Experimental Parasitology 122 (2009) 233–241

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Toxoplasma gondii: Effects of *Artemisia annua* L. on susceptibility to infection in experimental models in vitro and in vivo

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ARTICLE INFO

Article history:
Received 5 December 2008
Received in revised form 8 April 2009
Accepted 12 April 2009
Available online 21 April 2009

Keywords:
Toxoplasma gondii
Artemisia annua L.
Infusion
In vitro effect
In vivo effect

ABSTRACT

Considering that the treatment for toxoplasmosis is based on drugs that show limited efficacy due to their substantial side effects, the purpose of the present study was to evaluate the effects of *Artemisia annua* on in vitro and in vivo *Toxoplasma gondii* infection. *A. annua* infusion was prepared from dried herb and tested in human foreskin fibroblasts (HFF) or mice that were infected with the parasite and compared with sulfadiazine treatment. For in vitro experiments, treatment was done on parasite before HFF infection or on cells previously infected with *T. gondii* and the inhibitory concentration (IC_{50}) values for each treatment condition were determined. Viability of HFF cells in the presence of different concentrations of *A. annua* infusion and sulfadiazine was above 72%, even when the highest concentrations from both treatments were tested. Also, the treatment of *T. gondii* tachyzoites with *A. annua* infusion before infection in HFF cells showed a dose–response inhibitory curve that reached up to 75% of inhibition, similarly to the results observed when parasites were treated with sulfadiazine. In vivo experiments with a cystogenic *T. gondii* strain demonstrated an effective control of infection using *A. annua* infusion. In conclusion, our results indicate that *A. annua* infusion is useful to control *T. gondii* infection, due to its low toxicity and its inhibitory action directly against the parasite, resulting in a well tolerated therapeutic tool.

1. Introduction

Toxoplasmosis is an infection caused by the obligate intracellular parasite *Toxoplasma gondii* that is widespread in all warm-blooded animals and humans (Tenter et al., 2000). In immunocompetent individuals, *Toxoplasma* infection causes little or no overt signs of disease in its hosts due to efficient protective immunity, but in immunocompromised patients or during pregnancy, *T. gondii* may emerge as a serious infection, which if not treated can lead to host death (Denkers and Gazzinelli, 1998). A number of studies with murine models have shown the important role of cytokines, such as IL-12, TNF-α, and IFN-γ, and generation of reactive nitrogen intermediates (RNI) as mediators of host resistance to early *T. gondii* infection (Liesenfeld et al., 1996; Denkers and Gazzinelli, 1998). Nevertheless, C57BL/6 mice orally infected with 100 cysts of *T. gondii* develop an exacerbated and fatal intestinal inflammatory response, associated with the production of high levels of IFN-γ, tumor necrosis factor (TNF), and RNI (Liesenfeld et al., 1996; Welter et al., 2006).

Sulfadiazine plus pyrimethamine are currently the drugs of first choice for treatment of most clinical presentations of toxoplasmosis (Petersen and Schmidt, 2003). Although this therapy is frequently successful, it is associated with many side effects including bone marrow suppression, which requires the concomitant administration of folinic acid (Martins-Duarte et al., 2006). In addition to the fact that it is frequently not well tolerated, it is necessary the substitution of sulfadiazine by other drugs such as clindamycin (Montoya and Liesenfeld, 2004). Thus, to find out less toxic drug active against all stages of the parasite is crucial to treat *T. gondii* infection.

For that reason new drugs have been intensively studied for the control of *Toxoplasma* infection. Among them, *Artemisia annua* L. is an annual herb belonging to the family Asteraceae and it is endemic to the northern parts of China (Bilia et al., 2006). Its active compound, artemisinin, and its derivates such as artemether, originally developed for the treatment of malaria (De Ridder et al., 2008), have shown some ability to inhibit *Toxoplasma* replication in vitro (Jones-Brando et al., 2006; D'Angelo et al., 2009). Artemisinin is generally present in the leaves and flowers of the plant at concentrations ranging from 0.01% to 1.4% of dry weight (Bilia et al., 2006) and the current pharmacopoeia of the People's Republic of China officially lists the dry herb *A. annua* L. as a

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doi:10.1016/j.exppara.2009.04.010

remedy for fever and malaria at daily dose from 4.5 to 9 g of dried herb to be prepared as a tea infusion with boiling water (Rath et al., 2004). Recently Lans et al. (2007) observed an extensive use for *A. annua* infusion in ethnoveterinary medicines, where it was used for the treatment of roundworms, pinworms, and giardia in pigs and was shown to be 81.6–83.2% suppressive towards the development of *Cryptosporidium parvum*.

Although previous studies have reported the effects of artemisinin or its derivates on infection with *T. gondii* (Ou-Yang et al., 1990; Holfels et al., 1994; Sarciron et al., 2000; Jones-Brando et al., 2006; Nagamune et al., 2007) or its closely related *Neospora caninum* (Kim et al., 2002), no data are available on the effects of *A. annua* infusion on *Toxoplasma* infection. In addition, the potential use of *A. annua* infusion instead of pure artemisinin, particularly in areas where large-scale pharmaceutical production is not possible, is clearly of interest due to its easy accessibility (Bilia et al., 2006). Therefore, the purpose of the present study was to evaluate the effects of *A. annua* infusion on in vitro and in vivo *T. gondii* infection.

2. Materials and methods

2.1. Animals

Female C57BL/6 and BALB/c mice (6–10 weeks old) and male Calomys callosus from Canabrava strain (8–12 weeks old) were kept in the Bioterism Center of the Animal Experimentation Laboratory, Federal University of Uberlandia, Brazil. All animals were kept under standard laboratory conditions (12-h light and 12-h dark cycle, controlled temperature of 22 ± 2 °C) with food and water ad libitum. All procedures were conducted according to institutional guidelines for animal ethics.

2.2. Cell culture

Human foreskin fibroblasts (HFF) were obtained from American Type Culture Collection (ATCC, Manassas, USA) and cultured in 25 cm^2 flasks until confluence in RPMI-1640 medium (Gibco, Paisley, UK), supplemented with 25 mM Hepes, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma Chemical Co., St. Louis, USA) and 10% heat-inactivated fetal calf serum (Cultilab, Campinas, Brazil) (complete RPMI) in a humidified incubator at 37 °C and 5% CO_2.

2.3. Parasites

Tachyzoites of the highly virulent RH strain of *T. gondii* were maintained in Swiss mice by intraperitoneal serial passage at regular 48 h intervals (Mineo et al., 1980). Mouse peritoneal exudates were harvested and washed twice (720 g, 10 min, 4 °C). Parasites were suspended in RPMI-1640 medium and the number of viable tachyzoites was determined by Trypan blue exclusion in hemocytometric chamber. For infection experiments, parasites were used within 30–40 min of their removal from the peritoneal cavity.

Cysts of the moderately virulent ME49 strain of *T. gondii* were obtained from brain tissues from *C. callosus* infected 30–45 days earlier by oral route (Barbosa et al., 2007). Brains were removed, washed in 0.01 M phosphate-buffered saline (PBS) pH 7.2, homogenized and cysts were counted under light microscopy.

2.4. Artemisia annua L. infusion

Artemisia annua L. seeds came from Center of Chemical, Biological and Agricultural Researches, University of Campinas, Brazil. Seeds were sown in a greenhouse and transferred to an experimental area when the seedlings were 15 cm high. The plants were harvested before the flowering period for the best artemisinin content. The aerial parts were dried and ground to be stored at low humidity. *A. annua* L. infusion was prepared from the infusion of 10 g of dried herb in 100 mL of boiling distilled water. The mixture was briefly stirred and covered for 20 min, and the plant material was removed by filtration, squeezed gently to release residual water and allowed to cool at room temperature (Rath et al., 2004). The *A. annua* L. infusion was stored as stock solution (100 mg/mL) at 4 °C in the dark until being analyzed with regards to specific content of artemisinin by HPLC and used in cytotoxicity assays and in vitro or in vivo experiments.

2.5. Cytotoxicity assay

Cytotoxicity of *A. annua* L. infusion was assessed by determining cellular viability using MTT assay as previously described (Mosmann, 1983). HFF cells were cultured in 96-well plates (1×10^5 cells/well) in triplicate in complete RPMI medium in the presence of twofold serial dilutions of *A. annua* infusion stock solution starting from 1:10 dilution (10,000 to 80 μg/mL) or sulfadiazine (Biopharma, Uberlândia, Brazil) ranging from 200 to 1.56 μg/mL in RPMI medium. As controls, cells were incubated with medium alone. After 24 h of incubation at 37 °C and 5% CO_2, cells were washed and pulsed with 10 μL of thiazolyl blue (MTT, Sigma Chemical Co.) at 5 mg/mL in 90 μL of complete RPMI medium 4 h prior to the end of the culture. Formazan particles were solubilized in 10% sodium dodecyl sulfate (SDS) and 50% *N*,*N*-dimethyl formamide. The optical density was read after 30 min at 570 nm in a plate reader (Titertek Multiskan Plus, Flow Laboratories, McLean, USA). Results were expressed as percentage of cell viability in relation to controls.

2.6. Effect of drugs on T. gondii infection in vitro

HFF cells were cultured on 13-mm round glass coverslips into 24-well plates (1×10^5 cells/well/200 μL) for 24 h at 37 °C and 5% CO_2. In a first set of experiments (treatment before infection), parasites were pretreated for 1 h at 37 °C and 5% CO_2 with different concentrations of drugs in twofold serial dilutions, as follows: *A. annua* infusion (2500 to 80 μg/mL) or sulfadiazine (200 to 1.56 μg/mL), or with medium alone (control) and incubated with cell monolayers on coverslips at 2:1 (parasite: host cell) rate of infection (2×10^5 tachyzoites/well/200 μL) for 24 h at 37 °C and 5% CO_2. Cells were washed with PBS to remove non-adherent parasites, fixed in 10% buffered formalin for 2 h and stained with 1% toluidine blue (Sigma Chemical Co.) for 5 s. Coverslips were mounted on glass slides and cells were examined under light microscope with regards to *T. gondii* infection index (percentage of infected cells per 100 examined cells) and parasite intracellular replication (mean number of parasites per cell in 100 infected cells) (Oliveira et al., 2006).

In a second set of experiments (treatment after infection), cell monolayers were washed with medium and infected with *T. gondii* RH strain tachyzoites at a 2:1 (parasite: host cell) rate of infection (2×10^5 tachyzoites/well/200 μL). After 3 h of incubation, cells were again washed to remove non-adherent parasites and then treated with twofold serial dilutions for 24 h at 37 °C and 5% CO_2, as above described. As control, infected cells were incubated with medium alone. Cells were washed with PBS, stained, and examined as described for the first set of experiments. Three slides of each treatment condition were assessed by two independent observers.

Results were expressed as percentages of inhibition of infection as well as of parasite intracellular replication for each treatment in relation to controls. The median inhibitory concentration (IC_{50}) of each drug was calculated by extrapolation of the corresponding

dose–response curve on a log-linear plot employing the portions of the curve that transected the 50% response point (Jones-Brando et al., 2006).

2.7. Effect of drugs on T. gondii infection in vivo

Initially, 30 female C57BL/6 mice were infected intraperitoneally (i.p.) with 10^2 tachyzoites of *T. gondii* RH strain and distributed into three groups with 10 animals that were treated in the same day of inoculum, during 5 days at regular 8-h intervals as follows: (i) 10 mg/kg/day of *A. annua* infusion subcutaneously (s.c.); (ii) 400 mg/L/day of sulfadiazine in the drinking water (Jeroen et al., 2005); and (iii) PBS (200 μL, s.c.). In another set of experiments, 15 female C57BL/6 mice were infected i.p. with 20 cysts of *T. gondii* ME49 strain and distributed into three groups with 5 animals that were treated as above described. An additional group with 5 non-infected animals, but treated with 10 mg/kg/day of *A. annua* infusion by subcutaneous route, was also included.

The animals were observed daily for mortality and the morbidity was assessed with basis on scores (Bartley et al., 2006): sleek/glossy coat, weight maintained at pre-infection level, bright and active (score 0); ruffled coat, 10% weight loss, hunched, tottering gait, reluctance to move (score 1); starry stiff coat, 20% weight loss (score 2). All surviving animals were euthanized at 30 days post-infection (dpi), when brain and liver tissues were collected for immunohistochemical assay, mouse bioassay and polymerase chain reaction (PCR) for the detection of *T. gondii.*

2.8. Immunohistochemical assay

Tissue specimens were fixed in 10% buffered formalin and processed routinely for paraffin embedding. Tissue sections with 4 μm thickness were obtained and processed as previously described (Silva et al., 2002a). Briefly, sections were incubated (30 min at room temperature) with 3% hydrogen peroxide and then with 0.2 M citrate buffer, pH 6.0 (one 7-min cycle in microwaves) to rescue antigenic sites. Next, sections were incubated (30 min at 37 °C) with 1% bovine serum albumin followed by incubation with hyperimmune rabbit serum (overnight at 4 °C) obtained after immunization with soluble antigen of *T. gondii* as previously described (Mineo et al., 1986). As negative control, tissue sections were also incubated with non-immune rabbit serum. After incubation with secondary biotinylated goat anti-rabbit antibodies, streptavidin-biotinylated peroxidase complex (DAKO Corporation, Carpentaria, USA) was added an the reaction was developed with H_2O_2 plus 3,3′-diaminobenzidine tetrahydrochloride (DAB) tablets (Sigma Chemical Co.) for 5 min. Slides were counterstained with Harry's haematoxylin and examined under light microscopy.

2.9. Mouse bioassay

Detection of *T. gondii* was evaluated by mouse bioassay as described elsewhere (Freyre et al., 2006). Brain tissues were homogenized in PBS and separately inoculated i.p. in BALB/c mice. Animals were daily observed for morbidity or mortality and blood samples from surviving mice were collected and analyzed for seroconversion through the detection of IgG antibodies to *T. gondii* in ELISA (Barbosa et al., 2007).

2.10. PCR for detection of T. gondii DNA in tissues

The presence of *T. gondii* DNA was investigated by PCR as previously described (Grigg and Boothroyd, 2001) with some modifications (Silva et al., 2002b). Brain tissues were treated with proteinase K (Invitrogen Life Technologies, São Paulo, Brazil) and submitted to phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) extraction. Qualitative PCR detecting the B1 gene of *T. gondii* was performed using the primers 5′-TCTTCCCAGAGGTGGATTTC-3′ and 5′-CTCGACAATACGCTGCTTG-3′ (Invitrogen Life Technologies), which should amplify a 531 bp fragment under predetermined conditions. DNA extracted from 10^7 RH strain tachyzoites and brain tissue from non-infected mice were included as positive and negative controls, respectively. PCR products were analyzed in 1% agarose gel containing ethidium bromide and visualized under UV illumination.

2.11. Determination of nitrite and cytokine measurements

C57BL/6 mice were inoculated i.p. with 1 mL of sterile 3% (w/v) thioglycolate medium. After four days, peritoneal macrophages were harvested and cultured on 24-well plates (1×10^5 cells/well) for 24 h in complete RPMI medium at 37 °C and 5% CO_2. Cells were stimulated in triplicate with *A. annua* infusion at twofold serial dilutions from 10 to 0.65 mg/mL. As positive and negative controls, cells were stimulated with 1 ng/mL of IFN-γ (PharMingen, California, USA) plus 10 μg/mL of lipopolysaccharide (LPS; Sigma Chemical Co.) and medium alone, respectively. Supernatants were collected after 24 and 48 h of incubation at 37 °C and 5% CO_2 and analyzed for nitrite determination (Seabra et al., 2002). Briefly, 100 μL of supernatant from each well was mixed (v/v) to Griess reagent (0.1% *N*-1-naphthyl-ethylenediamine in distilled water and 1% sulfanilamide in 5% phosphoric acid) on a 96-well plate. Absorbance was read at 540 nm and nitrite concentration was calculated from a standard curve using sodium nitrite. IL-12 and TNF-α levels were quantified in 24- and 48-h supernatants from macrophage cultures treated with different concentrations of *A. annua* infusion as above described. Cytokine levels were determined by sandwich ELISA according to the manufacturer's instructions (PeProtech, Veracruz, México) in comparison to a standard curve built with the respective recombinant murine cytokines. The lower limit of detection for each assay was 78 pg/ml for IL-12 and 390 pg/ml for TNF-α.

2.12. Statistical analysis

The Kaplan–Meier method was used to compare the survival rates of the studied groups (Fletcher et al., 1996) and survival curves were compared using logrank and χ^2-tests. Cytokine levels were analyzed by Student *t*-test. Statistical analysis and graphs were performed using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, USA). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Cytotoxicity activity of A. annua infusion

The specific content of artemisinin in the *A. annua* L. infusion was 24.53 μg/mL (0.2% of total) corresponding to starting concentration of 10,000 μg/mL of herb infusion, as determined by HPLC (data not shown). Viability of HFF cells in the presence of different concentrations of *A. annua* infusion (Fig. 1A) and sulfadiazine (Fig. 1B) was above 72%, even when the highest concentrations from both treatments (10,000 and 200 μg/mL, respectively) were tested.

3.2. Effect of A. annua infusion on T. gondii infection in vitro

Effect of *A. annua* infusion and sulfadiazine against *T. gondii* infection in HFF cells was evaluated in two different conditions of treatment (Fig. 2). First, the treatment of *T. gondii* tachyzoites with *A.*

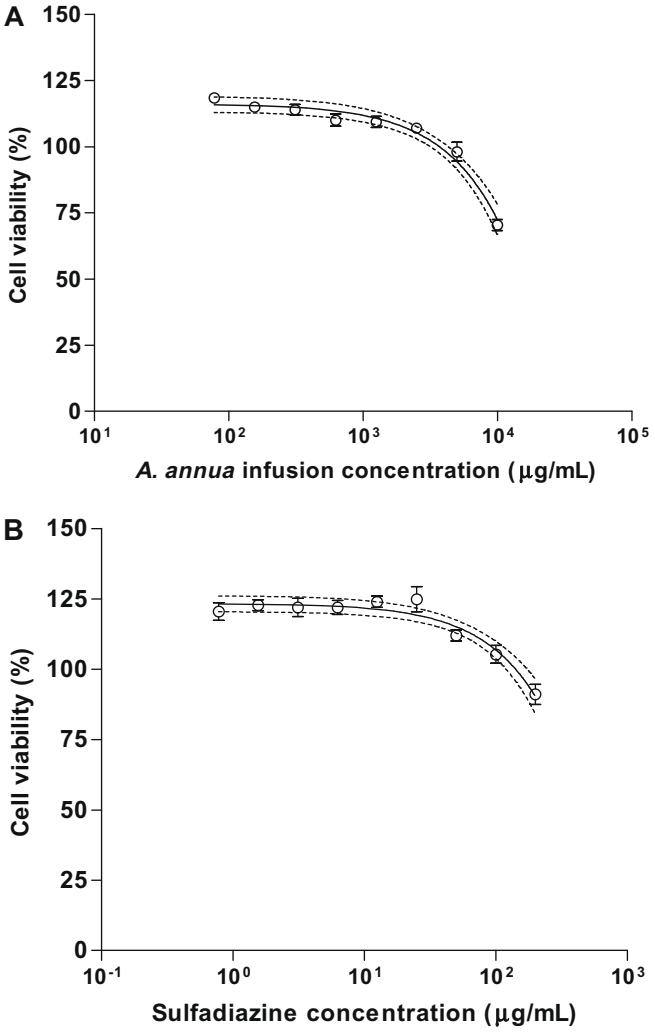

Fig. 1. In vitro cytotoxicity activity determined by MTT assay. Human foreskin fibroblasts were cultured in 96-well plates in the absence (control) or presence of different concentrations of (A) *A. annua* infusion (from 10,000 to 80 µg/mL) or (B) sulfadiazine (from 200 to 1.56 µg/mL) for 24 h. The results were expressed as the percentage of viable cells in relation to the control.

annua infusion before infection in HFF cells showed a dose–response inhibitory curve that reached up to 75% of inhibition (Fig. 2A). Similar results were observed when parasites were pretreated with sulfadiazine (Fig. 2B). Second, the treatment of *T. gondii*-infected HFF cells with *A. annua* infusion also showed inhibitory dose–response curve that reached 30% of inhibition (Fig. 2C). Also, the treatment of infected cells with sulfadiazine showed similar results (Fig. 2D).

When *T. gondii* treatment was performed before infection in HFF cells, *A. annua* infusion showed an IC_{50} of 95 µg/mL for the infection index, while the treatment with sulfadiazine resulted in an IC_{50} of 3 µg/ml. On the other hand, when the treatment with *A. annua* infusion was done after *T. gondii* infection of HFF, the IC_{50} could not be detected, whereas this parameter was calculated as 70 µg/mL for sulfadiazine treatment.

Concerning the inhibition of intracellular parasite replication, the treatment of *T. gondii* tachyzoites with *A. annua* infusion before infection of HFF cells showed a dose-dependent inhibition, reaching rates of 55% (Fig. 3A). HFF treated with *A. annua* infusion after infection showed inhibition dose–response curve with rates of 30% (Fig. 3C). Treatment with sulfadiazine showed results slightly lower (Fig. 3B and D). Also, IC_{50} was possible to be determined only when *T. gondii* tachyzoites were treated with *A. annua* infusion before HFF infection (IC_{50} = 188 µg/mL).

3.3. Effect of A. annua infusion on T. gondii infection in vivo

Survival curves of mice infected with the virulent RH strain (Fig. 4A) were not significantly different between the treatments with *A. annua* infusion (survival rate of 20% at 30 dpi and median survival of 12 days) and sulfadiazine (survival rate of 50% at 30 dpi and median survival of 25.5 days) ($P = 0.0820$). However, the survival curves of both treatments were significantly higher than that of control infected animals (PBS) that died all at 10th dpi (median survival of 9.0 days) ($P = 0.0007$). As expected, all non-infected animals and treated with *A. annua* infusion alone survive throughout the experiment. When analyzing the morbidity score curves for the same strain (Fig. 4C), animals treated with *A. annua* infusion developed ruffled coat at 6 dpi and from the 7th dpi additional clinical symptoms were observed including hunching and reluctance to move. A drop of 10% in mean body weight of group animals was observed at 10th dpi and from the 15th dpi forward; surviving animals remained with no clinical signs up to the end of experiment. Similar morbidity score curve was observed for the treatment with sulfadiazine, even though with clinical signs being noted up to 21 dpi. Animals of the control infected group (PBS) showed earlier clinical signs (3 dpi) and reached the maximum scores between 6 and 9 dpi, when all animals died.

Survival curves of mice infected with the ME49 strain (Fig. 4B) were identical for both treatments with *A. annua* infusion and sulfadiazine (survival rate of 100% at 30 dpi), but significantly different from that of control infected animals (PBS) that died all at 25th dpi (median survival of 10 days) ($P = 0.0001$). Also, morbidity score curves for the same strain were similar for both drug treatments (Fig. 4D), even though animals presented clinical signs from 6 to 8 dpi with sulfadiazine and from 6 to 13 dpi with *A. annua* infusion treatment. Again, control infected animals showed earlier, higher and long-term morbidity scores (3–24 dpi).

3.4. Detection of T. gondii in tissues

No tissue parasitism was observed in fresh brain tissues under light microscopy or brain and liver tissues examined by immunohistochemical assay in animals infected with RH strain of *T. gondii* and treated with both *A. annua* infusion and sulfadiazine (Table 1). However, all animals infected with the ME49 strain and treated with *A. annua* infusion showed brain tissue cysts by immunohistochemical assay (Fig. 5A), in contrast to animals treated with sulfadiazine (Fig. 5B). In mouse bioassay, all surviving animals infected with both RH and ME49 strains of *T. gondii* and treated with both *A. annua* infusion and sulfadiazine showed that brain tissues had viable parasites or *T. gondii* antigens as demonstrated by positive results found for mortality and seroconversion, respectively (Table 1). PCR results confirmed the presence of *T. gondii* DNA in brain tissues from all surviving animals infected whit both RH and ME49 strains and treated with both drugs (Table 1 and Fig. 6).

3.5. Cytokine measurements and nitrite determination in supernatants from murine macrophages after A. annua infusion stimulation

Culture supernatants from murine peritoneal macrophages were assessed for detection of cytokines or nitrite after stimulation with different concentrations of *A. annua* infusion (Fig. 7). Levels of IL-12 were significantly increased when macrophages were stimulated with the highest concentrations (10, 5, and 2.5 mg/mL) of *A. annua* infusion for 24 h only as compared with medium alone (Fig. 7A). In contrast, no detectable level of TNF-α was found after stimulation of macrophages with any concentration of *A. annua* infusion for 24 or 48 h (Fig. 7B). Positive controls (LPS + IFN-γ) showed significantly increased IL-12 levels after 24 and 48 h of stimulation with *A. annua* infusion while TNF-α levels were signif-

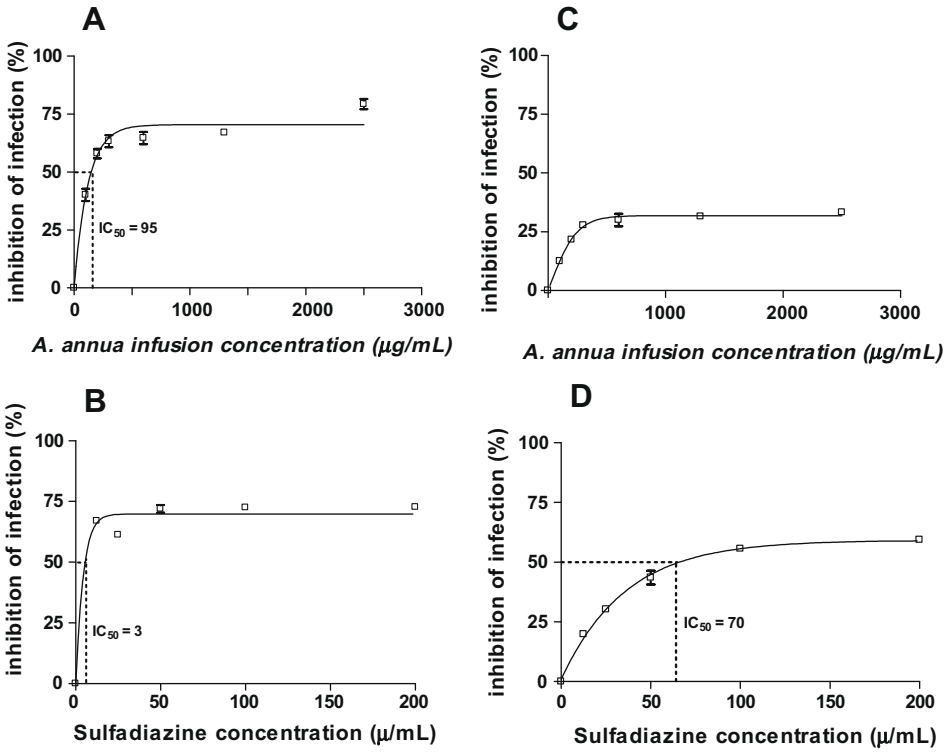

Fig. 2. Effect of treatment with *A. annua* infusion or sulfadiazine on *T. gondii* infection index in human foreskin fibroblasts (HFF). Treatments were carried out on *T. gondii* tachyzoites before HFF infection (A and B) or on HFF after *T. gondii* infection (C and D). Results are expressed as percentage of inhibition of infection related to controls. Dotted lines show the inhibitory concentration of 50% (IC_{50}) and bars represent standard deviations.

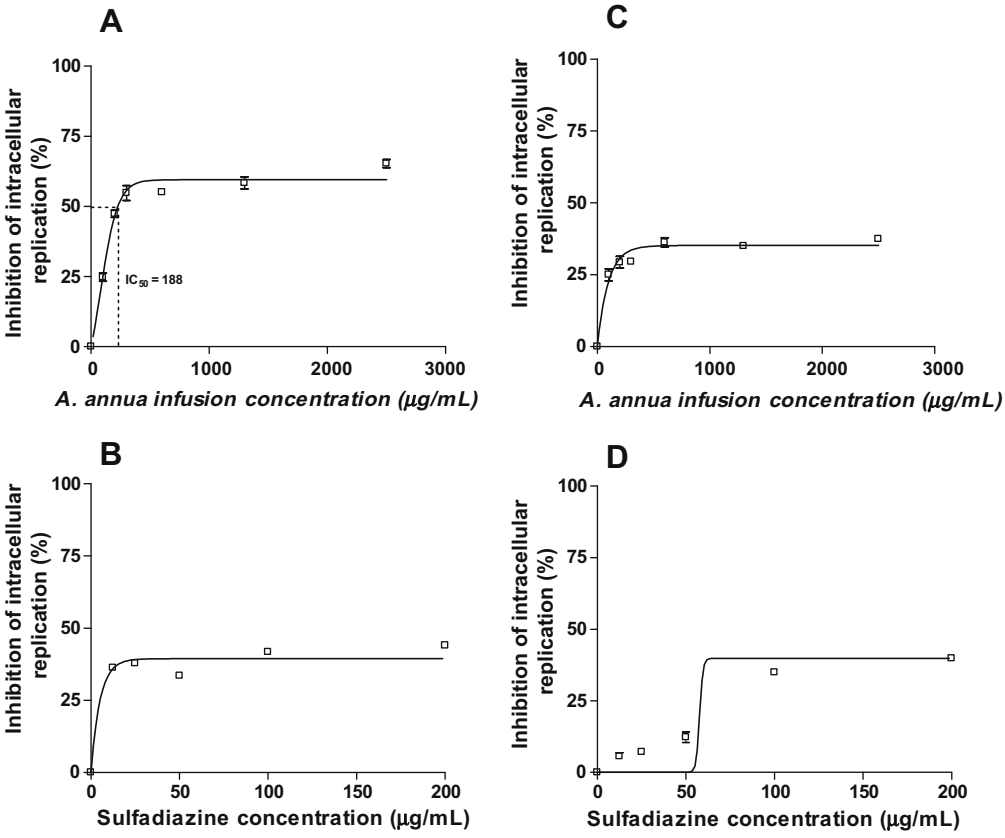

Fig. 3. Effect of treatment with *A. annua* infusion or sulfadiazine on *T. gondii* intracellular replication in human foreskin fibroblasts (HFF). Treatments were carried out on *T. gondii* tachyzoites before HFF infection (A and B) or on HFF after *T. gondii* infection (C and D). Results are expressed as percentage of inhibition of intracellular replication to controls. Dotted lines show the inhibitory concentration of 50% (IC_{50}) and bars represent standard deviations.

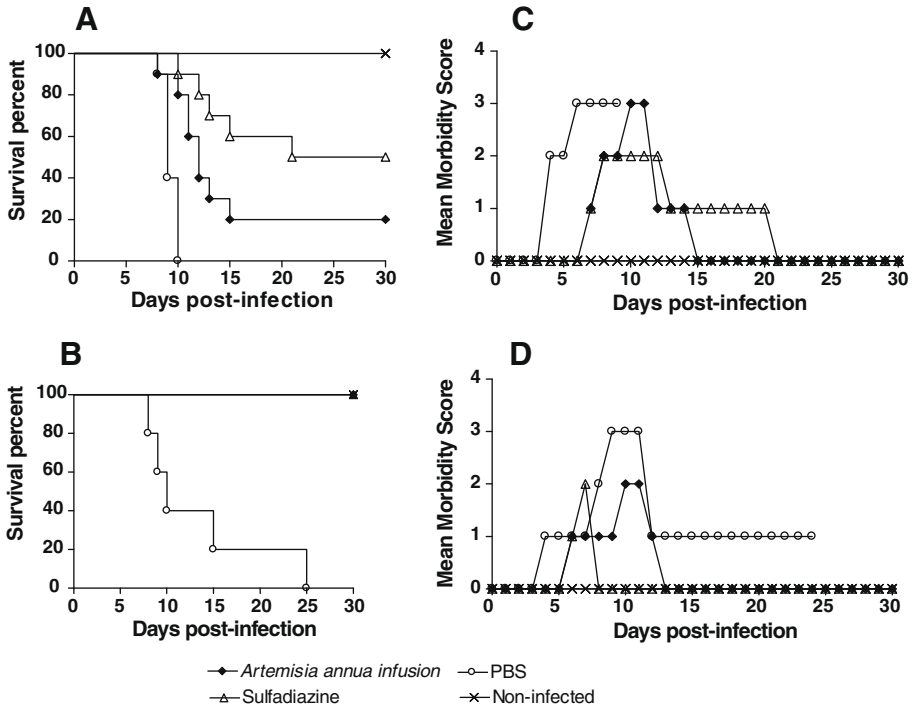

Fig. 4. Survival curves and mean morbidity scores of C57BL/6 mice after infection with virulent RH strain of *T. gondii* (A and C, respectively) or with ME49 strain of *T. gondii* (B and D, respectively). Animals were treated with *A. annua* infusion (10 mg/kg/day) or sulfadiazine (400 mg/L/day) during 5 days, three times a day. As controls, animals were infected with the same strains, but treated with phosphate-buffered saline (PBS) or they were not infected, but treated with *A. annua* infusion.

Table 1
Comparative results of immunohistochemistry, mouse bioassay and PCR in surviving C57BL6 mice infected with *T. gondii* and treated with *A. annua* infusion or sulfadiazine.

T. gondii infection[a]	Treatment[b]	Surviving animals	Immunohistochemistry		Mouse bioassay		PCR[d]
			Brain	Liver	Mortality	Seroconversion[c]	
RH strain	*A. annua* infusion	2/10	0/2	0/2	2/2	ND	2/2
	Sulfadiazine	5/10	0/5	0/5	4/5	1/1	5/5
ME49 strain	*A. annua* infusion	5/5	5/5	0/5	4/5	1/1	5/5
	Sulfadiazine	5/5	0/5	0/5	4/5	1/1	5/5

ND, not done.
[a] Infection was performed with 10^2 tachyzoites of RH strain or 20 cysts of ME49 strain of *T. gondii* by intraperitoneal route.
[b] Treatment was performed with *A. annua* infusion (10 mg/kg/day at 8-h intervals for 5 days) or sulfadiazine (400 mg/L/day in drinking water for 5 days).
[c] Seroconversion was analyzed in surviving animals after the bioassay, that is, in only one animal.
[d] PCR was performed in brain tissue only as described in Section 2.

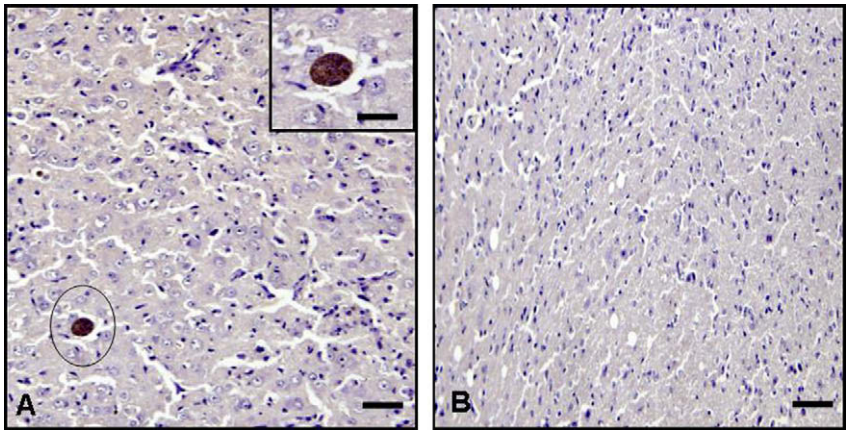

Fig. 5. Representative photomicrographs of immunohistochemical assays in brain tissues from C57BL/6 mice infected with ME49 strain of *T. gondii* and treated with (A) *A. annua* infusion at 10 mg/kg/day or (B) sulfadiazine at 400 mg/L/day during 5 days, three times a day. Tissue sections were analyzed at 30 days post-infection. Inset represents the circled area in (A), showing a strongly stained *T. gondii* cyst. No cyst staining was observed in brain tissues from mice infected and treated with sulfadiazine (B). Bar scales: (A) = 42 μm; Inset (A) = 21 μm; (B) = 84 μm.

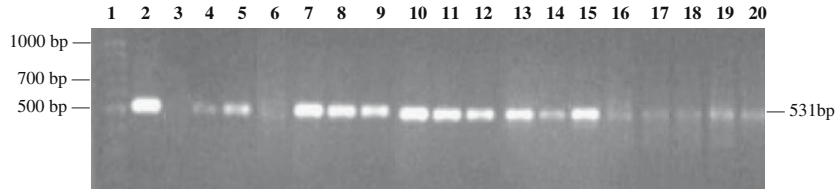

Fig. 6. Representative PCR analysis of the *T. gondii* 35-copy B1 gene in brain tissues of C57BL/6 mice collected after 30 days of infection with RH or ME49 strains of *T. gondii* submitted to *A. annua* or sulfadiazine treatment. (1) DNA ladder; (2) *T. gondii* (RH strain) tachyzoite DNA (positive control); (3) brain tissue from non-infected mice (negative control); (4 and 5) brain tissues from mice infected with *T. gondii* RH strain and treated with *A. annua* infusion; (6–10) brain tissues from mice infected with *T. gondii* RH strain and treated with sulfadiazine; (11–15) brain tissues from mice infected with *T. gondii* ME49 strain and treated with *A. annua* infusion; (16–20) brain tissues from mice infected with *T. gondii* ME49 strain and treated with sulfadiazine.

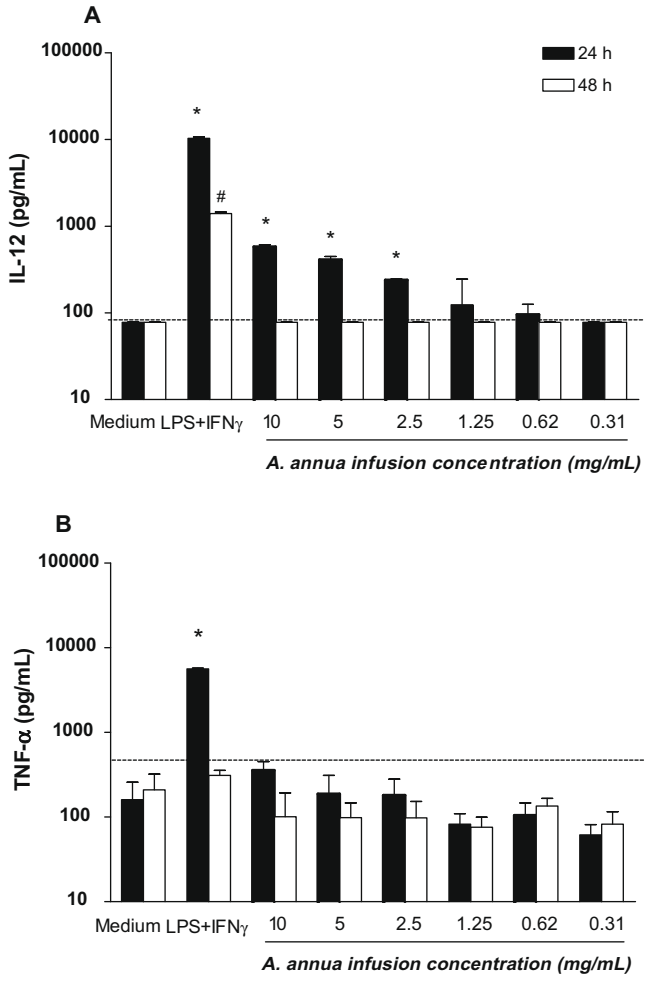

Fig. 7. Cytokine measurements in supernatants from peritoneal macrophage cultures after 24 and 48 h of stimulation with different concentrations of *A. annua* infusion. Levels of IL-12 (A) and TNF-α (B) were determined by sandwich ELISA. Results were expressed as means ± SD in triplicate cultures. $^{*}P < 0.0001$ in relation to medium for 24 h-culture supernatants; $^{\#}P < 0.0001$ in relation to medium for 48 h-culture supernatants.

icantly higher only after 24 h of stimulation. Nitrite levels were not detected after macrophage stimulation with any concentration of *A. annua* infusion (data not shown).

4. Discussion

The effects of artemisinin or its derivates on infection with *T. gondii* have been already investigated (Sarciron et al., 2000; Jones-Brando et al., 2006; Nagamune et al., 2007; El Zawawy, 2008), but there is limited information focusing in the effect of *A. annua* infusion as tea infusion on *Toxoplasma* infection, contrasting to its current use as a remedy for fever and malaria (Rath et al., 2004). In the present study, in vitro and in vivo effects of *A. annua* infusion were evaluated on *T. gondii* infection. Cytotoxicity assays in vitro revealed that both *A. annua* infusion and sulfadiazine, in the concentrations here tested, displayed similar and high cell viability for human fibroblasts. However, several conventional as well as new designed drugs used for the treatment of *Toxoplasma* infection in humans, such as antifolate and atovaquone compounds have increased anti-*Toxoplasma* activity, but their toxicity precludes the widespread usage, particularly over prolonged periods of administration (Jones-Brando et al., 2006).

In the present study, when treatment of *T. gondii* tachyzoites was performed before infection on HFF cells, both herb infusion and sulfadiazine showed high efficacy to control the infection, as demonstrated by dose-dependent inhibition curves and considerably low IC_{50} values obtained for each drug. On the other hand, a high inhibition of parasite intracellular replication was found only for the herb infusion treatment that showed relatively low IC_{50} values, whereas no IC_{50} value could be determined for the sulfadiazine treatment. These findings indicate that the herb infusion showed to be effective when tested directly against the parasite in both infection parameters, with slightly higher efficacy on the infection index than the parasite replication. A previous study analyzing the effect of artemisinin derivates on the cultures of human myelomonocytic cell line (THP-1) infected with an avirulent (DUR) strain of *T. gondii* also showed that the growth of the parasite was not completely inhibited, reaching a maximum inhibition of 70% (Sarciron et al., 2000). However, an efficient dose-dependent inhibition of *T. gondii* (RH strain) infection in enterocyte cell lines was evidenced after the treatment of infected cells with artemisinin derivates (Holfels et al. 1994).

In our in vitro experiments, it was evidenced that the pretreatment of *T. gondii* tachyzoites with *A. annua* infusion before infection of human fibroblasts demonstrated a marked effect as compared to the treatment of cells with herb infusion after *T. gondii* infection. Contradictory results were reported in inhibition studies of growth of *T. gondii* by artemisinin (qinghaosu) that showed no effect of the pretreatment of fibroblasts or *T. gondii* with qinghaosu (Ou-Yang et al., 1990). Our findings suggest that *A. annua* infusion seems to affect more directly the parasite than the infected cells and further studies should be required to elucidate these mechanisms of action. Indeed, it was described recently that artemisinin induces calcium-dependent protein secretion in *T. gondii* tachyzoites, perturbing their calcium homeostasis and supporting the hypothesis that Ca^{2+}-ATPases are potential drug targets to control the spreading of this parasite during infection (Nagamune et al., 2007). Taken together, our results provided a foundation to study the efficacy of *A. annua* infusion by using in vivo models, considering that the conventional treatment is responsible for severe side-effects.

In vivo experiments performed in the present study using a highly virulent strain of *T. gondii* (RH strain) evidenced that neither *A. annua* infusion nor sulfadiazine were able to control completely the infection, since survival rates ranged from 20% to 50%, although they were significantly higher than those obtained for untreated infected controls. Despite a tendency of higher survival rate observed for sulfadiazine in relation to herb infusion, although not statistically significant, the low toxicity of *A. annua* infusion makes it a potential therapeutic candidate in additional studies. A recent study using alternative drugs (PHNQ6) alone or combined with sulfadiazine to control *T. gondii* infection with the RH strain in mice showed that sulfadiazine alone was able to control partially the infection with survival rate of 60% and when combined it reached 70% of survival (Ferreira et al., 2006). However, the use of *A. annua* infusion combined with sulfadiazine should be cautiously investigated, since allergies to sulfa drugs are a serious problem in addition to their side-effects of toxicity, particularly when used for prolonged periods.

When the ME49 strain of *T. gondii* was used in the present study, it was observed an effective control of infection with the use of both *A. annua* infusion and conventional drug. Nevertheless, morbidity scores obtained for both drugs showed slight variations, with the herb infusion presenting later and longer scores than those observed for sulfadiazine. For the RH strain, however, the *A. annua* infusion presented higher, but shorter scores than the sulfadiazine, reflecting in the lower survival curves noted for the herb infusion treatment. These findings indicate that the differential response for treatments may be considered predominantly strain-dependent rather than drug-dependent. Thus, the moderately virulent ME49 strain was susceptible to both drugs while the highly virulent RH strain was more susceptible to the activity of sulfadiazine and more resistant to herb infusion. Accordingly, a previous study showed that the susceptibility of *T. gondii* to atovaquone and to sulfadiazine was different according to the parasite strain (Alves and Vitor, 2005), since type I strains were more susceptible to the activity of sulfadiazine and more resistant to atovaquone, whereas type III strains were susceptible to atovaquone and to sulfadiazine. Comparative results of immunohistochemistry, mouse bioassay and PCR in surviving animals after *T. gondii* infection with RH or ME49 strains and treatment with *A. annua* infusion or sulfadiazine showed that both treatments were unable to clearance the tissue parasitism. These findings were particularly evidenced in mouse bioassay and PCR, indicating the presence of viable parasites or *T. gondii* DNA or antigens. Thus, both sulfadiazine and herb infusion here investigated seem to be ineffective on cystic stages of the parasite. Searching for drugs that are able to control all stages of parasite still constitutes a major challenge (Grujić et al., 2005). Concerning the effect of artemisinin derivates on chronic infection of *T. gondii* in mice, it was described a possible recrudescence of parasite growth, which could limit the use of these drugs in the treatment of toxoplasmic encephalitis (Sarciron et al., 2000). Our results, however, demonstrated an effective control of infection using *A. annua* infusion in the early phase of infection with a *T. gondii* cystogenic strain.

In an attempt to elucidate the probable immunomodulation effects of the *A. annua* infusion in our experimental model, murine peritoneal macrophages previously elicited with thyoglycolate medium were stimulated with different concentrations of herb infusion and cytokine and nitrite production was evaluated. It was observed that the herb infusion was able to induce significant levels of IL-12 in a dose-dependent manner, but not of TNF-α and nitrite in culture supernatants, suggesting that RNI-independent mechanisms may be involved in the control of early *T. gondii* infection. A previous study evaluated the role of an artemisinin derivative (SM735) on the proliferation of murine splenocytes and cytokine production, showing that SM735 strongly inhibited the splenocyte proliferation and proinflammatory cytokine production, including IL-12, IL-6, and IFN-γ, but not IL-2 (Zhou et al., 2005). Recently, Wang et al. (2007) demonstrated that another artemisinin semi-synthetic derivative (SM905) had a high inhibitory activity in splenocyte proliferation and mixed lymphocyte reaction as well as in IL-2 and IFN-γ production, and exerted an inhibitory action on the phosphorylation of mitogen-activate protein (MAP) kinases. Therefore, these artemisinin derivatives exhibit potent immunosuppressive activity in vitro and in vivo in contrast to our results obtained with the stimulation of *A. annua* infusion on murine macrophages, showing a stimulatory rather than inhibitory effect on IL-12 production.

Recent studies have emphasized that artemisinin is not the only active compound in *A. annua* and that the effectiveness of an infusion preparation may be partly due to other components (Willcox et al., 2007). Therefore, *A. annua* infusion may be considered as a natural artemisinin combination therapy, as a result of the presence of other active components. Accordingly, it is worthy to note that the *A. annua* infusion herein used contained only 0.2% of artemisinin and showed to be able to partially control in vitro and in vivo *T. gondii* infection. These data are in agreement with previous reports showing that artemisinin is generally present in the leaves and flowers of the plant at concentrations ranging from 0.01% to 1.4% of dry weight (Bilia et al., 2006). In addition, *A. annua* infusion may exert an immunomodulatory role on the immunosuppressive effects of pure artemisinin and its derivatives, thus contributing to control *T. gondii* infection, as observed in the present study. Further studies will be required to determine the total composition of the *A. annua* infusion herein study and the role of each specific component towards parasite control. In conclusion, our results indicate a potential use of *A. annua* infusion to control *T. gondii* infection, due to its low toxicity and considerable inhibition of parasite infection and replication, resulting in a suitable alternative therapeutic tool.

Acknowledgments

We thank Dr. Janethe D.O. Pena for providing the necessary tools and technical assistance to perform PCR in her laboratory. This study was financially supported by Brazilian Research Agencies (CNPq, FAPEMIG and CAPES).

References

Alves, C.F., Vitor, R.W., 2005. Efficacy of atovaquone and sulfadiazine in the treatment of mice infected with *Toxoplasma gondii* strains isolated in Brazil. Parasite 12, 171–177.

Barbosa, B.F., Silva, D.A.O., Costa, I.N., Pena, J.D.O., Mineo, J.R., Ferro, E.A.V., 2007. Susceptibility to vertical transmission of *Toxoplasma gondii* is temporally dependent on the preconceptional infection in *Calomys callosus*. Placenta 28, 624–630.

Bartley, P.M., Wright, J., Sales, J., Chianini, F., Buxton, D., Innes, E.A., 2006. Long-term passage of tachyzoites in tissue culture can attenuate virulence of *Neospora caninum* in vivo. Parasitology 133, 421–432.

Bilia, A.R., Melillo de Malgalhaes, P., Bergonzi, M.C., Vincieri, F.F., 2006. Simultaneous analysis of artemisinin and flavonoids of several extracts of *Artemisia annua* L. obtained from a commercial sample and a selected cultivar. Phytomedicine 13, 487–493.

D'Angelo, J.G., Bordón, C., Posner, G.H., Yolken, R., Jones-Brando, L., 2009. Artemisinin derivates inhibit *Toxoplasma gondii* in vitro at multiple steps in the lytic cycle. Journal of Antimicrobial Chemotherapy 63, 146–150.

Denkers, E.Y., Gazzinelli, R.T., 1998. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. Clinical Microbiology Reviews 11, 569–588.

De Ridder, S., Van der Kooy, F., Verpoorte, R., 2008. *Artemisia annua* as a self-reliant treatment for malaria in developing countries. Journal of Ethnopharmacology 120, 302–314.

El Zawawy, L.A., 2008. Effect of artesunate on *Toxoplasma gondii*: in vitro and in vivo studies. Journal of the Egyptian Society of Parasitology 38, 185–201.

Ferreira, R.A., Oliveira, A.B., Ribeiro, M.F., Tafuri, W.L., Vitor, R.W., 2006. *Toxoplasma gondii*: in vitro and in vivo activities of the hydroxynaphthoquinone 2-hydroxy-

3-(1′-propen-3-phenyl)-1,4-naphthoquinone alone or combined with sulfadiazine. Experimental Parasitology 113, 125–129.
Fletcher, R.H., Fletcher, S.W., Wagner, E.H., 1996. Clinical Epidemiology: The essentials, 3rd ed. Williams and Wilkins, Baltimore.
Freyre, A., Falcon, J., Mendez, J., Rodriguez, A., Correa, L., Gonzalez, M., 2006. *Toxoplasma gondii*: partial cross-protection among several strains of the parasite against congenital transmission in a rat model. Experimental Parasitology 112, 8–12.
Grigg, M.E., Boothroyd, J.C., 2001. Rapid identification of virulent type 1 strains of the protozoan pathogen *Toxoplasma gondii* by PCR-restriction fragment length polymorphism analysis at the B1 gene. Journal of Clinical Microbiology 39, 398–400.
Grujić, J., Djurković-Djaković, O., Nikolić, A., Klun, I., Bobić, B., 2005. Effectiveness of spiramycin in murine models of acute and chronic toxoplasmosis. International Journal Agents Antimicrobial 25, 226–230.
Holfels, E., McAuley, J., Mack, D., Milhous, W.K., McLeod, R., 1994. In vitro effects of artemisinin ether, cycloguanil hydrochloride (alone and in combination with sulfadiazine), quinine sulfate, mefloquine, primaquine phosphate, trifluoperazine hydrochloride, and verapamil on *Toxoplasma gondii*. Antimicrobial Agents Chemotherapy 38, 1392–1396.
Jeroen, P.J., Saeij, J.P., Boyle, M.E., Grigg, G.A., Boothroyd, J.C., 2005. Bioluminescence imaging of *Toxoplasma gondii* infection in living mice reveals dramatic differences between strains. Infection and Immunity 73, 695–702.
Jones-Brando, L., D'Angelo, J., Posner, G.H., Yolken, R., 2006. In vitro inhibition of *Toxoplasma gondii* by four new derivatives of artemisinin. Antimicrobial Agents Chemotherapy 50, 4206–4208.
Kim, J.T., Park, J.Y., Seo, H.S., Oh, H.G., Noh, J.W., Kim, J.H., Kim, D.Y., Youn, H.J., 2002. In vitro antiprotozoal effects of artemisinin on *Neospora caninum*. Veterinary Parasitology 103, 53–63.
Lans, C., Turner, N., Khan, T., Brauer, G., 2007. Ethnoveterinary medicines used to treat endoparasites and stomach problems in pigs and pets in British Columbia. Canada Veterinary Parasitology 148, 325–340.
Liesenfeld, O., Kosek, J., Remington, J.S., Suzuki, Y., 1996. Association of $CD4^+$ T cell-dependent, interferon-γ-mediated necrosis of the small intestine with genetic susceptibility of mice to peroral infection with *Toxoplasma gondii*. Journal of Experimental Medicine 184, 597–607.
Martins-Duarte, E.S., Urbina, J.A., Souza, W., Vommaro, R.C., 2006. Antiproliferative activities of two novel quinuclidine inhibitors against *Toxoplasma gondii* tachyzoites in vitro. Journal of Antimicrobial Chemotherapy 58, 59–65.
Mineo, J.R., Camargo, M.E., Ferreira, A.W., 1980. Enzyme-linked immunosorbent assay for antibodies to *Toxoplasma gondii* polysaccharides in human toxoplasmosis. Infection and Immunity 27, 283–287.
Mineo, J.R., Camargo, M.E., Ferreira, A.W., Almeida, G., 1986. Research on IgM anti-*Toxoplasma gondii* antibodies by using a reverse immunoenzymatic technique. Revista do Instituto de Medicina Tropical de São Paulo 28, 6–11.
Montoya, J.G., Liesenfeld, O., 2004. Toxoplasmosis. Lancet 363, 1965–1976.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55–63.
Nagamune, K., Beatty, W.L., Sibley, L.D., 2007. Artemisinin induces calcium-dependent protein secretion in the protozoan parasite *Toxoplasma gondii*. Eukaryotic Cell 6, 2147–2156.
Oliveira, J.G., Silva, N.M., Santos, A.A., Souza, M.A., Ferreira, G.L., Mineo, J.R., Ferro, E.A., 2006. BeWo trophoblasts are unable to control replication of *Toxoplasma gondii*, even in the presence of exogenous IFN-gamma. Placenta 27, 691–698.
Ou-Yang, K., Krug, E.C., Marr, J.J., Berens, R.L., 1990. Inhibition of growth of *Toxoplasma gondii* by Qinghaosu and derivatives. Antimicrobial Agents and Chemotherapy 34, 1961–1965.
Petersen, E., Schmidt, D.R., 2003. Sulfadiazine and pyrimethamine in the postnatal treatment of congenital toxoplasmosis: what are the options? Expert Review of Anti-Infective Therapy 1, 175–182.
Rath, K., Taxis, K., Walz, G., Gleiter, C.H., Li, S.-M., Heid, L., 2004. Pharmacokinetic study of artemisinin after oral intake of a traditional preparation of *Artemisia annua* L. (Annuam wormwood). American Journal of Tropical Medicine and Hygiene 70, 128–132.
Sarciron, M.E., Saccharin, C., Petavy, A.F., Peyron, F., 2000. Effects of artesunate, dihydroartemisinin, and artesunate-dihydroartemisinin combination against *Toxoplasma gondii*. The American Society of Tropical Medicine and Hygiene 62, 73–76.
Seabra, S.H., Souza, W., DaMata, R., 2002. *Toxoplasma gondii* partially inhibits nitric oxide production of activated murine macrophages. Experimental Parasitology 100, 62–70.
Silva, N.M., Tafuri, W.L., Alvarez-Leite, J.I., Mineo, J.R., Gazzinelli, R.T., 2002a. *Toxoplasma gondii*: in vivo expression of BAG-5 and cyst formation is independent of TNF p55 receptor and inducible nitric oxide synthase functions. Microbes and Infection 4, 261–270.
Silva, N.M., Rodrigues, C.V., Santoro, M.M., Reis, L.F., Alvarez-Leite, J.I., Gazzinelli, R.T., 2002b. Expression of indoleamine 2,3-dioxygenase, tryptophan degradation, and kynurenine formation during in vivo infection with *Toxoplasma gondii*: induction by endogenous gamma interferon and requirement of interferon regulatory factor 1. Infection and Immunity 70, 859–868.
Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. International Journal for Parasitology 30, 1217–1258.
Wang, J.X., Tang, W., Yang, Z.S., Wan, J., Shi, L.P., Zhang, Y., Zhou, R., Ni, J., Hou, L.F., Zhou, Y., He, P.L., Yang, I.F., Li, Y., Zuo, J.P., 2007. Suppressive effect of a novel water-soluble artemisinin derivative SM905 on T cell activation and proliferation in vitro and in vivo. European Journal of Pharmacology 14, 211–218.
Welter, A., Mineo, J.R., Silva, D.A.O., Lourenço, E.V., Ferro, E.A.V., Roque-Barreira, M.C., Silva, N.M., 2006. An opposite role is exerted by the acarian *Myocoptes musculinus* in the outcome of *Toxoplasma gondii* infection according to the route of the protozoa inoculation. Microbes and Infection 8, 2618–2628.
Willcox, M., Falquet, J., Ferreira, J.F.S., Gilbert, B., Hsu, E., Melillo Magalhães, P., Plaizier-Vercammen, J., Sharma, V.P., Wright, C.W., Yaode, W., 2007. *Artemisia annua* as a herbal tea for malaria. The African Journal Traditional, Complementary and Alternative Medicines 4, 121–123.
Zhou, W.L., Wu, J.M., Wu, Q.L., Wang, J.X., Zhou, Y., Zhou, R., He, PL., Li, X.Y., Yang, Y.F., Zhang, Y., Li, Y., Zuo, J.P., 2005. A novel artemisinin derivative, 3-(12-beta-artemisininoxy) phenoxyl succinic acid (SM735), mediates immunosuppressive effects in vitro and in vivo. Acta Pharmacologica Sinica 26, 1352–1358.