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INTRACELLULAR *TOXOPLASMA GONDII* ELIMINATION IN PRESENCE OF JcTI-I, A PROTEASE INHIBITOR FROM *JATROPHA CURCAS* SEED CAKE

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ABSTRACT

Toxoplasma gondii is the agent of toxoplasmosis, a widespread disease of medical and veterinary importance where the parasites infect all eukaryotic cells of vertebrate animals. There is no effective and low toxic treatment to eliminate the intracellular parasites, perpetuating the infection. The *Jatropha* species, including *Jatropha curcas*, acquired high economic importance as a source of high-quality biofuel and medicinal plant. Nevertheless, the anti-parasite activity of the *Jatropha* species has not been reported to date. In this study, the effects of a protease inhibitor from *Jatropha curcas* seed cake, named JcTI-I, on extra- and intracellular tachyzoites of *T. gondii* were assessed *in vitro*. Extracellular tachyzoites treated before invasion had morphological modifications but did not altered the establishment of infection. The incubation of infected host cells with 5, 10, 50, and 100 µM JcTI-I for 24 h decreased the intracellular parasite number (IC_{50} = 4.57 µM), without causing a significant reduction in the infected host cell population. Moreover, JcTI-I led ultrastructural alterations on intracellular tachyzoites as assessed by light and transmission electron microscopy. In summary, JcTI-I is a promising candidate for drug development to treat toxoplasmosis and other intracellular parasites.

KEYWORDS: Physic nut; anti-*Toxoplasma gondii* action; serine protease inhibitor; antiprotozoal agent.

INTRODUCTION

Toxoplasma gondii, the agent of toxoplasmosis, is a widespread zoonotic parasite[1] of medical and veterinary importance that infects all vertebrate animals with high prevalence in humans.[2] *T. gondii* exists in two main morphological forms during its complex parasite life cycle and uses birds and mammals as intermediates and felines as definitive hosts.[3] Tachyzoites, the infective form, utilize secretory organelles and apical structures to invade all host cells, leading to the success of infection. Once located in the intracellular environment, *T. gondii* resides and proliferates into the parasitophorous vacuole (PV) until the host cell undergoes lysis, and the released parasite infects new cells.[4] This PV is formed mainly by the host cell plasma membrane, but it is constantly modified by products secreted by the parasite.[5,6] The PV membrane is permeable to metabolics, nutrients, and proteins, facilitating the replication of the parasite.[7] During the intracellular development on parasitophorous vacuole, the tachyzoites secretes molecules and associates with host cell organelles, to maintain the infection.[8,9] Among these molecules responsible for parasite invasion and infection development, the proteases have received special attention. Shaw and colleagues were the first to report that proteases are important to host cell invasion by Apicomplexa.[10] Currently, it is well established that *T. gondii* uses proteases for host cell invasion, nutrient acquisition, avoidance of host microbicidal responses, evasion from the parasitophorous vacuole, differentiation, and other activities.[11]

Jatropha curcas, a small tree that belongs to the *Euphorbiaceae* family, is an economic resource that provides oiled seeds for biodiesel production.[12] *Jatropha* species are native of Caribbean regions, Africa and North America where they have been used in popular medicine to treat skin, cancer, digestive and infectious diseases.[13] Although *J. curcas* has been used in phytomedicine[13], little is known regarding the

parasiticidal properties of its plant products and isolated molecules. For instance, *J. curcas* seeds contain highly toxic phorbol esters and curcin[14] in addition to the antinutritional factors protease inhibitors, lectin, saponin, and phytate were described.[15] Recently, a trypsin protease inhibitor was isolated from *J. curcas* seed cakes (Jc-TI-I) and tested against pathogenic bacteria species with promising results.[16]

In this study, we investigated whether the JcTI-I[16], has anti-*Toxoplasma gondii* activity against extracellular and intracellular *T. gondii* tachyzoites.

MATERIALS AND METHODS

Host cells and *Toxoplasma gondii* maintenance

Vero cells were maintained in a DMEM 1152 medium (DULBECCO`S MODIFIED EAGLE'S MEDIUM, SIGMA-ALDRICH, ST. LOUIS, MISSOURI, EUA), supplemented with 5% (v/v) FBS (fetal bovine serum) (SIGMA) and streptomycin (1 µg/mL) in sterile plastic flasks (60 mL). *T. gondii* tachyzoites (RH strain) were maintained in infected host cells at a ratio of 5:1 (parasites: Vero cells).[17]

Parasite – Host cell interaction

The Vero cell cultures were treated with trypsin (SIGMA) and transferred to sterile 24-well plates containing 3 x 10^4 cells per well. The cell infection with *T. gondii* tachyzoites was performed as previously described.[17]

JcTI-I purification

JcTI-I was purified according to.[16] Briefly, soluble proteins were extracted from defatted flour from *J. curcas* seeds cake with 0.1 M sodium borate buffer, pH 10.0, in a 1:10 (w/v) proportion, for 4 h at 4 ˚C. After centrifugation, the proteins in the crude extract were fractionated by precipitation with 2.5% (v/v) trichloroacetic acid (TCA). The suspension was centrifuged and the supernatant, containing 30 mg protein, was loaded onto a trypsin-Sepharose 4B affinity column. After the complete removal of the non-retained proteins, the bound-proteins were eluted with 0.1 M glycine-HCl buffer, pH 2.6. After an exhaustive dialysis against Milli-Q grade water and lyophilization, the protein fraction obtained was loaded onto a Sephacryl S-200 column and the protease inhibitor JcTI-I was eluted with 0.050 M sodium phosphate buffer, pH 7.5, containing 0.2 M NaCl.

Anti-*T. gondii* assay

JcTI-I, comprising 5, 10, 50, and 100 µM final concentrations, prepared in a DMEM 1152 medium supplemented with 5% (v/v) FBS and 1 µg/mL streptomycin, was incubated with the Vero cells that were previously infected (24 h) with *T. gondii* tachyzoites. In addition, free tachyzoites that were pre-incubated with JcTI-I at 100 µM for 30 min and washed with the culture medium to remove the JcTI-I, were incubated with the host Vero cells for 24 h. Furthermore, free tachyzoites were treated with JcTI-I (100 µM) but were immediately incubated with the host cells for 1 h, washed and analyzed. As the control, tachyzoites and infected Vero cells were incubated with the culture medium in the absence of JcTI-I. For the morphological analysis, the samples were processed and visualized as previously described.[17] At least 400 Vero cells from the same sample were taken from three randomly selected fields of eight separate glass slides and counted under an optical microscope (Zeiss Axiovert 135) at 400× magnification.[18]

Ultrastructural studies

For the ultrastructural analyses, free tachyzoites and Vero cells previously infected with *T. gondii* tachyzoites were treated with 6 µM JcTI-I as described above. After 24 h, the samples were processed for the ultrastructural analysis as previously described.[19]

Statistical analysis

Data were expressed as the mean±SEM and analyzed with GraphPad Prism 5.0 (analysis of variance – ANOVA), followed by a Tukey multiple comparison test. Differences between the mean values were considered significant at $p \leq 0.01$. The value of the half-maximal inhibitory concentration (IC_{50}) of JcTI-I was calculated from three independent experiments with the transformation of the JCTI-I concentration data and normalization of the parasite number to construct the dose-response curve (GraphPad Prism 5.0).

RESULTS AND DISCUSSION

Effect of JcTI-I on *in vitro* infection and intracellular parasite development

JcTI-I was not cytotoxic to Vero cells since none of the four concentrations (5, 10, 50, and 100 µM) promoted cell death and elimination (Table 1). This finding indicated that JcTI-I could be used to assess intracellular parasite.

The Vero cells that were previously infected with *T. gondii* and harvested 24 h later contained tachyzoites in the proliferative stage and an increasing parasite number in the parasitophorous vacuole, which characterized the establishment and progression of the infection. In contrast, the incubation of JcTI-I with the Vero cells that were pre-infected (24 h) with tachyzoites decreased ($p \leq 0.01$) both the infection and intracellular parasites. Indeed, particularly at the 10, 50 and 100 µM concentrations, JcTI-I promoted a 33%, 64% and 67% reduction in the infected Vero cell numbers and a 63%, 78%, and approximately 90% reduction in the intracellular parasite numbers, respectively (Table 1). Here, half-moon shaped tachyzoites were considered viable although cells containing parasites with different shapes were considered infected. Nevertheless, JcTI-I presented an IC_{50} of 4.57 µM (Table 1).

Table 1: Mean number of uninfected and infected cells treated with JcTI-I for 24 h at different concentrations. ± Standard deviation of six different samples.

JcTI-I (µM)	Uninfected cells	Infected cells	Intracellular tachyzoites
0	214.0 ± 17.4	60.5 ± 5	504.0 ± 29.0
5	226.7 ± 17.4	60.1 ± 13	275.7 ± 29.0
10	198.1 ± 14.4	40.5 ± 2	186.5 ± 4.0
50	227.8 ± 20.0	27.8 ± 2	113.8 ± 10.0
100	220.5 ± 13.1	20.0 ± 3	58.0 ± 7.0

Several plant extracts and natural compounds were tested against *T. gondii*.[20] Studies exploring anti-protozoan activity of *Jatropha* genus showed three microcyclic lathyrane diterpenoids purified from the methanolic extract of *Jatropha multifida* stem bark exhibited *in vitro* anti-parasite activity against extracellular *Leishmania donovani* and *Plasmodium falciparum*.[21]

Regarding *J. curcas*, extracts prepared from various plant parts showed antimicrobial activity against a variety of microorganisms, including bacterial and fungal species of medical importance as *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, *Microsporum gypseum*.[16,17, 21-25] Soares et al.[17] reported the effects of an aqueous protein extract from *J. curcas* seed cake and its derived cysteine protease inhibitor enriched fraction on intracellular *T. gondii*. This cysteine protease inhibitor enriched preparation decreased the number of infected Vero cells and intracellular parasites (IC_{50} of 1.59 mg/mL) after 24 h of treatment with no toxic effect on the host cells.

Effect of the pretreatment of *T. gondii* tachyzoites with JcTI-I before parasite invasion on Vero cells

The effects of the pretreatment (30 min) of *T. gondii* free tachyzoites with JcTI-I (100 µM) on the posterior parasite invasion and replication in Vero cells were assessed to verify if the protease activity inhibits parasite invasion and development. The number of infected cells in the control (152 ± 21) was not different from that in the cells treated with JcTI-I (158 ± 25) 24 h after the parasite infection, and the parasite number remained unaltered (862 ± 65 in the control and 823 ± 49 after the treatment). In the assay in which the parasites were treated with 100 µM JcTI-I and immediately incubated with Vero cells for 1 h, no difference was observed in the infected cell numbers in the control (193 ± 27) compared with that in the JcTI-I-treated group (185 ± 19) or the intracellular parasite number (733 ± 58, control; 711 ± 45, treated-group).

Proteases play important roles in protozoan parasites since they participate in host cell invasion, metabolism, parasite development and the activation or turnover of parasite enzymes or regulatory proteins.[26] Specifically, in *T. gondii*, the invasion step involves the coordinated release of specialized secretory organelles, and serine and cysteine proteases subtypes may mediate both events.[10] *T. gondii* also has catalytic complexes and proteasomes with proteolytic activities, such as trypsin- and chymotrypsin-like serine proteases to sustain the infection.[27] Based on the information above, it is plausible to speculate that protease inhibitors affect both the parasite invasion process and intracellular development.[28] For example, Conseil et al. showed that irreversible serine protease inhibitors of a non-protein nature, i.e., 3,4-dichloroisocoumarin and 4-(2-aminoethyl)-benzenesulfonyl fluoride, prevented *T. gondii* invasion without affecting its morphology, basic metabolism, or gliding motility.[29] Other study showed the treatment of extracellular tachyzoites with the cysteine protease inhibitors pepstatin and E64 (up to 100 µM) did not alter the parasite growth, but, at the concentration used, both inhibitors reduced the percentage of parasites that successfully penetrated the cells. Nonetheless, our results showed that the pretreatment of the tachyzoites did not affect the invasion, although the infection development was reduced when infected cells were treated.

In the present study, the pre-treatment of *T. gondii* tachyzoites with JcTI-I for 30 min followed by washings to eliminate the inhibitor did not prevent the parasite replication in an exponential manner similarly to the control culture. This finding suggests that structural disorders, if present, were not sufficient for preventing host cell invasion by the *T. gondii* tachyzoites. Indeed, when the *T. gondii* tachyzoites were treated with JcTI-I and immediately incubated with the Vero cells, no differences were observed in comparison with the control. Cysteine proteinase inhibitors inhibit *T. gondii* invasion, growth[30] and gliding motility at low-micromolar concentrations by preventing the secretion of microneme-derived adhesins to the parasite surface.[9] Moreover, cysteine protease peptidyl inhibitors and chymotrypsin-like serine protease inhibitors, at micromolar concentrations, decreased *T. gondii* growth and replication 24 h after host cell entry. However, these inhibitors were highly toxic to the host cells after incubation for 72 h. Furthermore, some of these inhibitors failed to block *T. gondii* replication possibly because they present stage-specific differentiation similar to that of other protozoan parasites.[31-33] These results indicate that JcTI-I did not affect the organelles or proteins that are involved in host cell invasion and/or parasitophorous vacuole formation under the experimental conditions used. The DNAse activity of JcTI-I (unpublished data) likely contributes to the anti-*T. gondii* activity observed in this study.

Ultrastructural features of extracellular *T. gondii* and infected Vero cells

The untreated (control) cells infected with *T. gondii* showed the typical morphological characteristics without vacuolization in the cytoplasm and parasitophorous vacuoles containing proliferative tachyzoites (Fig. 1A). Following all JcTI-I treatments, the Vero cells presented the same features as the control cultures. However,

significant differences were observed among the parasites. Indeed, tachyzoites were progressively destroyed or eliminated as the JcTI-I concentrations increased, particularly at 100 µM (Fig. 1B). For the *T. gondii* previously incubated for 30 min with 100 µM JcTI-I prior to the Vero cell infection, no difference was observed among this group of cells and those of the control in which the Vero cells were incubated with the parasite without JcTI-I. In these two groups of cells, the parasitophorous vacuoles presented replicative tachyzoites and the percentage of infected cells and number of intracellular parasites were similar (data not shown). Nevertheless, the ultrastructural analyses of the parasites previously incubated for 30 min with 100 µM JcTI-I prior to the Vero cell infection revealed a disorganization and swelling of the tachyzoite endomembrane system in addition to multiple vacuolization in the cytoplasm (Fig. 1D). The morphology of *T. gondii*-infected Vero cell incubated with JcTI-I (6 µM) for 24 h was completely modified, and some cytoplasmic vesicles were formed (Fig. 1F).

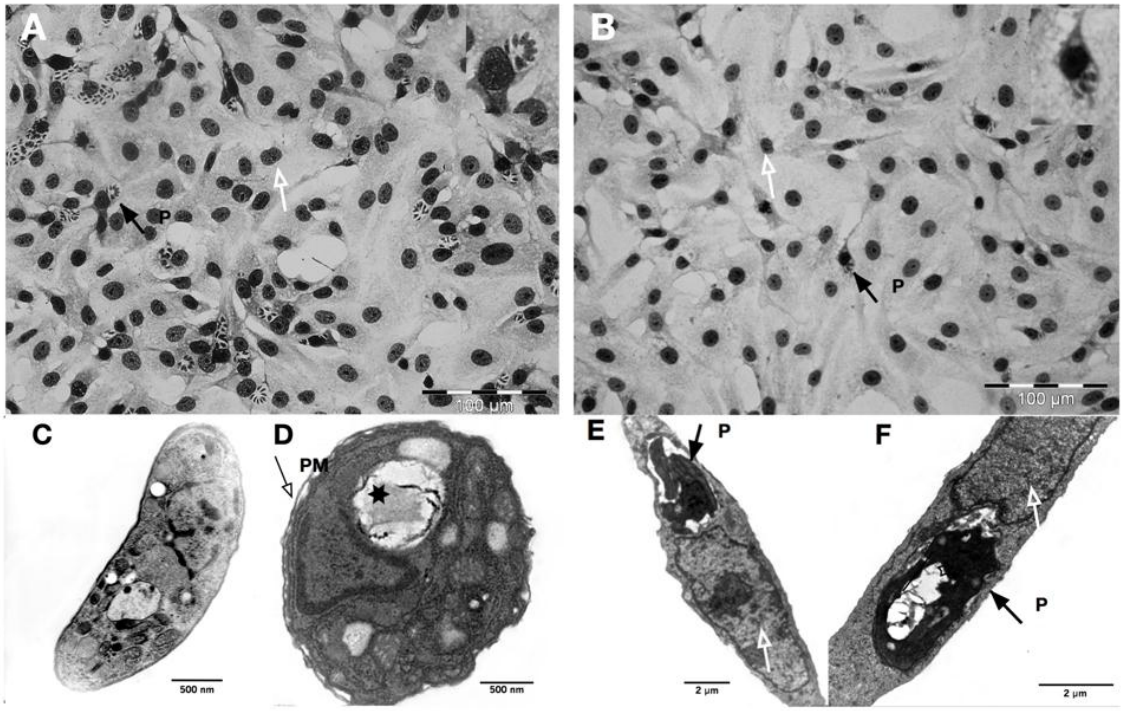

Fig. 1- Ultrastructural features of untreated and JcTI-I-treated *T. gondii* tachyzoite and *T. gondii*-infected Vero cells. (A) *T. gondii*-infected Vero cells incubated with the culture medium (control). (B) *T. gondii*-infected Vero cells incubated with 100 µM *JcTI-I* for 24 h. (C) Extracellular tachyzoite incubated with the culture medium. (D) Extracellular tachyzoite incubated with 100 µM JcTI-I for 30 min (E) *T. gondii*-infected Vero cells incubated with the medium culture. (F) *T. gondii*-infected Vero cells incubated with 6 µM JcTI-I for 24 h. White arrow: Vero cell nucleus. Black arrow: tachyzoites. PM: plasma membrane; Stars: vesicles. Scale bars: A and B: 100 µm. C and D: 500 nm. E and F: 2 µm.

Some studies showed that although some serine and cysteine protease inhibitors have little or no effect on the parasite morphology, growth and replication, they provoked changes in the host cells.[10] However, other protease inhibitors blocked intracellular parasite development, most caused only minor alterations in the parasite morphology independently of the effects on the host cells.[10] Nonetheless, cathepsin inhibitor III, N-Tosyl-L-Phenylalanine Chlroromethyl ketone (TPCK), and subtilisin inhibitor III caused drastic morphological changes in *T. gondii*, including swelling of the endomembrane systems in the tachyzoite protein trafficking pathway. In addition, rhoptry formation and disruption of the outer plasma membrane led to the release of the cytoplasmic contents into the vacuole. Moreover, treatment with cathepsin inhibitor III, TPCK, and subtilisin inhibitor III lead to the accumulation of materials that did not undergo proteolytic modification or degradation in the parasitophorous vacuole.[10] In our study, light microscopy analysis revealed that the morphology of Vero cells infected with *T. gondii* tachyzoites pre-treated with JcTI-I did not change, although intracellular tachyzoites suffered progressive destruction and elimination (Fig. 1 A-B). In addition,

electron microscopy revealed that the parasites underwent drastic morphological modifications, and many cytoplasmic vesicles appeared in both extra- and intracellular stages. Furthermore, the JcTI-I pre-treated tachyzoites showed a disorganization of both the inner pellicular membrane and plasma membrane (Fig. 1 C-D).

CONCLUSIONS

Our results showed that JcTI-I induced intracellular *T. gondii* elimination at low concentrations without causing any morphological modifications in the host cells. In addition, although extracellular parasites pre-treated with JcTI-I underwent cytoplasm vacuolization and plasma membrane disorganization, they invaded and proliferated inside the host cells. However, further studies are required for better understanding the main target of JcTI-I and the mechanism by which *T. gondii* is eliminated. Nevertheless, JcTI-I is a promising candidate for drug development.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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