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ORIGINAL PAPER

# Antimalarial potential of leaves of *Chenopodium ambrosioides* L.

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**Abstract** In an effort to identify novel therapeutic alternatives for the treatment of malaria, the present study evaluated the antimalarial effect of the crude hydroalcoholic extract (HCE) from the leaves of *Chenopodium ambrosioides* L. For this purpose, the molecular affinity between the total proteins from erythrocytes infected with *Plasmodium falciparum* and HCE or chloroquine was evaluated by surface plasmon resonance (SPR). Subsequently, the plasmodicidal potential of HCE was assessed in a *P. falciparum* culture. Using BALB/c mice infected with *Plasmodium berghei* intraperitoneally (ip.), we evaluated the effects of ip. treatment, for three consecutive days (day 7, 8, and 9 after infection), with chloroquine (45 mg/kg) or HCE (5 mg/kg), considering the survival index and the parasitaemia. The groups were compared to an untreated control group that receives only PBS at the same periods. The results indicated that HCE could bind to the total proteins of infected erythrocytes and could inhibit the parasite growth in vitro ($IC_{50}$ = 25.4 g/mL). The in vivo therapeutic treatment with HCE increased the survival and decreased the parasitaemia in the infected animals. Therefore, the HCE treatment exhibited a significant antiplasmodial effect and may be considered as a potential candidate for the development of new antimalarial drugs.

**Keywords** *Chenopodium ambrosioides* · *Plasmodium* · Malaria · Treatment

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## Introduction

Malaria continues to pose a challenge to global public health (Liu et al. 2013). In 2013, approximately 198 million cases and 584,000 deaths due to malaria were reported worldwide (World Health Organization 2014). Five species of *Plasmodium* parasites are known to cause this disease in humans (Kantele and Jokiranta 2011). Despite the greater geographical distribution of *Plasmodium vivax*, *Plasmodium falciparum* deserves attention because it is responsible for the largest number of severe cases and deaths (World Health Organization 2014).

Currently, artemisinin-based combination therapies are the treatment of choice for falciparum malaria (World Health Organization 2014) because of the reported resistance of malaria parasites to other antimalarials, including chloroquine (Hyde 2002; Juge et al. 2015). However, resistance to artemisinin has been reported in five Asian countries: Cambodia, Laos, Myanmar, Thailand, and Vietnam (World Health Organization 2013, 2014). Moreover, a safe, effective, and globally available vaccine against the disease is not yet available. For this reason, the search for new antimalarial drugs is crucial and necessary.

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Natural products are promising sources of new active ingredients for the treatment of malaria (Batista et al. 2009; Oliveira et al. 2009). This notion is corroborated by the fact that the antimalarial drugs quinine and artemisinin are plant-derived antimalarials (Saxena et al. 2003; Wright 2005). Therefore, several research groups have explored the antimalarial potential of plant species in vitro and in vivo (Muñoz et al. 2000; Deharo et al. 2001; Hilou et al. 2006; Bantie et al. 2014). Pollack et al. (1990), for example, demonstrated that ascaridole, isolated from the essential oil of the plant species *Chenopodium ambrosioides*, has great antiplasmodial potential in vitro. Likewise, Monzote et al. (2014) demonstrated the in vitro activity of the essential oil of this plant species against *P. falciparum*.

*C. ambrosioides* L. (Amaranthaceae) is popularly known in Brazil as "erva-de-santa-maria," "mentrasto," "mentruço," or "mastruz" (França et al. 1996; BRASIL 2008) and in other American countries as "wormseed," "goosefoot," "paico," and "epazote" (Conway and Slocumb 1979; Kliks 1985). It is popularly and extensively used as an anthelmintic (Kliks 1985), anti-inflammatory (Di Stasi and Hiruma-Lima 2002), anti-*Leishmania* drug (França et al. 1996), and antidiarrheal/antidysenteric (Neiva et al. 2014), among other uses. This therapeutic potential has been recognized to such an extent that, in 2008, the Brazilian Ministry of Health included this plant species in the National List of Medicinal Plants of Interest to the National Health System (RENISUS) (BRASIL 2008).

Our research group has shown that the crude hydroalcoholic extract (HCE) of the *C. ambrosioides* leaves has a significant anti-tumor (Nascimento et al. 2006), anti-*Leishmania* (Bezerra et al. 2006; Patrício et al. 2008; Reis et al. 2012a; Lima-Júnior et al. 2014), anti-*Giardia* (Neiva et al. 2014), analgesic (Sousa et al. 2012) and immunomodulatory activity (Cruz et al. 2007).

Despite the extensive research performed on the biological activity of the HCE, no previous studies have evaluated the antimalarial activity of *C. ambrosioides* in animal models. Based on this, we aim to evaluate the antiplasmodial effects of the HCE in vitro and in vivo as a pre-clinical trial, using different experimental models.

## Materials and methods

### Plant material

The leaves of *C. ambrosioides* were collected in Canaã Garden, located in the municipality of Paço do Lumiar, Maranhão, Brazil (2°30′08.1″ S 44°08′39.2″ W). The plant was identified at the Ático Seabra Herbarium of the Federal University of Maranhão (UFMA), with specimen voucher number 1148/SLS017213. A total of 4.8 kg of plant material was collected, cleaned, dried, and powdered, resulting in 594 g of raw material. This material was percolated in a 70 % hydroalcoholic solution and mixed every 8 h, for 24 h, at a hydromodule of 1:5 (*w*/*w*). After this time, the HCE was filtered four times, and then it was filtered and concentrated under reduced pressure to obtain a dry extract (Neiva et al. 2011). The final yield was 16 % of the weight of the crushed dry leaves.

### In vitro assays

#### *P. falciparum culture*

Blood forms of *P. falciparum* strains W2 and 3D7 were maintained in suspension cultures of human erythrocytes from volunteer blood donors and 5 % hematocrit, as detailed by Trager and Jensen (1976). The culture was performed in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 5 % defibrinated human plasma (blood type: $O^+$) for a final concentration of 1 %, 22.8 mM Hepes (Promega, Madison, WI, USA), 11.1 mM glucose (Sigma, St. Louis, MO, USA) 0.36 mM (50 μg/mL) hypoxanthine (HPX) (Sigma, St. Louis, MO, USA), 23.8 mM NaHCO3 (Merck & Co., Inc., Whitehouse Station, New Jersey, USA), and 40 μg/mL gentamicin (Sigma, St. Louis, MO, USA) and was incubated in 25 $cm^2$ culture plastic bottles (Sarstedt, Nümbrecht, Germany), at 37 °C and 5 % $CO_2$. Parasite development was monitored by examining blood smears under an optical microscope. The slides were stained with Giemsa and observed at a magnification of ×1000.

#### *P. falciparum synchronization*

Ring-stage *P. falciparum* W2 or 3D7 cultures in human erythrocytes were used in the assays, as previously described (Lambros and Vanderberg 1979). Briefly, the culture medium was removed from the culture bottle, and 10 mL of 5 % sorbitol and 0.5 % glucose were added to the sediment containing the parasitized blood. The content was transferred to a conical-bottom tube (15 mL) and incubated at 37 °C, for 10 min. After the incubation, the material was centrifuged at 1000 g, for 5 min, at room temperature. The supernatant was removed; the pellet was resuspended in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with inactivated human plasma ($O^+$), and the hematocrit was adjusted to 5 %. The suspension was transferred to a culture dish and left at 37 °C, for approximately 10 min, to allow the sedimentation of the erythrocytes. Subsequently, a blood smear was prepared and stained with Giemsa for the determination of parasitaemia under an optical microscope. For all in vitro assays, hematocrit and parasitaemia were adjusted to 5 and 1 %, respectively, with the addition of erythrocytes and RPMI.

*Evaluation of the molecular interactions by surface plasmon resonance*

Molecular interactions on CM5 sensor chips, supported with a ligand molecule, were analyzed using the Biacore T200 biosensor system (GE Healthcare, NJ, USA) and the Biacore T200 Evaluation Software version 1.0. Amine coupling was used to couple the ligand to the chip surface.

The interaction between the immobilized ligand and the analytes of interest occurred under physiological conditions using HBS-P buffer (0.1 M HEPES, 1.5 M NaCl, 0.5 % P20 surfactant, pH 7.4) at a temperature of 37 °C and under other conditions that depended on the interaction model under study.

Molecular interaction assays were conducted using the total proteins of the erythrocytes infected with *P. falciparum* W2 as the ligand, at a pH of 5.0 (acetate 5.0) and a concentration of 150 μg/mL. The total proteins from the infected erythrocytes in the protein extract were quantitated using the Bradford method. The HCE and chloroquine were used as analytes at the concentrations of 25, 50, and 100 μg/mL.

*In vitro treatment of P. falciparum 3D7 culture with HCE*

After parasites were synchronized to the ring stage, the cultures were exposed to HCE solubilized in sterile RPMI 1640 medium (Sigma, St. Louis, MO, USA). The extract of *C. ambrosioides* was serially diluted at the following concentrations: 250, 125, 62.5, 31.25, 15.62, and 7.8 μg/mL. The concentration of HCE that inhibited 50 % of the parasite growth ($IC_{50}$) in the intra-erythrocytic stage was calculated as previously described (Jordão et al. 2011), and the in vitro treatment with HCE was performed for 48 h at 37 °C and 5 % $CO_2$. Parasitaemia was measured using Giemsa-stained smears immediately after the end of the HCE treatment and calculated according to the following formula:

$$\text{Parasitaemia}(\%) = \frac{(\text{Number of parasitized erythrocytes})}{\text{Total number of erythrocytes}} \times 100$$

**In vivo assays**

*Animals*

Female Balb/c mice weighing 20–25 g and aged 8–12 weeks were obtained from the animal facility of the University of São Paulo (USP). The animals were maintained in standard housing conditions at an animal facility at the Department of Parasitology - USP and were fed a regular diet. All performed procedures were authorized by the National Council for Animal Control and Experimentation (CONSEA) and approved by the Research Ethics Committee of the UFMA under protocol number 23115.005821/2012-92.

*Infection*

For the in vivo infection protocols, the *Plasmodium berghei* ANKA–GFP 15cy1 clone was used, which is a strain that constitutively expresses green fluorescent protein (GFP) (Janse et al. 2006a, b). The erythrocytes used for infection were obtained from in vivo passages in Balb/c mice when the percentage of parasitaemia reached approximately 10 %. Sixteen mice were intraperitoneally (ip.) inoculated with $10^4$ erythrocytes infected with *P. berghei* ANKA–GFP. The animals were randomly divided into three groups: the control group ($n = 5$), the chloroquine (CQ) group ($n = 5$), and the HCE group ($n = 6$).

*Treatment of P. berghei infected mice with HCE*

Treatment with HCE (5 mg/kg/day) was performed ip., daily, on days 7, 8, and 9 after infection. At the same periods, the control group received phosphate buffered saline (PBS) and the CQ group received chloroquine (45 mg/kg/day).

Parasitaemia was assessed by flow cytometry on the 6th, 9th, and 11th days after infection (Janse and Van Vianen 1994). Animal survival was monitored for 21 days, and on the basis of mortality during this period, the median survival time (MST) and the percentage increase in life span (ILS) were calculated according to the following formula (Gupta et al. 2000):

$$\text{ILS}(\%) = \frac{(\text{MST of the treated group} - \text{MST of the control group})}{\text{MST of the control group}} \times 100$$

*Statistical analysis*

All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). After normality was assessed using the D'Agostino-Pearson test, numeric variables were subjected to an analysis of variance (ANOVA), followed by the Newman-Keuls post-test or a two-way ANOVA. The Kaplan-Meier method and the log-rank test were used for the statistical analysis of the survival data. The inhibitory concentration of parasite growth ($IC_{50}$) was calculated using nonlinear regression. The significance level required to reject the null hypothesis was 5 % ($p < 0.05$).

## Results

### HCE can bind to the total proteins of erythrocytes infected with *P. falciparum*

The molecular interaction between the total proteins of erythrocytes infected with *P. falciparum* and HCE or CQ was evaluated using the association curve of the surface plasmon resonance (SPR) assays. The results indicated that

at all the concentrations evaluated (25 to 100 μg/mL), HCE showed 1.9- to 4.3-fold higher binding affinities than CQ to the total proteins of erythrocytes infected with *P. falciparum* in terms of resonance units (RU) (Fig. 1).

### HCE has an antiplasmodial effect in vitro

To evaluate the in vitro antiplasmodial effect of the HCE of *C. ambrosioides*, *P. falciparum* clones were grown in the absence and presence of increasing concentrations of the extract (Fig. 2). The HCE treatment inhibited the parasite growth, exhibiting an $IC_{50}$ of 25.4 μg/mL.

### Therapeutic treatment with HCE increases the survival and decreases parasitaemia in murine malaria

The survival of the animals from the HCE group was higher than that of the control group but lower than that of the CQ group because treatment with CQ resulted in 100 % survival until the last day of the evaluation (Fig. 3). The comparison of the MST between the HCE group and the control group indicated that the HCE treatment increased the life span by 33 % (Table 1).

On day 6 post-infection, i.e., 1 day before treatment initiation, the mice infected with *P. berghei* exhibited similar parasitaemia in all groups. On the ninth day after infection (the time point corresponding to the third and final treatment), parasitaemia in the HCE group was similar to that in the control group and was approximately sixfold higher than that of the CQ group. On the 11th day after infection, i.e., 2 days after the last treatment and 2 days before the death of the first animals, parasitaemia had nearly doubled in the control group, whereas in the HCE group, the level of parasitaemia was very similar to the levels detected on day 9 and was therefore 53 % lower than that of the control. Parasitaemia in the CQ group, in turn, was undetectable on the last day of the evaluation (Fig. 4).

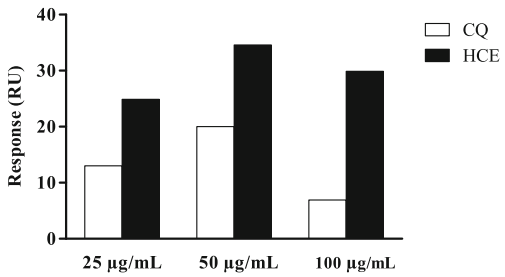


**Fig. 1** Molecular interactions between the total proteins of erythrocytes infected with *Plasmodium falciparum* and the crude hydroalcoholic extract (HCE) of *Chenopodium ambrosioides* analyzed using surface plasmon resonance (SPR). The total proteins of erythrocytes infected with *P. falciparum* W2 were quantified using the Bradford method to obtain a specified concentration (150 μg/mL) and after were immobilized on a CM5 sensor chips as ligand. In the other hand, HCE and chloroquine were used as analytes at different concentrations (25, 50, and 100 μg/mL). SPR was performed in a Biacore T200 biosensor system (GE Life Sciences). *RU* resonance unit

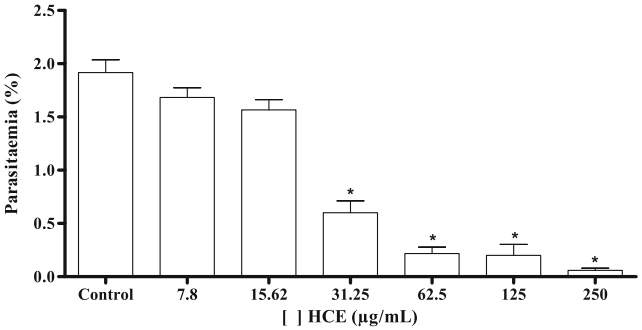


**Fig. 2** In vitro effect of the crude hydroalcoholic extract (HCE) of *Chenopodium ambrosioides* on the growth of *Plasmodium falciparum*. The inhibition of parasite growth was evaluated after the treatment with HCE at different concentrations (250, 125, 32.5, 62.5, 31.25, 15.62, and 7.8 μg/mL) in 48-h cultures. The *bars* represent the mean ± SD for each group. $*p < 0.05$ compared to the control

## Discussion

The need for therapeutic alternatives for the treatment of malaria is urgent, owing to the recognized parasite resistance to antimalarial drugs and the current worldwide impact of this disease (World Health Organization 2014; Juge et al. 2015). Regarding this need, the present study showed that the hydroalcoholic crude extract of *C. ambrosioides* leaves had a significant antiplasmodial potential in different in vitro and in vivo experimental models.

The molecular association between the total proteins of erythrocytes infected with *P. falciparum* and HCE or chloroquine was initially investigated using SPR. The results

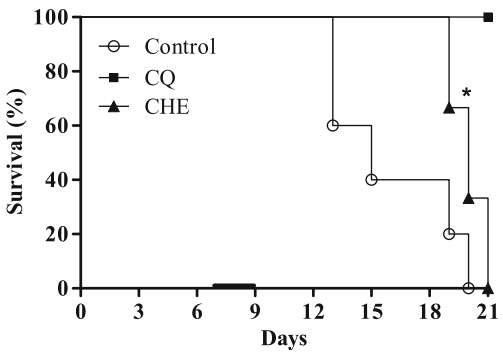


**Fig. 3** Survival of mice infected with *Plasmodium berghei* and treated with crude hydroalcoholic extract (HCE) of *Chenopodium ambrosioides*. Female Balb/c mice infected ip. with $10^4$ of *P. berghei* ANKA-GFP were treated ip. during three consecutive days (*horizontal bar*), starting at day 7 post-infection, with crude hydroalcoholic extract (HCE; 5 mg/kg; $n = 6$); chloroquine (CQ; 45 mg/kg; $n = 5$) and compared to the control group, which received PBS at the same periods (control; $n = 5$). Survival was evaluated until the 21st day after inoculation. $*p < 0.05$ compared to the control

**Table 1** Survival of mice infected with *Plasmodium berghei* and treated ip. with crude hydroalcoholic extract (HCE) for three consecutive days starting on day 7 post-infection

| Treatments | MST (days)[a] | ILS (%) |
|---|---|---|
| Control | 15 | – |
| CQ (45 mg/kg)[b] | – | – |
| HCE (5 mg/kg) | 20 | 33 |

[a] *MST* median survival time

[b] Animals treated with chloroquine (CQ) showed 100 % survival until the last day of monitoring

indicated that at all three concentrations tested, the binding affinity between those proteins and HCE was up to 4.3-fold higher than the binding affinity between the proteins of the infected erythrocytes and chloroquine (Fig. 1). Therefore, HCE is a potential therapeutic candidate with good affinity for *P. falciparum*-infected erythrocytes.

No previous reports have evaluated the affinity between a plant extract and *P. falciparum* using SPR, which makes these findings particularly relevant. In addition, the use of chloroquine in this assay served primarily as a comparative parameter because the affinity of this drug to total proteins from infected erythrocytes is not necessarily associated with its therapeutic efficacy. Chloroquine spreads through the membrane of the digestive vacuole of *Plasmodium*, prevents the subsequent polymerization of protoporphyrin IX, and leads to the accumulation of this molecule, which causes parasite death due to toxicity and oxidative damage to the parasite membranes (Fitch 2004). Based on the binding affinity profile achieved for HCE compared to chloroquine, it can be speculated that a different mechanism of action for controlling parasite's growth may occur.

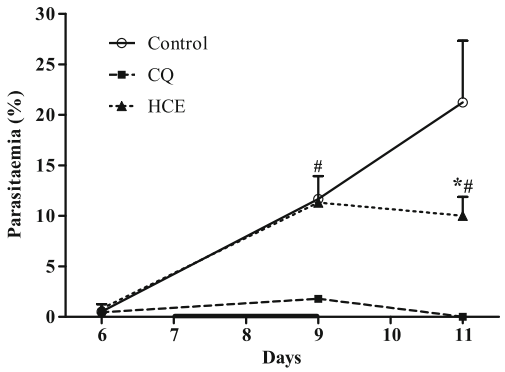


**Fig. 4** Effect of the crude hydroalcoholic extract (HCE) of *Chenopodium ambrosioides* on parasitaemia in mice infected with *Plasmodium berghei* ANKA-GFP. Female BALB/c mice infected i.p. with $10^4$ of an inoculum of *P. berghei* ANKA-GFP were treated i.p. starting at day 7 post-infection with PBS (control; $n = 5$), chloroquine at 45 mg/kg (CQ; $n = 5$) or crude hydroalcoholic extract at 5 mg/kg (HCE; $n = 6$) for three consecutive days (*horizontal bar*). Parasitaemia was assessed by flow cytometry on the 6th, 9th, and 11th days after infection. The *bars* represent the mean ± SD for each group. *$p < 0.05$ compared with control and (#) compared with the CQ group

Thus, to assess whether the molecular binding affinity of HCE to infected erythrocytes was correlated with its antiplasmodial activity, the in vitro effect of HCE on *P. falciparum* cultures was evaluated. HCE inhibited the parasite growth in a dose-dependent manner (Fig. 2), exhibiting an $IC_{50}$ of 25.4 μg/mL.

In order to compare the in vitro plasmodicidal activity of HCE with compounds from other plant species, we compared the $IC_{50}$ of this extract with the classification used in previous studies that evaluated the in vitro antimalarial potential of plant-derived natural products (Basco et al. 1994; Osorio et al. 2007; Dolabela et al. 2008; Batista et al. 2009). In the classification of plant extracts and constituents, an $IC_{50} <$ 10 μg/mL indicates high activity; an $IC_{50}$ between 10 and 50 μg/mL, moderate activity; an $IC_{50}$ between 50 and 100 μg/mL, low activity; and an $IC_{50} > 100$ μg/mL, lack of activity. Therefore, the $IC_{50}$ obtained in this study showed that HCE has moderate plasmodicidal activity.

This $IC_{50}$ value for *P. falciparum* was even lower than that found by our research group when the antimicrobial activity of the HCE was evaluated in vitro against other protozoa. Bezerra et al. (2006) found an $IC_{50}$ of 151.9 μg/mL for the HCE treatment against promastigotes of *Leishmania amazonensis*. Neiva et al. (2014) evaluated the anti-*Giardia* effect of HCE and found an $IC_{50}$ of 198.18 μg/mL of this extract prepared by percolation and 77.28 μg/mL when prepared by maceration against trophozoites of *Giardia lamblia*.

Ascaridole is one of the constituents present in HCE that may be responsible for the antiplasmodial activity observed in vitro. Previous studies have shown that this terpene was isolated from the essential oil of *C. ambrosioides* and is a major constituent of this plant species (MacDonald et al. 2004; Monzote et al. 2006). Ascaridole has also been found in the ethanol extract of leaves and stems of *C. ambrosioides* (Trivellato-Grassi et al. 2013) and in the hexane fraction of the crude extract of leaves, flowers, and stems of this plant (MacDonald et al. 2004), which suggests the presence of this terpene in HCE.

Pollack et al. (1990) reported that the ascaridole isolated from the essential oil of *C. ambrosioides* effectively inhibited the growth of malaria parasites in vitro. In addition, these investigators also found that the presence of the peroxide group in the chemical structure of the ascaridole was directly associated with its antiparasitic activity because the use of cineol, which has an epoxide group rather than a peroxide one, did not inhibit parasite growth at the same concentrations. These results assume greater importance because the presence of the peroxide group is essential to the mechanism of action of artemisinin (Cui and Su 2009), which, currently, is the most important compound for the treatment of malaria (World Health Organization 2014).

More recently, Monzote et al. (2014) reported the in vitro activity of the essential oil of *C. ambrosioides* against *P. falciparum* and other protozoa. These researchers also emphasized the importance of ascaridole as a constituent associated with this antimicrobial activity showed.

Based on the efficacy of HCE against *P. falciparum* in vitro, the effect of this compound on the survival and parasitaemia using an in vivo murine model of malaria was evaluated. The employed model was suitable for the analysis since that BALB/c mice infected with *P. berghei* ANKA have resistance to the development of severe malaria (cerebral) and frequently die later due to anemia and high parasitaemia (Moumaris et al. 1995; Hanum et al. 2003). It is important to emphasize that, in the current study, the treatment was initiated only after all the animals had detectable parasitaemia, on day 7 post-infection (Fig. 4), which allowed the evaluation of the therapeutic activity of HCE against malaria.

Our results indicated an increased survival, corresponded to 33 % of ILS, in the animals treated with HCE compared to the control group (Fig. 3, Table 1). The animals treated with chloroquine, in turn, remained alive until the end of follow-up. Furthermore, treatment with HCE decreased parasitaemia by 53 % compared with the control treatment at day 11 post-infection (Fig. 4). Despite the absence of the complete inhibition of parasitaemia, as observed for chloroquine, the HCE treatment continually reduced the parasite proliferation, even 48 h after the last treatment dose, showing a long lasting anti-*Plasmodium* effect.

To the best of our knowledge, no previous studies have evaluated the antimalarial potential of *C. ambrosioides* in vivo. Therefore, we compare the results presented herein with those reported by Hilou et al. (2006), who showed that the extract of *Amaranthus spinosus*, also a member of the Amaranthaceae family, suppressed parasitaemia in infected animals by approximately 53.1 % at a dose of 900 mg/kg. Other studies have evaluated the in vivo antimalarial potential of different plant extracts using doses 20 to 100 times higher (Muñoz et al. 2000; Deharo et al. 2001; Bantie et al. 2014) than those used in the present study. The results altogether indicate an antiparasitic effect of HCE at low doses and suggest that higher doses, or even a longer duration of treatment, could enhance the antiplasmodial activity of the extract; however, this hypothesis still needs to be tested.

Possibly, the increase in survival detected may be related to the plasmodicidal activity of HCE detected both in vitro and in vivo. To reinforce this hypothesis, previous studies using cerebral malaria models have highlighted the importance of decreasing the parasite load to increase the survival of the infected animals (Bienvenu and Picot 2008; Reis et al. 2012b).

An important consideration, however, is that in vivo experimental models also take into account the participation of the immune system in the control of parasitic infection (Waako et al. 2005). In this respect, previous studies from our research group have indicated that the HCE treatment has a significant immunomodulatory effect in mice both in the absence and presence of parasitic infection (Cruz et al. 2007; Patrício et al. 2008). For this reason, it is reasonable to suggest a relation between the modulation of the immune system and the increased survival and decreased parasitaemia showed in the current study.

Furthermore, previous reports of the anti-inflammatory and analgesic effect of *C. ambrosioides* (Sousa et al. 2012; Trivellato-Grassi et al. 2013) corroborate the potential use of this plant species as an antimalarial agent. Wright (2005), discussing about the development of plant-derived antimalarial drugs, highlighted that they can also be important in reducing the characteristic symptoms of malarial infection, including pain and fever.

In conclusion, this study is the first to report the ability of the HCE of *C. ambrosioides* to bind to the total proteins of erythrocytes infected with *P. falciparum* and the antiplasmodial activity of this extract in vitro. The antiparasitic effect of HCE was also observed in vivo, characterized by the increased survival of and decreased parasitaemia in animals treated with the extract. These results indicate that the HCE of *C. ambrosioides* can be considered as a tool for the development of new antimalarial drugs.

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**Authorship policy** Conceived and designed the experiments: DNC, TSF, CRFM, RN, and FRFN. Performed the experiments: DNC, TSF, ASR, and ASF. Analyzed the data: DNC, TSF, and BPR. Contributed reagents/materials/analysis tools: FMMA, CRFM, and RN. Wrote the paper: DNC, RNMG, RN and FRFN.

**Compliance with ethical standards** The authors declare that they have no conflict of interest. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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