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Original Article

In vitro activities of glycoalkaloids from the *Solanum lycocarpum* against *Leishmania infantum*

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ABSTRACT

Leishmania infantum is an etiologic agent of visceral leishmaniasis. This disease is a neglected disease that can be fatal if not treated and additionally, the few therapeutic option present several drawbacks, including difficult route of administration and toxicity, which turn the search for new therapeutic alternatives necessary. Herein, we evaluated the leishmanicidal *in vitro* activity of the solanum extract from *Solanum lycocarpum* A. St.-Hil., Solanaceae, and the isolated alkaloids solasodine, solamargine and solasonine against promastigotes and intracellular amastigotes of *L. infantum*. Solasodine ($IC_{50\text{-pro}}$ = 4.7 μg/ml; $IC_{50\text{-ama}}$ = 10.8 μg/ml) and solamargine ($IC_{50\text{-pro}}$ = 8.1 μg/ml; $IC_{50\text{-ama}}$ = 3.0 μg/ml) exhibited interesting leishmanicidal ativity. Solasonine was approximately four-times (Selective Index 3.7) more selective to the parasite than to the host cells. This data suggest that solasonine might be considered as a potential drug candidate for leishmaniasis treatment.

Introduction

Visceral leishmaniasis (VL), also known as kala-azar, is a neglected disease that reported 300,000 cases annually and leads 20,000 people per year to death around the world (WHO, 2018). More than twenty species of *Leishmania* can cause leishmaniasis, whereas *Leishmania (Leishmania) donovani* causes VL in India and other Asian and African countries and *Leishmania (Leishmania) infantum* or *Leishmania (Leishmania) infantum chagasi* cause VL in America and Europe (Lindoso et al., 2016). These parasitic diseases affect spleen, liver, bone marrow and lymph nodes, producing fever and anemia and usually is fatal if left untreated (Murray et al., 2005). Visceral leishmaniasis has emerged as an important opportunistic infection associated with HIV. *Leishmania*–HIV coinfection has been reported in 35 countries and VL-HIV has increased in the last years (Lindoso et al., 2016).

Pentavalent antimonials, paromomycin, amphotericin B or miltefosine are the commonly used drugs. However, treatment with these drugs requires long periods of administration leading to serious adverse effects, poor tolerance and development of resistant strains (Souza-Silva et al., 2015). Moreover, increasing resistance of the parasites contributes for the ineffectiveness of therapeutic regimens (Croft et al., 2006; Natera et al., 2007). Putting all these together, it is urgent the developing of new therapeutic strategies for VL.

Plants are interesting source of natural products and can be explored as hits for antileishmanial drug development (Onocha and Ali, 2010; González-Coloma et al., 2012; Mansour et al., 2013; Machado et al., 2014; Torres et al., 2014; Coqueiro et al., 2014; Funari et al., 2016). Recent studies showed that various plant species possess leishmanicidal activity against many different types of *Leishmania* such as *L. amazonensis* (Guimaraes et al., 2010; Miranda, 2010; Miranda et al., 2013; Coqueiro et al., 2014; Funari et al., 2016), *L. infantum* (Mansour et al., 2013; Machado et al., 2014), *L. braziliensis* (Munoz et al., 1994; Yamamoto et al., 2014), *L. chagasi* (Rondon et al., 2012), *L. major* (Ogeto et al., 2013), *L. tropica* (Iqbal et al., 2012), *L. aethiopica* (Bekele et al., 2013), *L. mexicana* (Gamboa-Leon et al., 2014), and *L. donovani* (Sachdeva et al., 2014a,b).

This is the case from the plants containing alkaloids (Munoz et al., 1994; Waechter et al., 1999; Miranda, 2010; Santos

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et al., 2012; Mansour et al., 2013; Miranda et al., 2013). Mishra et al. (2009) has reported the leishmanicidal activity of various alkaloids from Apocynaceae (*Kopsia griffithii*, *Peschirea australis*, *Aspidosperma ramiflorum*, *Peschiera van heurkii)*, Rubiaceae (*Corynanthe pachyceras*), Annonaceae (*Guatteria boliviana*, *Pseudoxandra sclerocarpa*, *Annona foetida*, *Guatteria foliosa Guatteria dumetorum Rollinia emarginata*, *Guatteria* sp. and *Unonopsis buchtienii*), Ancistrocladaceae (*Ancistrocladus griffithii*, *Ancistrocladus likoko*, *Ancistrocladaceae* sp., *Ancistrocladus tanzaniensis)*, Hernandiaceae (*Gyrocarpus americanus*), Menispermaceae (*Albertisia papuana*, *Caryomene olivasans*, *Limaciopsis loangensis*) Rutaceae (*Galipea longiflora*, *Dictyoloma peruviana*).

Plants containing steroidal alkaloids from Solanaceae also are cited in the literature as containing antileishmanial activity such as *Saracha punctata* against *Leishmania braziliensis*, *Solanum glabratum* against *L. infantum* and *Solanum lycocarpum* against *L. amazonensis* promastigotes forms (Miranda et al., 2013) among others. *S. lycocarpum* A. St.-Hil. is a common plant that grows spontaneously in tropical and temperate zones, including the Cerrado of Brazil (Cruz and Silva, 1995; Lorenzi, 2000), where is popularly known as "lobeira" or "fruta-do-lobo", having great importance as food and as traditional remedy because of its alleged hypoglycemic effect (Dall'Agnol and Von Poser, 2000). Furthermore, their biological activities have been intensively investigated, particularly anti-viral, diuretic, anti-fungi, anti-spasmodic and anti-inflammatory (Fewell et al., 1994; Vieira et al., 2003; Balasubramanian et al., 2007; Martins, 2013). Several studies also evidenced that extracts of *S. lycocarpum* are active against flagellated protozoa, such as *Giardia lamblia* (Martins, 2013), *L. amazonensis* (Miranda et al., 2013) and *Trypanosoma cruzi* (Hall et al., 2006; Moreira et al., 2013), as well as, against helminthes, *Strongyloides stercoralis* (Miranda, 2010) and *Schistosoma mansoni* (Miranda et al., 2012). The glycoalkaloids solasonine (**1**) and solamargine (**2**) are pointed as phytochemicals responsible for such activities, although the probable contribution of several other compounds, such as phenolic acids, tannins, flavonoids, steroids and triterpenes (Tiossi et al., 2012; Martins, 2013).

The compounds **1** and **2** are steroid glycosides sharing the same aglycone but differing in the sugar moieties, rhamnose–galactose–glucose in the first and rhamnose-glucose-rhamnose in the last. The difference among the sugar moieties may influence how these compounds cross cell membranes (Udalova et al., 2004; Tiossi et al., 2012). As previously mentioned, **1** and **2** as well as the equimolar mixture of both were proved to be active against *L. amazonensis* (Miranda et al., 2013). For this reason and considering the epidemiological relevance of VL, we report here on the *in vitro* activity of these glycoalkaloids against *L. infantum.*

Materials and methods

Plant material

Fruits of *Solanum lycocarpum* A. St.-Hil., Solanaceae, were collected in Barretos, São Paulo, Brazil, S 20° 34 × 15.898″/W 48° 34 × 29.989″. A voucher specimen (SPFR 11.308) was deposited at the Herbarium of the Faculty of Philosophy Science and Letters, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. The fruits of *S. lycocarpum* were dried, reduced to powder and extracted with ethanol 96%. Extract corresponds to the ethanol 96% dry extract. *Solanum* extract, **1**, **2** and **3** were used for all the experiments were previously prepared and fully characterized. Details on the preparation and composition were previously reported (Martins et al., 2015).

Parasites and cultures

Promastigotes of *Leishmania infantum* strain (MHOM/BR/1972/LD), provided by Prof. José Angelo Lindoso of University of São Paulo, Brazil, were maintained at 28 °C in Schneider's medium (Sigma), supplemented with 10% heat-inactivated fetal calf serum (hi-FCS, Gibco), 10% male human urine, 1% penicilin/streptomycin (Pen/Strep, Sigma–Aldrich).

In vitro *antileishmanial activity against* Leishmania infantum *promastigotes*

The efficacy of compounds and extract against *L. infantum* were tested according methodology previously described with some modifications (Velásquez et al., 2016; De Almeida et al., 2017). Briefly, 1×10^7 parasites/ml of *L. infantum* promastigotes were seeded at in 96-well flat-bottom plates (TPP; Sigma-Aldrich). The tested compounds were dissolved in DMSO (the highest concentration was 3%, which was not hazardous to the parasites) and amphotericin B (AmpB) was used at positive control for assay. Control parasites were incubated with Schneider's medium alone. 3 μl of compounds and control were tested in 97 μl/well of parasites diluted in a different concentrations (100 μg/ml to 1.56 μg/ml) and incubated at 28 °C for 72 h. Leishmanicidal effects were assessed by counting motile promastigotes in a Neubauer chamber and the concentration that caused a 50% decrease in parasite viability compared to the control was calculated by non-linear regression expressed as the $IC_{50\text{-PRO}}$ in μg/ml.

Cytotoxicity assay

The cytotoxicity against murine macrophages was determined as previously described with some modifications (Dutra et al., 2014). In summary, for collected of mouse peritoneal macrophages, adult male Swiss albino mice were stimulated with 3% thioglycolate to collect the cells in concordance to protocol approved by the Institutional Ethics Committee, protocol CEUA/FCF/CAr No. 42/2016. Immediately, the cells were seeded in 96-well flat-bottom plates at a density of 10^5 cells/well (100 μl/well) in RPMI 1640 medium supplemented with 10% hi-FCS, 25 mM HEPES, and 2 mM L-glutamine, 1% Pen/Strep and incubated for 4 h at 37 ± 2 °C in a 5% CO_2–air mixture. Then, different concentrations of compounds diluted in RPMI medium were tested (100 μg/ml to 1.56 μg/ml) against the murine macrophages for 24 h and incubated under the same conditions. Cells without compounds were used as a negative control and with AmpB were used as a positive control. Finally, the MTT colorimetric assay was carried out and the absorbance was read at 540 nm using the Tecan Infinite M200 PRO microplate reader. The drug concentration that corresponds to 50% of cell growth inhibition is expressed as the 50% cytotoxic concentration (CC_{50}). The cytotoxicity for host cells and *L. infantum* were compared and expressed as the selectivity index ($SI = CC_{50\text{macrophages}}/IC_{50\text{leishmania}}$), which was defined as the ratio of the CC_{50} for macrophages to the IC_{50} for parasite.

In vitro *antileishmanial activity against* Leishmania infantum *intracellular amastigotes forms*

The activity compounds and extract of *S. lycocarpum* against intracellular amastigotes was evaluated in mouse peritoneal macrophages infected with *L. infantum* according methodology previously described with some modifications (De Almeida et al., 2017). Murine macrophages were collected from the peritoneal cavity of Swiss mice after thioglycolate-stimulation and plated at 3×10^5 cells/well on coverslips (13-mm diameter), previously arranged in a 24-well plate containing RPMI1640 medium

supplemented with 10% of hiFCS, 25 mM HEPES, 2 mM L-glutamine and 1% Pen/Strep, and allowed to adhere for 6 h at 37 °C in 5% CO_2–air mixture. Adherent macrophages were infected with promastigotes forms in the stationary growth phase using a ratio of 10:1 parasites per macrophage at 37 °C in 5% CO_2 for 18 h to allow parasite multiplication. The non-internalize parasites were removed by washed using 1× PBS. Then, infected cells were treated with different concentrations of each compound, extract and AmpB (20 μg/ml to 2.5 μg/ml) for 24 h. After incubation, the cells were fixed with methanol, Giemsa stained and examined by light microscopy. The number of amastigotes/100 macrophage cells and the percentage of infected cells were determined. The concentration that caused 50% growth inhibition compared to the control was expressed as the $IC_{50\text{-AMA}}$ in μg/ml. The infection index was calculated by multiplying the percentage of infected macrophages by the mean number of amastigotes per infected cells (Velásquez et al., 2017).

Statistical analysis

The concentrations of compounds that inhibited culture growth (parasites or mammalian cells) by 50% compared to the control were determined by non-linear dose-response regression analysis using software origin 7.0. The assays were carried out in biological and experimental duplicates. The statistical differences between groups were evaluated using one-way analysis of variance, followed by the Student–Newman–Keuls multiple comparison and Tukey tests (using GraphPad InStat software). Differences were considered significant when p values were ≤ 0.05 (Mean 95% Confidence Interval).

Results and discussion

Visceral leishmaniasis is the most severe life-threatening form of leishmaniasis and treatment of patients bearing this parasitic disease is mandatory. The unavailability of vaccines for human use and the few chemotherapeutical options associated with long-term, severe side effects, high cost and the appearance of resistant strains prove the importance of find new therapeutic alternatives against this extremely neglected disease (Chung et al., 2007; Pham et al., 2013). In order to contribute to the discovery of new antileishmanial agents, the *in vitro* anti-*L. infantum* promastigote activity was determined and the glycoalkaloids **3** and **2** were the most active compounds ($IC_{50\text{-PRO}}$ 4.7 μg/ml and 8.1 μg/ml, respectively) when compared with **1** ($IC_{50\text{-PRO}}$ 22.7 μg/ml) and solanum extract from *S. lycocarpum* ($IC_{50\text{-PRO}}$ 16.7 μg/ml) (Table 1). On the other hand, for the most clinically relevant life cycle stage form of the parasite, **1** and **2** also exhibited good anti-*L. infantum* amastigote activity ($IC_{50\text{-AMA}}$ 3.2 μg/ml and 3 μg/ml, respectively), and **3** ($IC_{50\text{-AMA}}$ 10.8 μg/ml), only mild activity, which was twice lower than AmpB (2.3 μg/ml).

Others studies also reported the antileishmanial activity for extracts from other *Solanum* species (Abdel-Sattar et al., 2010; Hubert et al., 2013; Estevez et al., 2007; Filho et al., 2013; Miranda et al., 2013). Hubert et al. (2013) reported that extract of *Solanum torvum* (IC_{50} 96.1 μg/ml) inhibited the proliferation of promastigotes of *L. donovani*. Filho et al. (2013) demonstrated inhibition of promastigotes forms of *L. amazonensis* and *L. brasiliensis* in the presence of extracts from *Solanumsisym briifolim* (IC_{50} 33.8 μg/ml and 20.5 μg/ml). Mothana et al. (2014) showed that the methanol extract of *Solanum glabratum* presented IC_{50} values equal 8.1 μg/ml against *L. infantum*. Miranda et al. (2013) isolated **2** and **1** from the fruits of *S. lycocarpum* and showed *in vitro* leishmanicidal activity against promastigotes forms of *L. amazonensis*. In this work, we observed that the isolated compounds, solamargine and solasonine, were high toxic against the amastigote forms of parasites as well as against macrophages, additionally the solanum extract (with a pool of molecules included solasonine (**1**), solamargine (**2**) and solasodine (**3**)) had been showed too highly toxic against macrophages, for these reasons we decided not tested it. The susceptibility of *L. infantum* to the solanum extract points out to a potential addictive effect in the antiparasitic activity. The cytotoxicity was evaluated against murine peritoneal macrophages and the anti-amastigote effect of the compounds and solanum extract on intracellular amastigotes. The selective index (SI) was determined using the relationship between CC_{50} and IC_{50} of both parasites forms of *L. infantum*, Table 1. The compound **1** reduced the amount of intracellular amastigote similarly to AmpB (Fig. 1) and was the most selective (SI ~4) to the intracellular amastigotes forms of *L. infantum* when compared to **2** and **3**, Table 1.

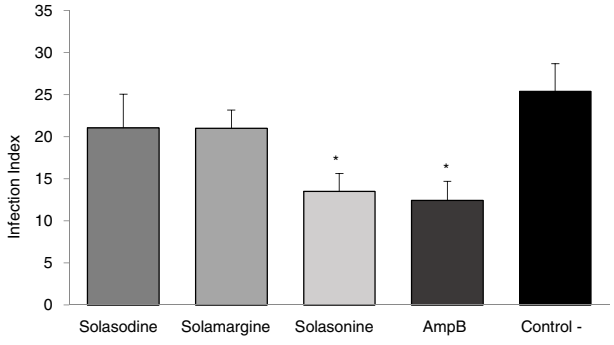
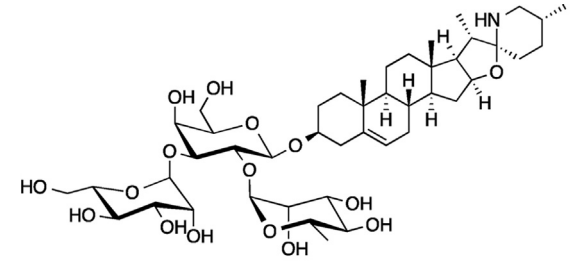


Fig. 1. *In vitro* effect of solasodine, solamargine, solasonine and amphotericin B on *L. infantum* intracellular amastigotes. The infection index (% of infected cells × number of the intracellular parasites) was calculated after 24 h of treatment with 5 μg/ml of each compounds. The negative control is *L. infantum* intracellular amastigotes not treated. Data are expressed as averages plus the standard deviations (SD) for two independent experiments. *: statistically significant difference relative to the negative control ($p < 0.05$).

Table 1
Leishmanicidal activities (IC_{50} concentration that caused a 50% decrease in parasite viability), mammalian cell toxicities (CC_{50} drug concentration that corresponds to 50% of cell growth inhibition), and selective indices ($SI = CC_{50\text{-macrophages}}/IC_{50\text{-}Leishmania}$) of extracts and glycoalkaloids of *Solanum lycocarpum*.

Compound	$IC_{50\text{-PRO}}$ (μg/ml)	SI	$IC_{50\text{-AMA}}$ (μg/ml)	SI	CC_{50} (μg/ml)
Solasonine (**1**)	$22.7 \pm 2.9^{A,b}$	(0.5)	3.2 ± 0.4	(3.7)	12.0 ± 1.3^{A}
Solamargine (**2**)	$8.1 \pm 0.9^{a,b,\gamma,*}$	(0.4)	$3.0 \pm 0.2^{*}$	(1.1)	$3.4 \pm 0.01^{A,c}$
Solasodine (**3**)	$4.7 \pm 0.1^{\beta,\gamma}$	(2.5)	$10.8 \pm 0.5^{A,\gamma}$	(1.1)	$11.5 \pm 0.5^{\alpha,c}$
Solanum extract	16.7 ± 1.3^{A}	(0.5)	ND	ND	8.3 ± 2.9
Amphotericin B	0.8 ± 0.1	(26.2)	2.3 ± 0.5	(9.2)	21.3 ± 2.5

Data are expressed as averages plus the standard deviations (SD) for two independent experiments. a,α,A: statistically significant difference relative to amphotericin B ($p < 0.05$, $p < 0.01$, $p < 0.001$). b,β: statistically significant difference relative to solanum extract ($p < 0.05$, $p < 0.01$). c,γ: statistically significant difference relative to solasonine ($p < 0.05$, $p < 0.001$).*: statistically significant difference relative to solasodine ($p < 0.001$). ND, not determinated.



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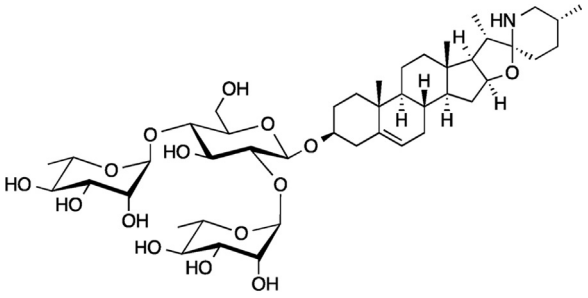

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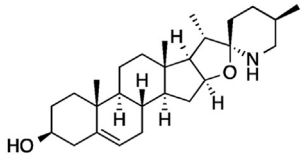

3

The observed antileishmanial activity might be attributed to the steroidal alkaloids, which represent the major constituents in *Solanum* species. It is interesting to mention that Devkota et al. (2007) reported that the varieties of functionalities present in ring A of the steroidal alkaloids might be playing a role in the antileishmanial activity. It is well known that glycosylphosphatidylinositol-anchored glycoproteins are the most prevalent cell-surface molecule present in *Leishmania* surface (Cham and Daunter, 1990; Ndjakou Lenta et al., 2007). Thus, the steroidal glycoalkaloids that contain chacotriose chain (rhamnose-glucose-rhamnose), as **1**, might be easily diffusing through *Leishmania* membranes and interacting with intracellular targets (Cham and Daunter, 1990).

Conclusions

The present study describes for the first time the activity of **1** against *L. infantum* and its potential to be considered a hit for the antileishmanial drug development efforts. Data herein reported suggest that **1** showed the best potential of antileishmanial activity against intracellular amastigotes forms of *L. infantum*, the most relevant forms of VL. Therefore, it is suggested that these compounds can be considered as templates for drug design and development of novel leishmanicidal therapeutic agents. However, further studies are necessary in order to fully comprehend their potential as hits for leishmaniasis treatment.

Authors' contributions

RRDM analyzed the data and drafted the paper. GZM contributed in collecting plant sample and identification, confection of herbarium. MASG designed the study, critically read the manuscript and wrote the manuscript. AMAV critically read the manuscript and wrote the manuscript. LS, MCS and CC critically read the manuscript. LCC, TGP and LA performed the biological assays. All the authors have read the final manuscript and approved the submission.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest.

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