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BIOTECHNOLOGY

Behavior of α-tomatine and tomatidine against several genera of trypanosomatids from insects and plants and *Trypanosoma cruzi*

Adriane Feijó Evangelista[1], Erica Akemi Kavati[2], Jose Vitor Jankevicius[3] and Rafael Andrade Menolli[4*]

*[1]Centro de Pesquisa em Oncologia Molecular, Hospital de Câncer de Barretos, Barretos, São Paulo, Brazil. [2]Laboratório de Genética, Instituto Butantan, São Paulo, São Paulo, Brazil. [3]Departamento de Microbiologia, Universidade Estadual de Londrina, Londrina, Paraná, Brazil. [4]Centro de Ciências Médicas e Farmacêuticas, Universidade Estadual do Oeste do Paraná, Rua Universitária, 2069, 85819-110, Cascavel, Paraná, Brazil. *Author for correspondence. E-mail: rafael.menolli@unioeste.br*

ABSTRACT. Glycoalkaloids are important secondary metabolites accumulated by plants as protection against pathogens. One of them, α-tomatine, is found in high concentrations in green tomato fruits, while in the ripe fruits, its aglycone form, tomatidine, does not present a protective effect, and it is usual to find parasites of tomatoes like *Phytomonas serpens* in these ripe fruits. To investigate the sensitivity of trypanosomatids to the action of α-tomatine, we used logarithmic growth phase culture of 20 trypanosomatids from insects and plants and *Trypanosoma cruzi*. The lethal dose 50% (LD50) was determined by mixing 10^7 cells of the different isolates with α-tomatine at concentrations ranging from 10^{-3} to 10^{-8} M for 30 min at room temperature. The same tests performed with the tomatidine as a control showed no detectable toxicity against the same trypanosomatid cultures. The tests involved determination of the percentage (%) survival of the protozoan cultures in a Neubauer chamber using optical microscopy. The LD50 values varied from 10^{-4} to 10^{-6} M α-tomatine. Slight differences were detected among the LD50 values of the analyzed samples, and none of them showed evidence of resistance to the action of tomatinase, as shown by some pathogenic fungi.

Keywords: tomato alkaloids; trypanocidal activity; *Phytomonas*; *Leptomonas*; *Crithidia; Herpetomonas*.

Comportamento de α-tomatina e tomatidina contra vários gêneros de tripanossomatídeos de insetos e plantas e *Trypanosoma cruzi*

RESUMO. Os glicoalcaloides são metabólitos secundários importantes produzidos pelas plantas e estão envolvidos em sua proteção contra agentes patogênicos. Um deles, α-tomatina, é encontrado em altas concentrações em frutos de tomate verde, enquanto que, nos frutos maduros, sua forma aglicona, tomatidina, não apresenta um efeito protetor, sendo comum encontrar parasitas de tomates como *Phytomonas serpens* nesses frutos maduros. Para investigar a sensibilidade dos tripanossomatídeos à ação da α-tomatina, utilizamos formas de cultura em fase logarítmica de 20 tripanossomatídeos de plantas e insetos e *Trypanosoma cruzi*. A dose letal 50% (DL50) foi determinada, misturando 10^7 células das formas de cultura com concentrações de 10^{-3} a 10^{-8} M de α-tomatina durante trinta minutos a temperatura ambiente. Testes realizados com a tomatidina como controle não mostraram toxicidade detectável contra os mesmos tripanossomatídeos. Os testes foram avaliados pela porcentagem (%) de sobrevivência das formas de cultura dos protozoários observados por microscopia óptica em câmara de Neubauer. Os resultados da determinação de DL50 mostraram que esta variou entre 10^{-4} a 10^{-6} M de α-tomatina. Pequenas diferenças foram observadas entre os valores de DL50 das amostras analisadas, e nenhuma delas mostrou evidência de resistência pela ação da tomatinidase, como demonstrado em alguns fungos patogênicos.

Palavras-chave: alcaloides de tomate; atividade tripanocida; *Phytomonas*; *Leptomonas*; *Crithidia; Herpetomonas*.

Introduction

Some species of the family Trypanosomatidae are responsible for diseases that affect humans, animals (*Leishmania* and *Trypanosoma*) and plants (*Phytomonas*). *Trypanosoma cruzi* is the etiologic agent of Chagas disease (Chagas, 1909) and various species are responsible for leishmaniasis, illnesses that affects millions of people, particularly in Latin America (Moncayo & Silveira, 2017). There is no vaccine against infections with *T. cruzi* and *Leishmania sp.* and chemotherapy remains the only means of treatment for Chagas disease and leishmaniasis. Meanwhile, the drugs available for treatment are few and their efficacy are limited, mainly due to the development of resistance and the

lack of host specificity (Silva-Jardim, Thiemann, & Anibal, 2014). Many plants harbor trypanosomatids, which primarily reside in the xylem, phloem tubes (Dollet, 2001), fruits and/or seeds (Jankevicius et al., 1989; Jankevicius et al., 1993) of infected plants.

The investigation of chemicals as candidate substances causing cytotoxic effects to trypanosomatids is a challenging area of research. However, plant secondary metabolites, known to be involved in plant chemical defense systems, could be active against endoparasites, including protozoa. There is recent evidence of growth inhibition of *Phytomonas serpens* by α-tomatine and tomatidine. In the present study, *in vitro* assays were carried out to show, for the first time, the toxicity of α-tomatine, but not tomatidine, against a wide range of plant and insect trypanosomatids and *T. cruzi*.

Plant secondary metabolites play an essential role in plant resistance to parasitic infections caused by bacteria, fungi, viruses and insects (Friedman, 2002). These specialized compounds include phenolic compounds, phytoalexins, protease inhibitors and alkaloids.

Glycoalkaloids are a family of chemical compounds derived from alkaloids. In tomato plants (*Solanum lycopersicum*), an important glycoalkaloid called α-tomatine has demonstrated the capacity to kill microorganisms *in vitro* (Kaup, Gräfen, Zellermann, Eichenlaub, & Gartemann, 2005; Sandrock & Vanetten, 1998). The carbohydrate parts of this molecule potentially play an important role, while the aglycone form, tomatidine, shows poor antibiotic characteristics (Chagnon et al., 2014).

The α-tomatine structure has been shown to contain a hydrophilic part, constituted by a tetrasaccharide side chain called lycotetraose, which consists of two d-glucose units, one d-xylose and one d-galactose unit; a hydrophobic part, comprised of the steroidal moiety of glycoalkaloid; and a polar -NH group. A tomatine can be partially or completely hydrolyzed by acid, and the resulting substances are called β_1-, β_2-, γ-, δ-tomatine and tomatidine (Friedman, 2002).

The ability to lyse cells has been demonstrated previously (Roddick & Drysdale, 1984) and was linked to adherence to sterols in membranes. This characteristic makes tomatine a lethal weapon in the defense mechanism of tomato plants against microorganisms, previously demonstrated against fungi, bacteria and protozoa, such as *Beauveria bassiana* (Costa & Gaugler, 1989), *Fusarium oxysporum* (Smith & MacHardy, 1982), *Phytophora megasperma* (Steel & Drysdale, 1988), *Pseudomonas solanacearum* (Arwiyanto, Sakata, Goto, Tsuyumu, & Takikawa, 1994) and *Tetrahihmena pyriformis* (Surak & Schifanella, 1979).

The aim of this study was to analyze the activity of α-tomatine against flagellate protozoans that act as etiological agents of diseases in plants with great economic importance, such as tomato, grape and coffee plants.

Material and methods

Glycoalkaloids - Commercial tomatine and tomatidine (Sigma Chemical Company™) were used, diluted in phosphate buffered saline (PBS; Gibco™), pH 7.2, containing 10% ethanol in serial dilutions of 10^{-3} to 10^{-8} M. Treatment with 10% ethanol alone had no effect on cell proliferation.

Protozoans - Twenty trypanosomatids, isolated from fruits, seeds and insects, were characterized as described for Batistoti et al. (2001), Catarino et al. (2001) and Serrano et al. (1999) using immunological methods (monoclonal antibodies) and molecular techniques (polymerase chain reaction (PCR), hybridization and random amplified polymorphic DNA (RAPD)). All isolates are characterized as belonging to four genera (*Phytomonas, Crithidia, Herpetomonas*, *Leptomonas*), and were grown at 28ºC in GYPMI medium (Jankevicius et al., 1993), maintained in the logarithmic growth phase through weekly subcultures. Epimastigotes of *Trypanosoma cruzi* (Y strain), also in the logarithmic growth phase, were maintained in this phase by weekly transfer in liver-infusion tryptose (LIT) medium.

Effects on growth - Determination of the LD50 involved mixing equal volumes of 10^7 protozoans, washed and resuspended in PBS, with the glycoalkaloids. After 30 min., cell densities were evaluated in a Neubauer chamber at room temperature. The lethality of both substances was determined by observing the motility of trypanosomatids, and the LD50 was determined by the % protozoan mortality, according to the method of Reed and Muench (1938). After the time of exposure, aliquots of the mixture were mounted on slides, fixed with methanol and stained with Giemsa.

Statistical analysis - Data from at least three independent experiments were expressed as mean values. Statistical significance was calculated using two-way ANOVA followed by Tukey's post-hoc-test. A difference was significant when $p \leq 0.05$. Analyses were performed using GraphPad 5.0 software.

Results and discussion

The trypanocidal effect of α-tomatine was demonstrated by mixing a commercial glycoalkaloid with a PBS containing 10% ethanol at different dilutions and determining the LD50. As shown in Table 1, the 21 isolates tested were divided into five genera, *Phytomonas, Leptomonas, Crithidia* and *Herpetomonas* (which alternate their life cycle between fruits and insects) and *Trypanosoma* (which alternates its life cycle between mammals and insects). These showed some differences in the concentrations of α-tomatine that determined the final LD50. The concentrations varied between 10^{-4} and 10^{-6} M, specifically between $10^{-4.5}$ and $10^{-5.5}$ M. This difference, ranging almost ten times, showed that some isolates had a little, but not significant resistance to tomatine. No significant differences were detected for any strain evaluated.

Mortality occurred at the three highest concentrations and rates were similar for most isolates. However, with 215 Ma, I24G and *T. cruzi* at 10^{-4} M, a very small proportion (< 10%) of live specimens remained and this was significantly different from the other isolates (Figure 1). Surprisingly, these three isolates (two *Phytomonas* species and one *Trypanosoma*) are not from tomato plants and had a slightly lower LD50 than the other isolates. This is notable because the presence of a more susceptible group (including *T. cruzi*) can indicate evolutionary differences among trypanosomatids. Another interesting point is that *T. cruzi* is usually grown on medium that contain sterols (LIT), but trypanosomatids from insects and plants are not; this make sterols more abundant in *T. cruzi* than in the other isolates tested (Nakamura et al., 1999; Rodrigues et al., 2001), which makes *T. cruzi* susceptible to tomatine. However, at the α-tomatine concentration of 10^{-3} M, all isolates showed very high mortality rates (> 90%).

The effect of α-tomatine on parasites is illustrated in Figure 2A, B and C, where high concentrations of glycoalkaloid (10^{-4} and 10^{-5} M) lysed the microorganisms and, consequently, the protozoans were almost impossible to see. The inverse situation occurred at low concentrations of tomatine (10^{-6} M), where little perturbation of the trypanosomatids was observed. The highest concentration (10^{-3}) caused total disruption of parasites and the lowest concentrations (10^{-7} and 10^{-8}) had the inverse effect, i.e., nothing was observed.

As expected, tomatidine, the aglycone form of α-tomatine, showed no microbicidal activity, as illustrated in Figure 2D. When a high concentration (10^{-4} M) of this alkaloid was used, the structure of the trypanosomatids was completely unaffected. The same results were observed at all concentrations tested.

Previous studies have demonstrated the efficacy of α-tomatine in disrupting eukaryotic cell membranes and in protecting plants against pathogens (Pareja-Jaime, Roncero, & Ruiz-Roldán, 2008; Steel & Drysdale, 1988). Our findings have shown that trypanosomatids are susceptible to the action of α-tomatine, with a LD50 for all tested strains in a low concentration range.

Table 1. Anti-trypanosomatid activity (LD50) of the glycoalkaloid, α-tomatine, against insects and plants trypanosomatids and *Trypanosoma cruzi*

Isolate	Genus	Source host	Concentration of tomatine (M)
15T	*Phytomonas*	tomato	$10^{-4.80}$
215 Ma	*Phytomonas*	maize	$10^{-5.50}$
270T	*Phytomonas*	tomato	$10^{-4.52}$
I24G	*Phytomonas*	insect	$10^{-5.47}$
748T	*Phytomonas*	tomato	$10^{-4.86}$
252Td	*Phytomonas*	tomato	$10^{-4.61}$
667	*Phytomonas*	tomato	$10^{-4.82}$
9T	*Phytomonas*	tomato	$10^{-4.58}$
490Ad	*Phytomonas*	blackberry	$10^{-4.73}$
6G	*Phytomonas*	insect	$10^{-4.51}$
163	*Phytomonas*	maize	$10^{-4.70}$
268T	*Leptomonas*	tomato	$10^{-4.70}$
563Td	*Leptomonas*	insect	$10^{-4.85}$
715Td	*Leptomonas*	insect	$10^{-4.50}$
274Ta	*Leptomonas*	tomato	$10^{-4.72}$
C. fasciculata	*Crithidia*	insect	$10^{-4.46}$
Crithidia sp.	*Crithidia*	insect	$10^{-4.32}$
C. acanthocephali	*Crithidia*	insect	$10^{-4.61}$
C. termophila	*Crithidia*	insect	$10^{-4.89}$
H. anglusteri	*Herpetomonas*	insect	$10^{-4.63}$
T. cruzi (Y)	*Trypanosoma*	human	$10^{-5.61}$

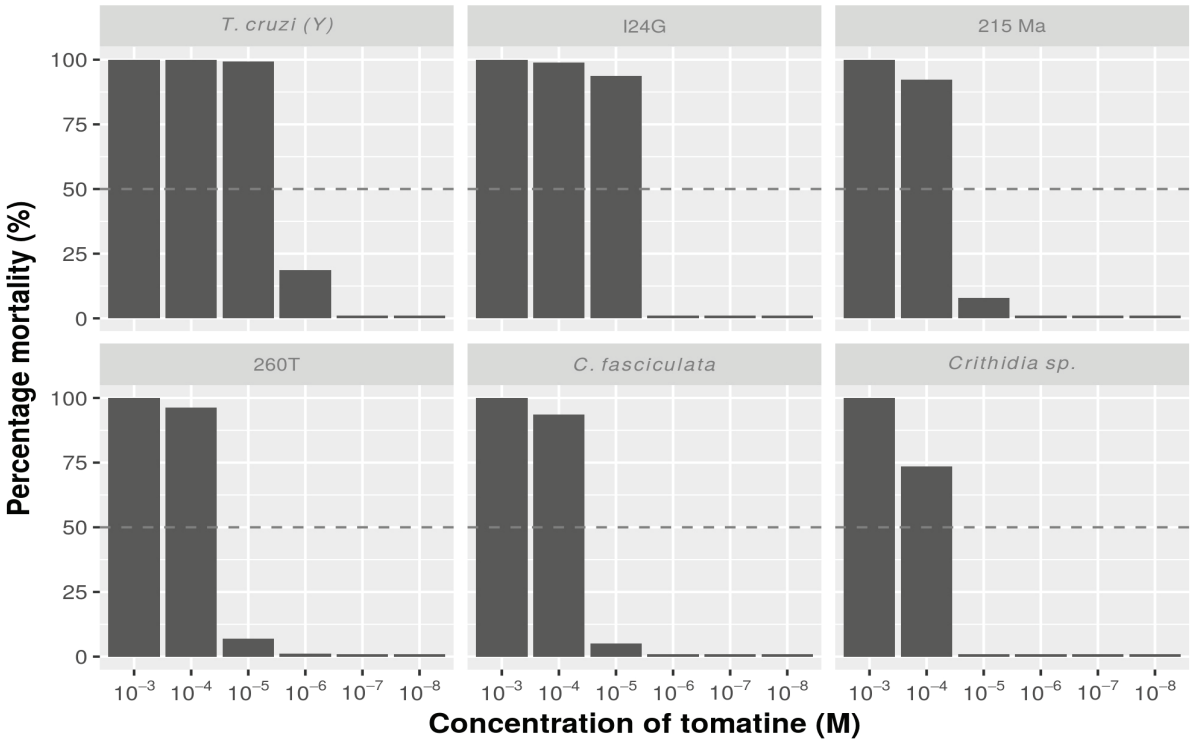

Figure 1. Percentage mortality (%) of five trypanosomatids from insects and plants and *T. cruzi* in the presence of α-tomatine at concentrations of 10^{-3}, 10^{-4} and 10^{-5} M. Percentage mortality was determined as described in Material and Methods. 215Ma, 24G and 260T are *Phytomonas* species.

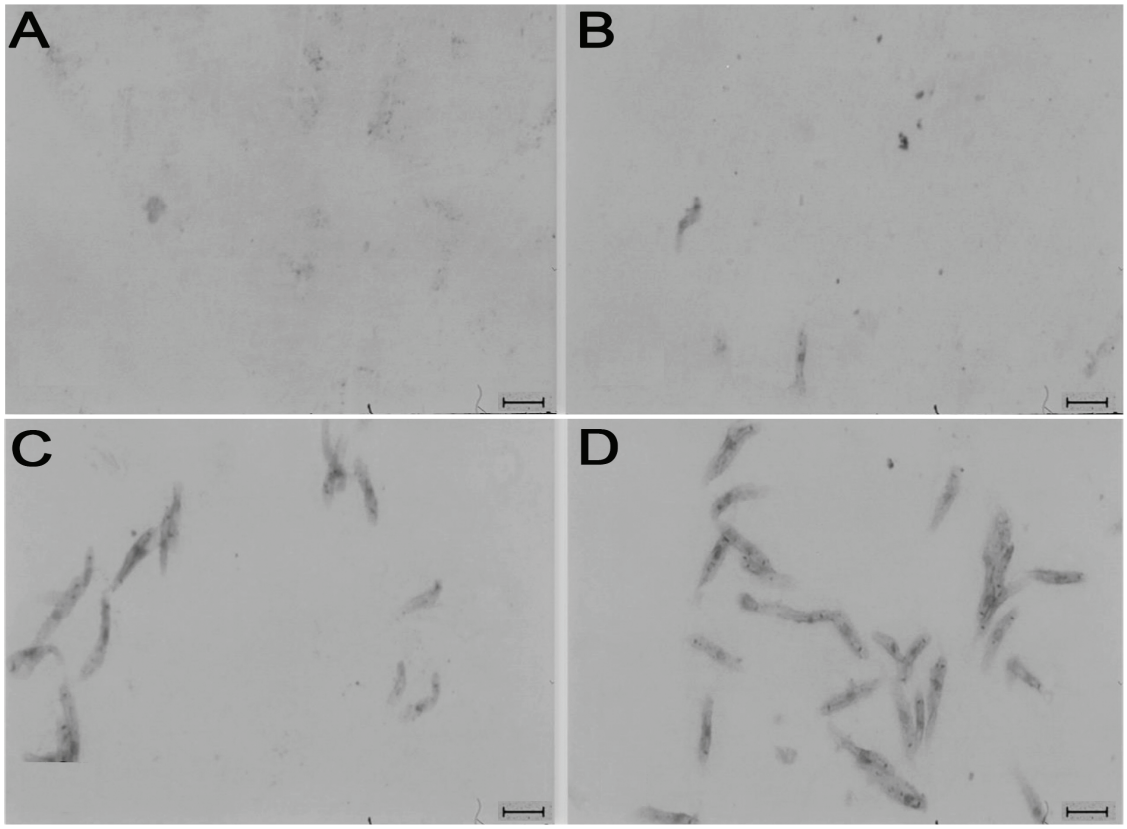

Figure 2. *Phytomonas sp.* (isolate 6G) exposed to α-tomatine at concentrations of 10^{-4} M (A), 10^{-5} M (B) and 10^{-6} M (C) and exposed to tomatidine at a concentration of 10^{-4} M (D). After exposure to the alkaloids for 30 min., cultures on slides were fixed with methanol and stained with Giemsa.

The life cycle of trypanosomatids from insects and plants has been shown to rely on insects biting plants, enabling parasites in the salivary glands to be transferred to fruits (Jankevicius et al., 1989). In tomatoes, however, only mature fruit can be parasitized once α-tomatine is present at high concentrations (approximately 5×10^{-4} M; 500 mg Kg^{-1}) in green fruits (Friedman & Levin, 1995). These data are consistent with our *in vitro* results, with the tomato plant protecting its fruits against the protozoan. In view of the high lethality achieved with 10^{-3} M α-tomatine with exposure for 30 min., an extended exposure time can lead to total lethality of the parasites in fruits, providing significant protection for the plant.

Trypanosomatids from insects and plants that cause death of plants are scarce; most of them cause economic losses due to parasitized fruits. It is clear that α-tomatine is not transported to fruits from other locations but is generated there, and the disappearance of α-tomatine from mature tomatoes is related to the synthesis of pigments (Eltayeb & Roddick, 1984a; 1984b). This provides two situations: an initial protection of green fruits, which attract insects at a later stage with red or yellow coloration and a small amount of protection from the residual tomatine.

Other studies have been performed involving the family Trypanosomatidae and tomato glycoalkaloids. For example, Medina et al. (2015) reported an IC50 of 9.9×10^{-6} M for *P. serpens* after 48h incubation with α-tomatine. However, with 24h incubation, they verified that all parasites were killed when incubated with tomatine in a concentration of 5×10^{-5} M, showing that high concentrations of this glycoalkaloids can lyse trypanosomatids in a short time, which is comparable with the data obtained in this investigation. A study on the effect of tomatine on *T. cruzi* growth (Chataing, Concepción, Lobatón, & Usubillaga, 1998) showed inhibition of *in vitro* growth after four days of incubation with 5.7×10^{-6} M, reaching a maximal inhibition after seven days (63.9%). Despite differences in methodology and strain, it was evident that tomatine was able to kill *T. cruzi*, as in our results.

Tomatidine did not cause lethality, but this result may be due to the short exposure time used in our study as other authors have shown that, with a prolonged exposure time, tomatidine can diminish trypanosomatid growth (Medina, Rodrigues, De Souza, Atella, & Barrabin, 2012; Medina et al., 2015).

Conclusion

In conclusion, the results indicate that α-tomatine was able to lyse various isolates of trypanosomatids (belonging to five different genera) in a short exposure time, varying the IC50 reached by them between $10^{-4.32}$ and $10^{-5.61}$. On the other hand, the aglycone form of tomatine, tomatidine, showed no activity against protozoans even with high concentrations of the alkaloid.

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