Article/Artigo

In vitro action of antiparasitic drugs, especially artesunate, against *Toxoplasma gondii*

Ação *in vitro* de drogas antiparasitárias, especialmente artesunato, contra *Toxoplasma gondii*

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ABSTRACT

Introduction: Toxoplasmosis is usually a benign infection, except in the event of ocular, central nervous system (CNS), or congenital disease and particularly when the patient is immunocompromised. Treatment consists of drugs that frequently cause adverse effects; thus, newer, more effective drugs are needed. In this study, the possible activity of artesunate, a drug successfully being used for the treatment of malaria, on *Toxoplasma gondii* growth in cell culture is evaluated and compared with the action of drugs that are already being used against this parasite. **Methods:** LLC-MK2 cells were cultivated in RPMI medium, kept in disposable plastic bottles, and incubated at 36ºC with 5% CO_2. Tachyzoites of the RH strain were used. The following drugs were tested: artesunate, cotrimoxazole, pentamidine, pyrimethamine, quinine, and trimethoprim. The effects of these drugs on tachyzoites and LLC-MK2 cells were analyzed using nonlinear regression analysis with Prism 3.0 software. **Results:** Artesunate showed a mean tachyzoite inhibitory concentration (IC_{50}) of 0.075µM and an LLC MK2 toxicity of 2.003µM. Pyrimethamine was effective at an IC_{50} of 0.482µM and a toxicity of 11.178µM. Trimethoprim alone was effective against the *in vitro* parasite. Cotrimoxazole also was effective against the parasite but at higher concentrations than those observed for artesunate and pyrimethamine. Pentamidine and quinine had no inhibitory effect over tachyzoites. **Conclusions:** Artesunate is proven *in vitro* to be a useful alternative for the treatment of toxoplasmosis, implying a subsequent *in vivo* effect and suggesting the mechanism of this drug against the parasite.

Keywords: Artesunate. Treatment. Anti-*Toxoplasma* activity. Toxicity.

RESUMO

Introdução: Toxoplasmose é geralmente uma infecção benigna, exceto nos eventos de doença ocular, congênito e do sistema nervoso central, e particularmente quando o paciente é imunocomprometido. O tratamento consiste de drogas que frequentemente causam efeitos adversos, então novas drogas, mais efetivas são necessárias. Neste estudo, a possível atividade de artesunato, uma droga usada com sucesso no tratamento da malária, sobre o crescimento de *Toxoplasma gondii* em cultura celular é avaliado e comparado à ação de drogas que já estão sendo utilizadas contra este parasita. **Métodos:** Células LLC-MK2 foram cultivadas em meio RPMI, mantidas em garrafas plásticas descartáveis e incubados a 36ºC com 5% CO_2. Taquizoítos da cepa RH foram usados. As seguintes drogas foram testadas: artesunato, cotrimoxazol, pentamidina, pirimetamina, quinino e trimetoprima. Os efeitos dessas drogas sobre taquizoítos foram analisados por análise regressiva não linear com o *software* Prism 3.0. **Resultados:** Artesunato mostrou uma concentração inibitória media (IC_{50}) de 0,075µM e uma toxicidade sobre células LLC MK2 de 2,003µM. Pirimetamina foi efetiva a uma IC_{50} de 0,482µM e uma toxicidade de 11,178µM. Trimetoprima sozinha foi efetiva contra o parasita *in vitro*. Cotrimoxazol também foi efetivo contra o parasita, mas a concentrações mais altas que aquelas observadas para artesunato e pirimetamina. Pentamidina e quinino não tiveram efeitos inibitórios sobre os taquizoítos. **Conclusões:** Provou-se que artesunato *in vitro* pode ser uma alternativa útil para o tratamento da toxoplasmose, implicando um subsequente efeito *in vivo* e sugerindo o mecanismo desta droga contra o parasita.

Palavras-chaves: Artesunato. Tratamento. Atividade anti-*Toxoplasma.* Toxicidade.

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INTRODUCTION

Toxoplasmosis is a highly prevalent cosmopolitan infection, but the disease occurs in only a fraction of infected people, mainly as a nonspecific immune activation syndrome, chronically in ocular forms as chorioretinitis. The main problems are congenital disease and the infection of immunocompromised people, especially those with acquired immunodeficiency syndrome (AIDS) or those undergoing chemotherapy for cancer or transplant rejection[1].

The etiological agent of toxoplasmosis is *Toxoplasma gondii*, the development of which has many forms. Tachyzoites are found in the acute phase of the disease and are responsible for clinical manifestations. They are susceptible to the immune response of the host and to drug action. Cysts are the resistant form of the parasite, persisting for the host's entire life. Cyst walls are resistant to both drugs and the immune system[2].

Felids are the definitive hosts for the parasite, with other mammalians and birds acting as intermediate hosts. Humans can be infected either congenitally or through ingestion of raw or undercooked meat; manipulation of infected meat containing tissue cysts; or consumption of water, fresh vegetables, or other food contaminated by oocysts eliminated in cat feces[1,2].

The most effective treatment against toxoplasmosis is a combination of the drugs sulfadiazine and pyrimethamine, which can cause hematological effects that are controlled with the administration of folinic acid. An association of great interest is the one between trimethoprim and sulfamethoxazole. Known as cotrimoxazole, its active compounds act synergistically, inhibiting two consecutive steps of folinic acid biosynthesis in a manner similar to that observed for pyrimethamine-sulfadoxine. Cotrimoxazole is well tolerated and less toxic to hematopoiesis. Human immunodeficiency virus–acquired immunodeficiency syndrome (HIV-AIDS) patients taking cotrimoxazole show a high incidence of adverse effects, and its use is discouraged in pregnant women because it crosses the placental barrier[3].

Although antifolate compounds, such as pyrimethamine, exhibit good anti-*Toxoplasma* activity, their toxicity limits widespread use, particularly for extended treatment periods. The discovery of viable low-toxicity compounds capable of preventing and treating *T. gondii* would represent a great advance in the treatment of infections in immunocompromised patients. Some compounds that are effective against species of *Plasmodium* could be effective against *T. gondii*. Those agents were selected for further testing in the present study because malaria and toxoplasmosis are caused by protozoans belonging to the phylum Apicomplexa, and antimicrobial agents that have been effective for the treatment of malaria, such as artemisinin and its derivatives, also have been effective for the treatment of toxoplasmosis. Artemisinin (qinghaosu) is a product extracted from the plant *Artemisia annua* L. Despite the fact that artemisinin has produced teratogenic effects in laboratory animals, precluding its use in pregnant women, few adverse effects have been observed in humans[4,5].

In the present study, the toxicity of artesunate and its effectiveness for the treatment of toxoplasmosis were studied *in vitro* and compared with the actions of three drugs: pyrimethamine, trimethoprim, and cotrimoxazole, which are currently in use against toxoplasmosis. Pentamidine and quinine, used for the treatment of other protozoans, also were evaluated and compared. Pentamidine is an organic compound and derivative of guanidine that has shown activity against *Leishmania sp.*, African trypanosomiasis, and pneumonia caused by *Pneumocystis carinii (jiroveci)*. Quinine is an alkaloid extracted from species of the genus *Cinchona*, the application of which is limited to cases of malaria caused by *Plasmodium falciparum*.

METHODS

Parasites

Tachyzoites of the type I RH strain of *T. gondii* were routinely maintained by intraperitoneal passage in BALB/c mice.

Drugs

All drugs were obtained from commercial sources (Sigma, USA) or as human use drugs, supplied by the pharmacy of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HC-FMUSP). Artesunate and quinine dichlorohydrate were supplied by Cipla Medpro (Belville, SA), pentamidine was supplied by Itaca Labs (Rio de Janeiro, BR), and cotrimoxazole was supplied by Ducto (Anapolis, BR).

Cell culture

The epithelial cell line LLC-MK2, derived from rhesus monkey (*Macaca mulatta*) kidneys, was used. Cells were cultivated in RPMI medium with the addition of 10% inactivated bovine fetal serum and gentamicin. Cultures were kept in disposable plastic bottles and incubated at 36ºC with 5% CO_2.

In vitro assays of drug effectiveness and toxicity

The assays were conducted in four stages, the first three on consecutive days. On the first day, LLC-MK2 cells were extracted from a plastic bottle with ATV enzyme and counted in a Neubauer counting chamber. Cells were diluted in RPMI medium containing 10% inactivated bovine fetal serum until a concentration of 1×10^4 was obtained. One hundred microliters of the mixture was added to each well of a 96-well plate and placed inside the CO_2 incubator. On the second day, the supernatant from all the wells was aspirated. Tachyzoites from the peritoneal fluid of BALB/c mice were extracted with a syringe and diluted in complete RPMI medium containing 10% bovine fetal serum until a concentration of 1×10^4 was obtained. One hundred microliters of the mixture was added to the wells in rows A to E and columns 1 to 12 of the plate and put into a CO_2 incubator. The remaining wells were filled with complete RPMI containing 10% bovine fetal serum. On the third day, the plate was washed with complete RPMI medium containing 10% inactivated bovine fetal serum. A mother solution of each drug was prepared by diluting each one in a solution of complete RPMI medium containing 10% inactivated bovine fetal serum until a 200µL/mL concentration was obtained. Two-fold serial dilutions of all compounds were performed, starting with initial concentrations of 100µg/mL. Therefore, the concentrations used were as follows: 100, 50, 25, 12.5, 6.25, 3.1, 1.6, 0.8, 0.4, 0.2, and 0.1µg/mL. For pyrimethamine and trimethoprim, the first dilution was made in dimethyl sulfoxide, and the remaining dilutions were made in RPMI because of the low solubility of these compounds in water. On another 96-well plate, 200µL of each drug solution was pipetted into wells in column 1, whereas 100µL of RPMI medium containing 10% inactivated bovine fetal serum solution was pipetted into the wells in columns 2 to 12. A two-fold serial dilution was performed by transferring 100µL from column 1 to those in column 2 and repeating this procedure until column 11. The contents of the second plate were transferred to the first plate and placed inside the CO_2 incubator. The reaction was interrupted when the tachyzoites had destroyed all the cells from the positive control wells. After that, the supernatant was moved, and the plate was washed with PBS, fixed with methanol, and stained with 1% aqueous crystal violet solution. After being washed, the plate was dried, and 200µL of methanol was added to dissolve the stain. The A_{620} was measured with an ELISA microplate reader. Adherent live cells were stained. The A_{620} is proportional to the number of viable cells. This allowed both the measurement of *T. gondii* cell destruction, or cell toxicity, and detection of infected or non-infected cell layers.

Statistical analysis

Drug effects on both tachyzoites *in vitro* and LLC-MK2 cells were analyzed using nonlinear regression analysis with Prism 3.0 software, yielding mean inhibitory concentrations (IC_{50}) for the studied compounds.

RESULTS

Artesunate at a concentration of 100µg/mL killed all cells in the culture. Therefore, the experiment was performed again with a maximum concentration of 10µg/mL. It was observed that artesunate was effective against tachyzoites at an IC_{50} of 0.075µM, resulting in preservation of the cell line (**Figure 1A** and **1B**). The mean toxicity of the drug was 2.003µM (**Figure 2A** and **2B**).

Cell death also was observed with pyrimethamine at 100µg/mL in the cell culture so that experiment also was performed again with a maximum concentration of 10µg/mL. It was observed that pyrimethamine was effective against tachyzoites at an IC_{50} of 0.482µM, resulting in the preservation of the cell line. The mean toxicity of the drug was 11.178µM (**Figure 3A** and **3B**).

Cotrimoxazole proved to be effective against tachyzoites at an IC_{50} of 11.884µM, whereas trimethoprim was effective against

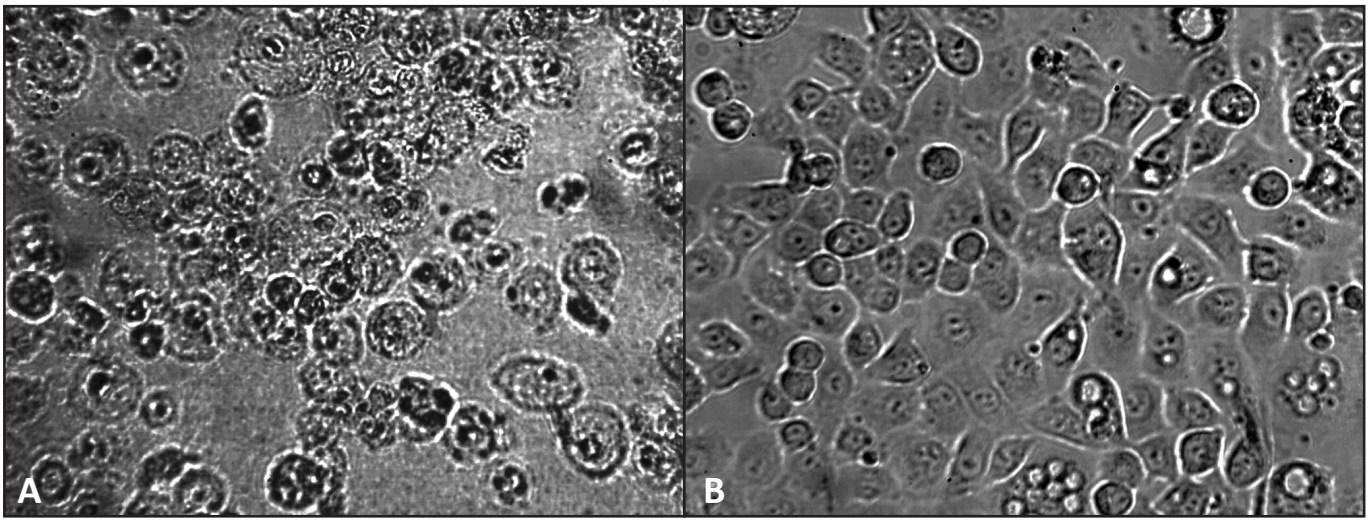

FIGURE 1 – Morphology of LLC-MK2 cells after (A) *Toxoplasma gondii* infection and (B) artesunate treatment following parasite challenge.
Digital images are from inverted microscope phase-contrast microscopy with 20 × objective.

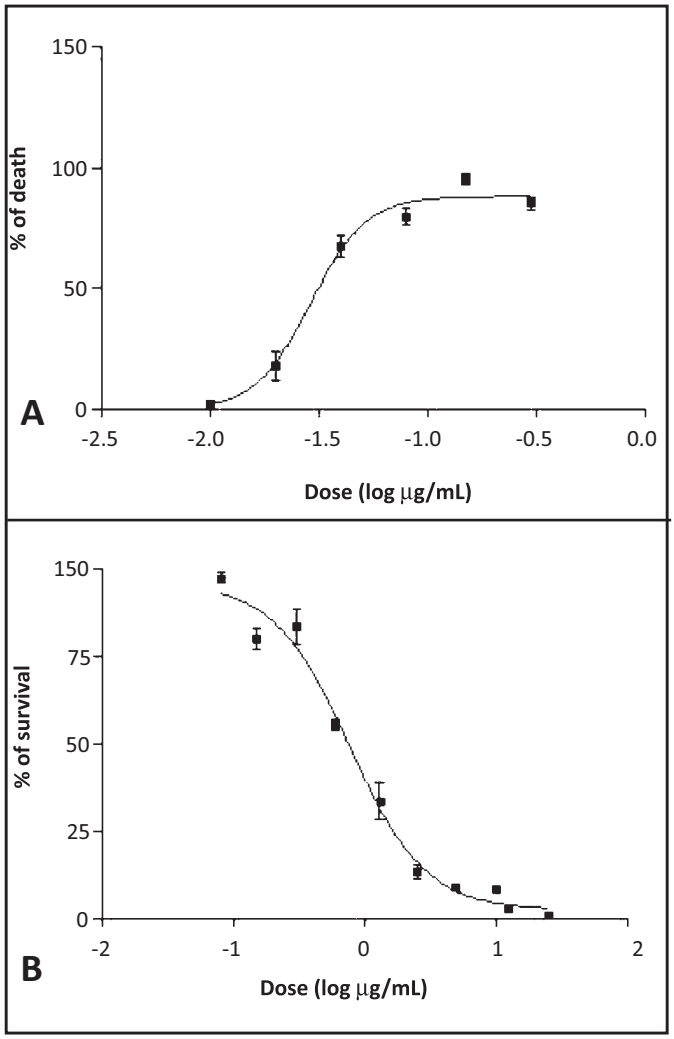

FIGURE 2 - A) Artesunate action in cell culture infected with *Toxoplasma gondii* at concentrations of 10 to 0.01μg/mL. B) Artesunate toxicity in cell culture, at concentrations of 10 to 0.01μg/mL.

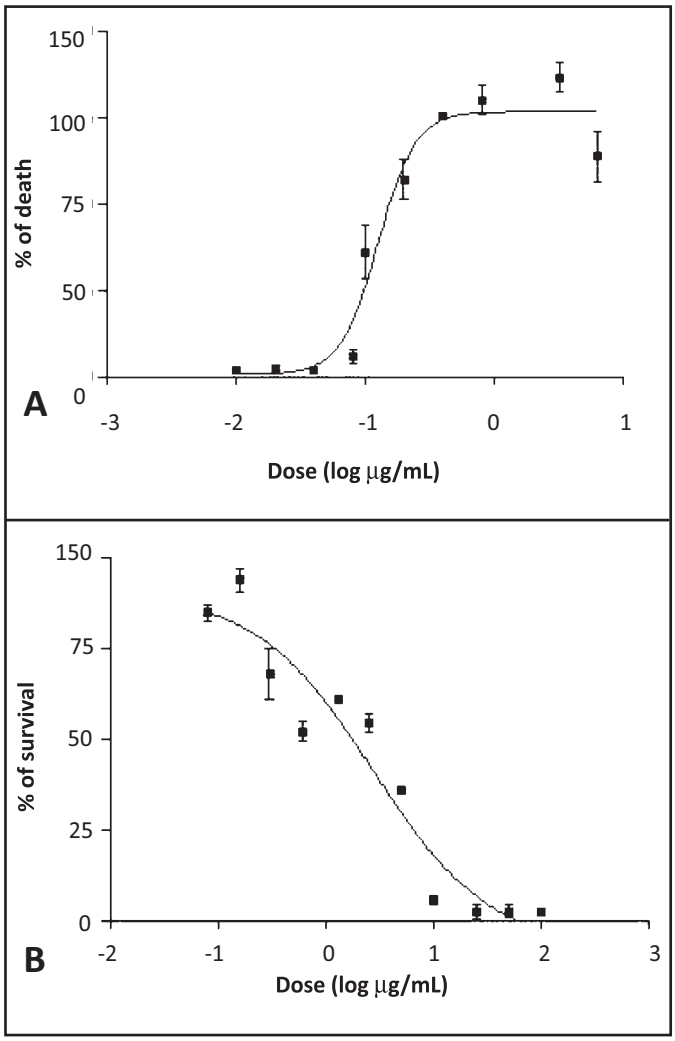

FIGURE 3 - A) Pyrimethamine action in cell culture infected with *Toxoplasma gondii* at concentrations of 10 to 0.01μg/mL. B) Pyrimethamine toxicity in cell culture at concentrations of 10 to 0.01μg/mL.

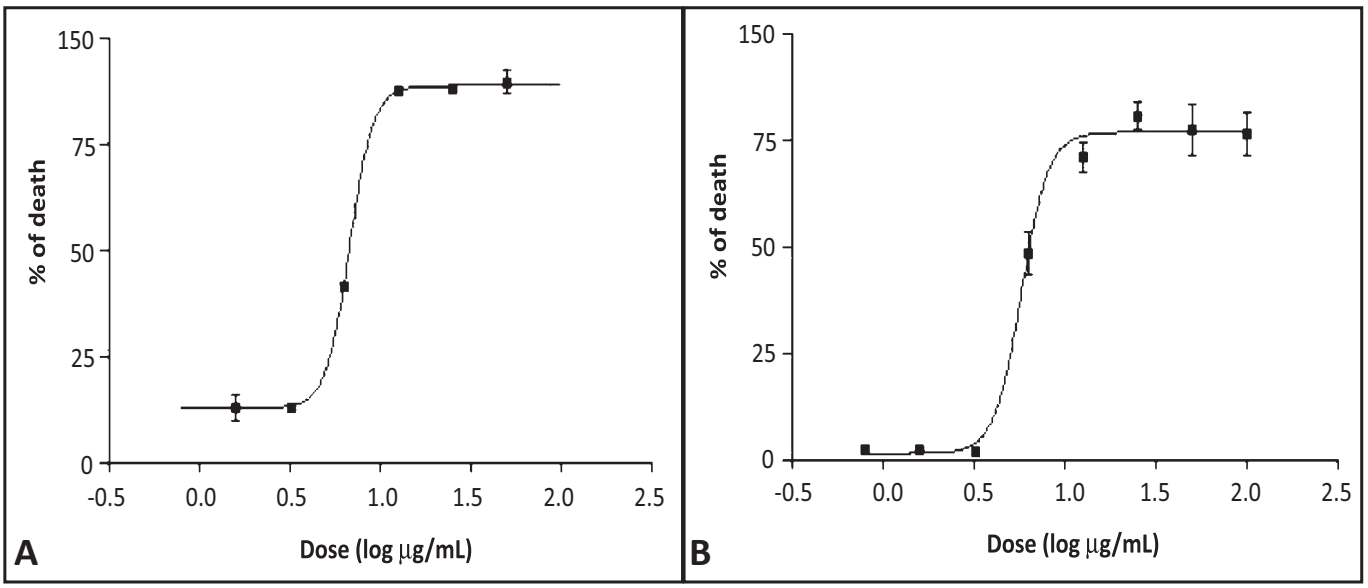

FIGURE 4 - A) Cotrimoxazole action in cell culture infected with *Toxoplasma gondii* at concentrations of 100 to 0.1µg/mL. B) Trimethoprim action in cell culture infected with *Toxoplasma gondii* at concentrations of 100 to 0.1µg/mL.

Toxoplasma gondii at an IC_{50} of 20.116µM. Neither drug was toxic to the cell culture at the tested concentrations (**Figure 4A** and **4B**).

Pentamidine had no effect on tachyzoites at the tested concentrations, resulting in destruction of the cell line. The same results were observed for quinine. The mean toxicity of pentamidine was 1,316µM, and for quinine, it was 91.030µM.

The selectivity index (SI) could be calculated only for artesunate and pyrimethamine. The SI for the first one was 26.707, and that for the latter was 23.191.

In the present study, artesunate showed the highest efficacy, followed by pyrimethamine. Moreover, artesunate presented the highest toxicity among the studied compounds, again followed by pyrimethamine.

An absence of cotrimoxazole and trimethoprim was observed, yet each showed a weaker effect than artesunate and pyrimethamine. Pentamidine and quinine had no inhibitory effects over *T. gondii* in this study.

The mean inhibitory concentrations and toxicities, 95% confidence intervals, and r^2 values for all drugs used in the study are shown in **Table 1**. The selectivity index for two of the six drugs could be calculated and also is shown. As can be seen, the most effective drugs against *T. gondii* were pyrimethamine and artesunate.

TABLE 1 - Values of inhibitory concentrations and toxic concentrations in LLC-MK2 cells (µg/mL and µM) for the tested drugs and selectivity index.

Drugs	IC50 (µM)	IC50 (µg/ml)	95% IC (µM)	R^2	IC50 tox (µM)	IC50 tox (µg/ml)	95% IC (µM)	R^2	SI
Artesunate	0.075	0.029	0.062 to 0.088	0.94	2.003	0.77	1.613 to 2.471	0.97	26.707
Cotrimoxazole	11.884	6.78	12.472 to 13.208	0.99	-	>100	-	-	-
Pentamidine	-	-	-	-	1.316	0.76	10.832 to 15.523	0.89	-
Pyrimethamine	0.482	0.12	0.402 to 0.603	0.89	11.178	2.78	5.790 to 21.672	0.91	23.191
Quininum	-	-	-	-	91.030	36.12	28.363 to 291.438	0.77	-
Trimethoprim	20.116	5.8	17.842 to 22.354	0.95	-	>100	-	-	-

IC50: *half maximal inhibitory concentration*; **95%CI:** 95% Confidence interval**; IC50 tox:** *half maximal* toxic concentrations; **R^2:** *Correlation coefficient* **SI:** Selectivity index.

DISCUSSION

An *in vitro* effect of artesunate against *T. gondii* was found. Previous reports had demonstrated that this compound showed action against other parasites, such as *Plasmodium*[6] and *Fasciola*[7]. Although inhibitory concentrations were higher than those used against the parasites that cause malaria, artemisinin and many of its derivatives were effective against *T. gondii*[5]. In this experiment, artesunate exhibited a strong effect against *T. gondii* tachyzoites in *in vitro* assays. This work represents an initial step toward future studies of the *in vivo* action of this compound and its effect on the cystic form of the parasite. Artesunate could be an alternative to the standard pyrimethamine-sulfonamide treatment.

Effective action of artesunate against tachyzoites was observed, and it was the highest among all studied compounds. Several studies demonstrating the action of artemisinin derivatives have been conducted, including Ke Ou-Yang et al.[8], D'Angelo et al.[9], and Sarciron et al.[10]. El Zawary, in 2008, studied the *in vitro* action of artesunate against RH strain *Toxoplasma* and observed a significant reduction in the viability and effect of tachyzoites exposed to drugs compared with a no-treatment control. Although the efficacy of artesunate was demonstrated both here and in El Zawary, the inhibitory concentration values for artesunate found by El Zawary were higher than those observed in the present work. However, because there is no standard methodology for drug testing in cell lines, different outcomes could result[11].

Clark et al.[12,13] observed that artesunate is toxic to the embryos of mice, rabbits, and nonhuman primates, causing cardiovascular and skeletal problems, even death, when given at higher doses and over longer periods than recommended for the treatment of malaria[12,13].

At this time, no adverse effects related to the drug have been reported in pregnant women treated with artemisinin, including artesunate. Although the number of pregnant women exposed to artemisinin during the first trimester is considered too small to demonstrate safety, the absence of any adverse effects to the babies in these limited published clinical studies is encouraging.

In this work, cotrimoxazole was effective against *T. gondii* and not toxic to the cell culture at tested concentrations. The synergistic *in vitro* effect between trimethoprim and sulfamethoxazole was demonstrated many decades ago, by Grossman and Remington[14] and Derouin and Chastang[18]. More recent studies such as those by Dumas et al.[15] and Soheilian et al.[16] support the use of this combination to prevent cerebral and ocular toxoplasmosis[14-16].

Lindsay et al.[17] examined the ability of pentamidine and nine of its analogs to inhibit the replication of RH strain *T. gondii* in Vero cell cultures. In that study, pentamidine at 25 and 10μg/mL was shown to have significant effects over tachyzoite replication. Lindsay et al.[17] obtained a different result from that in the present work, where no anti-*Toxoplasma* activity of pentamidine was observed at tested concentrations. The conflicting results between these studies may be due to the different cell lines used or differences in methodology because there is no standard model among the authors who perform assays with drugs[17].

Pyrimethamine is the main drug of choice for the treatment of toxoplasmosis. It is well known that this drug exhibits *in vitro* activity against the parasite, as demonstrated by studies such as Derouin and Chastang[18], Cantin and Chamberland[19], Ven et al.[20], and Meneceur et al.[4]. The findings of the present study were very similar to those previously mentioned, all of them showing effective action at concentrations between 0.05 and 0.24μg/mL on RH strain. When compared with artesunate, pyrimethamine has been shown to be less effective but also less toxic. The selectivity index obtained for these drugs were similar; therefore, artesunate was shown to be a promising option for the treatment of toxoplasmosis[18-20].

No anti-*Toxoplasma* activity was observed for quinine at tested concentrations. These results are compatible with the *in vitro* experiments from Holfels, which tested quinine sulfate on RH strain *T. gondii* at 2, 10, and 20μg/mL and observed no inhibitory effects on intracellular tachyzoites.

Since the 1970s, several works have demonstrated the effectiveness of trimethoprim against *T. gondii in vitro*, among them, Grossman and Remington, Derouin and Chastang, Ven et al.[20], and D'Angelo et al.[9]. Most of these works used RH strain, and all of them found IC_{50} values between 2 and 10μg/mL. The authors of these studies point out that a murine model is inadequate to evaluate trimethoprim's efficacy because of the difference in the drug's half-life in human and rat sera with a mean toxicity of 60μg/mL, and more human *in vivo* studies are therefore needed. Because of the short half-life of trimethoprim, a significant inhibitory concentration may not be sustained in human sera, which could explain the poor efficacy of this agent alone. Therefore, besides its low toxicity, trimethoprim on its own is not considered an alternative to pyrimethamine for the treatment of toxoplasmosis[9,14,18,20].

In this study, artesunate showed the highest efficacy among the compounds studied, followed by pyrimethamine. Along with their higher efficacy, however, these drugs showed higher toxicity in cell culture. Trimethoprim has shown both efficacy and low toxicity, but treatment with this drug alone is not effective. It is combined with sulfamethoxazole to form cotrimoxazole, which also has been tested and shown to be effective and nontoxic at administered concentrations, lending support to the use of this drug as an alternative treatment to toxoplasmosis.

The data obtained in the present study suggest that artesunate could be a useful alternative to antifolates in the treatment of toxoplasmosis. Further study of artesunate is still required, specifically into its action against *T. gondii in vivo* and its efficacy against tissue cysts.

The possible toxic effects of artesunate on pregnant women who are being treated for malaria should continue to be investigated, keeping in mind that the dose necessary to kill *T. gondii* is higher than that for *Plasmodium* sp.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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