, L.,
Frédérick, M., 2008. Screening of medicinal plants from Reunion Island for antimalarial and cytotoxic activity. J. Ethnopharmacol. 120, 382-386.


Kongpatanakula, S., Chatsiricharoenkula, S., Sathirakulb, K., Suputtamongkolc, Y.,
of the safety and relative bioavailability of a new dihydroartemisinin tablet
formulation in healthy Thai volunteers. Trans. R. Soc. Trop. Med. Hyg.,101, 972-
979.

58, 533- 534.

Loiseau, P.M., Xuong, N.D., 1996. Plasmodium berghei mouse model: antimalarial
activity of new alkaloid salts and of thiosemicarbazone and acridine derivates. Trop.


Munõz, V., Sauvain, M., Bourdy, G., Callapa, J., Bergeron, S., Rojas, I., Bravo, J.A.,
bioactive compounds in Bolivia through a multidisciplinary approach Part I. Evaluation of the antimalarial activity of plants used by the Chacobo Indians. J. Ethnopharmacol. 69,127-137.


Silva, S.L., Figueiredo, P.M., Yano, T., 2007. Cytotoxic evaluation of essential oil from


Soriano-Agatón, F., Lagoutte, D., Poupon, E., Roblot, F., Fournet, A., Gantier, J.C.,
Hocquemiller, R., 2005. Extraction, hemisynthesis, and synthesis of canthin-6-one


*Zanthoxylum ovalifolium*. Phytochemistry 12, 729-730.

Thouvenel, C., Gantier, J.C., Duret, P., Fourneau, C., Hocquemiller, R., Ferreira, M.E.,


Venkataramanan, R., Komoroski, B., Strom, S., 2006. In vitro and in vivo assessment of


Witkowski, B., Berry, A., Benoit-Vical, F., 2009. Resistance to antimalarial compounds:


Legends to Figures