

Effects of the extract and glycoalkaloids of *Solanum lycocarpum* St. Hill on *Giardia lamblia* trophozoites

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ABSTRACT

Background: *Solanum lycocarpum* has great importance for food and medicinal traditional use. Recently, it was also evidenced that extracts of *S. lycocarpum* St. Hill (Solanaceae) and its glycoalkaloids, solamargine (Sg) and solasonine (Sn), are active against flagellated protozoa. **Objective:** The aim was to assess the effects of the extract of *S. lycocarpum* and its glycoalkaloids, Sn, and Sg, on *Giardia lamblia* trophozoites. **Materials and Methods:** A crude extract (96% ethanol) (EB) of fruits of *S. lycocarpum* was prepared and fractionated by partition with 40% ethanol and *n*-hexane: Ethyl acetate. Glycoalkaloids, Sn, and Sg were recognized in the ethanol fraction (EF) and further isolated by column chromatography. EB, EF, the isolated Sn and Sg and a mixture (1:1) of both glycoalkaloids were tested on cultures of *G. lamblia* trophozoites and macrophages. **Results:** EB, EF and glycoalkaloids of *S. lycocarpum* showed activity against *Giardia* ($95.0 < \text{Inhibitory concentration } 50 [\text{IC}_{50}] \leq 120.3 \mu\text{g/mL}$). The mixture of glycoalkaloids (1:1) was more active ($\text{IC}_{50} = 13.23 \mu\text{g/mL}$) than each one individually, suggesting a synergic effect. Moreover, the mixture is nontoxic to macrophage cells. **Conclusion:** Results are optimistic concerning the anti-*Giardia* potential of the mixture Sn + Sg. Further studies, *in vitro* and *in vivo*, will be required to consolidate the usefulness of the mixture of Sn + Sg in view of a new therapeutic strategy for giardiasis.

Key words: *Giardia lamblia*, glycoalkaloids, solamargine, *Solanum lycocarpum*, solasonine

INTRODUCTION

Solanum lycocarpum St. Hill is a plant species of the Solanaceae family, popularly known in Brazil as “lobeira”, “fruta-do-lobo”, “juribebão” or “maça-do-cerrado”. It grows spontaneously in tropical and temperate zones, including the Brazilian Cerrado biome, having great importance for food and medicinal use.^[1,2] At Brazil, a traditional remedy (“polvilho-da-lobeira”) is produced from the pulp of the fruit to be used by its alleged hypoglycemic effect.^[3]

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Biological activities have been intensively investigated, being proved the anti-viral, diuretic, anti-fungi, anti-spasmodic, anti-inflammatory and other pharmacodynamic proprieties.^[4-7] Recently it was also evidenced that extracts of *S. lycocarpum* and its glycoalkaloids, solamargine (Sg) and solasonine (Sn), are active against flagellated protozoa, *Trypanosoma cruzi*,^[8] *Leishmania infantum*,^[7] *Leishmania amazonensis*,^[9] as well as, against helminthes, *Strongyloides stercoralis*^[10] and *Schistosoma mansoni*.^[11] The anti-parasitic effect of *S. lycocarpum* seems to be also useful in wild nature: Ripen fruits are sought by the maned-wolf (*Chrysocyon brachyurus*), the largest canid of South America and some authors believe that eaten fruits help in the control of parasitic diseases that affect the maned-wolf and point glycoalkaloids as the active constituents.^[12,13]

Solamargine and Sn [Figure 1] are the typical metabolites of *S. lycocarpum*, however, several other classes of compounds,

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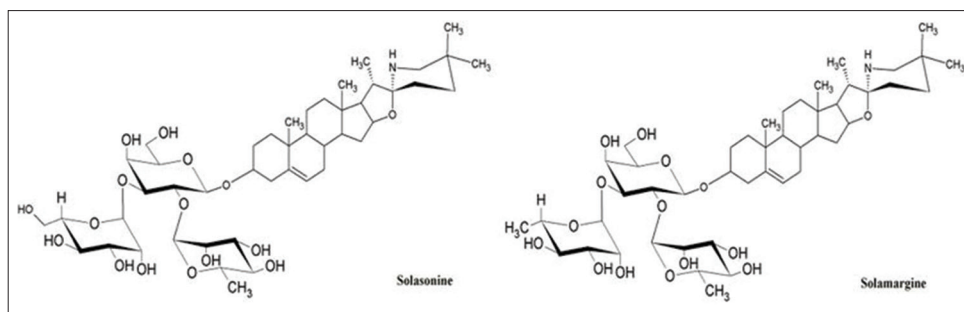


Figure 1: Structures of glycoalkaloids: Solasonine and solamargine

such as phenolic acids, tannins, flavonoids, steroids, and triterpenes were already recognized.^[14,15]

Considering the indications on the anti-parasitic usefulness of *S. lycocarpum* extracts and compounds we proposed to investigate their effects on *Giardia lamblia*, a flagellated protozoon responsible for an intestinal infection (giardiasis) prevalent in many parts of the world and endemic in countries with poor standards of hygiene and sanitation.^[16,17] In those countries, common drugs to treat giardiasis (nitroimidazoles, benzimidazoles, quinacrine, paromomycin or furazolidone) are not of easy access^[18] and together with increasing resistances, adverse effects, and toxicity, therapeutic alternatives are welcome.

Plants with alleged anti-parasitic activity are excellent starting point to be investigated on *G. lamblia* parasite.

MATERIALS AND METHODS

Plant material

Fruits of *S. lycocarpum* St. Hill were collected in Barretos, São Paulo State, 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 State, Brazil.

Fruits were dried in hot air flow at 60°C and then crushed and reduced to powder in a blade grinder.

Extraction

Fruits powder (35 g) of *S. lycocarpum* was submitted to extraction with 250 mL of 96% ethanol during 4 h at boiling temperature, under reflux, following the procedure described by Almeida and Rocca (1995).^[19] After filtration under reduced pressure, remainder powder was re-extracted with 200 mL of 96% ethanol. Extractive solutions were blended, concentrated under reduced pressure to achieve a syrupy consistency and then, dried at room temperature, yielding 8.12 g of dry extract (EB). A thin layer

chromatography (TLC) profile of EB was obtained, using 20 cm × 20 cm TLC aluminum plates pre-coated with a 0.25 mm layer of silica gel 60 F₂₅₄ (Macherey-Nagel GmbH and Co). Plate was activated at 105°C for 30 min prior to use. 15 µL of EB solution (1 mg/mL in 96% ethanol), as well as, 15 µL of solutions of Sn and Sg (1 mg/mL in 96% ethanol) were applied. Reference Sn and Sg (purity 96%) were kindly provided by Professor Adélia Emília de Almeida, Department of Drugs and Medicines, School of Pharmaceutical Sciences, UNESP, Araraquara, São Paulo State - Brazil. Plate was developed with a solution of *n*-butanol: Acetic acid: Water (6:3:1), then dried and revealed with sulfuric acid 10% (v/v) and heated at 150°C for 5 min. Five bands were observed in the chromatogram at retention factors (R_f) of 0.38, 0.46, 0.57, 0.67 and 0.78. Sn and Sg bands were revealed at R_fs 0.46 and 0.57, respectively.

Isolation of solamargine and solasonine

0.5 g of EB was solubilized in 10 mL of 40% ethanol and partitioned (v/v) with 3 × 50 mL of *n*-hexane: Ethyl acetate (9:1). The obtained two fractions, the ethanol fraction (EF) and the *n*-hexane: Ethyl acetate fraction (HF), were concentrated under reduced pressure and monitored by TLC in the condition described above. Sn and Sg [Figure 1] are two major glycoalkaloids found in at least 100 *Solanum* species.^[2,7] In the present study, glycoalkaloids (Sn and Sg) were found in EF and not detected in the HF. 1.0 g of EF was then solubilized in 10 mL of 40% ethanol (v/v) and submitted to open column chromatography, using a 40.0 cm × 5.0 cm glass column filled with a bed of neutral aluminum oxide (VETEC) (63–200 µm).

Elution was performed with 40% ethanol (v/v). Six sub-fractions (F0, F1, F2, F3, F4, and F5), 60 mL each were collected and inspected by TLC. Sn, and Sg were found in sub-fraction F2. Sub-fraction F2 was, then, submitted to preparative TLC, using 20 × 20 cm plates coated with silica gel 60 (0,75 µm layer thickness) activated at 105°C during 30 min 500 µL of the F2 solution were applied and developed with *n*-butanol: Glacial acetic acid: water (6:3:1). TLC bands corresponding to Sg and Sn were scraped-off

and then extracted with ethanol 96%. After centrifugation, the supernatant was evaporated under reduced pressure, and glycoalkaloids recovered.

Concentrations of Sg and Sn in EB and EF, as well as the purity of the isolated glycoalkaloids were estimated by high-performance liquid chromatography-photodiode array detection analysis, following the method described by Tiozzi *et al.*^[20]

Parasites and cultures

The antiparasitic activity assay was performed by growth inhibition assays of trophozoites *G. lamblia* according to the methodology described by Sousa and Piores-da-Silva.^[21]

Giardia lamblia (WBC6 strain [ATCC 30957] originally from a patient with chronic diarrhea) was obtained from the American Type Culture Collection, Rockville, Md. Trophozoites were maintained in axenic culture at 37°C in 10 mL of Diamond's TYI-S-33 medium, as modified by Keister (1983),^[22] in screw-cap cell culture vials. Penicillin G (250 µg/mL) and streptomycin sulfate (250 µg/mL) were added during routine culture. Log-phase cultures (2–3 days) were harvested by cooling culture vials (4°C/15 min) and centrifuged (1500 g/5 min). Trophozoites were washed three times and were then counted in a hemocytometer (Neubauer cell-counter chamber) (5.0×10^4 cells). These cells were used to study the effects of *S. lycocarpum* samples and glycoalkaloids on *G. lamblia* trophozoites growth.

Growth inhibition assay

Susceptibility of *G. lamblia* growth was determined as previously described by Sousa and Piores-da-Silva (1999).^[21] Samples, *S. lycocarpum* extract (EB), the ethanolic fraction (EF), the isolated Sg, the isolated and Sn and mixture of both glycoalkaloids (Sn + Sg) were diluted in dimethylsulfoxide (DMSO; Sigma Chemical) at 100 mg/mL and then in TYI-S-33 medium in order to get a range of concentrations from 10 to 200 µg/mL. Cultures of log-phase trophozoites (5×10^4) were incubated at 37°C for 48 h as a function of samples concentrations in fresh culture medium using 1.5 mL eppendorf vials. Controls were performed in similar experimental conditions with the DMSO solvent and in the absence of samples. After incubation, vials were cooled at 4°C/15 min and the total number of trophozoites were counted under the light microscope (Nikon Eclipse E100) using a Neubauer cell-counter chamber. The results were expressed as cell number, percentage of control, and the concentration that inhibited the growth at 50% inhibitory concentration 50 (IC₅₀) was determined. All experiments were performed in duplicate and in at least three independent assays.

Cytotoxicity assays

For cytotoxicity assays, late log phase of macrophages cells (J774) were trypsinized and was incubated at 37°C in 24-well tissue culture plates under microaerophilic condition. When the monolayers reached confluence (3–4 days), the medium was removed, and the cells incubated with *S. lycocarpum* samples for 48 h. The cells viability was evaluated by tetrazolium-dye (MTT) colorimetric method.^[23] All experiments were performed in duplicate and in at least three independent assays.

RESULTS AND DISCUSSION

Essayed samples from *S. lycocarpum* were characterized in what concerns to the concentrations of Sn, and Sg [Table 1]. In the extract EB, Sn, and Sg account, respectively, 4.6% and 4.4% of the composition. In the EF, these glycoalkaloids attain 15.3% and 35.7%, respectively. Isolated Sn, and Sg are, respectively, at 71.5% and 63.1% purity.

The biological activity of samples of *S. lycocarpum* was evaluated on *G. lamblia* trophozoites and the cytotoxicity on macrophage cells. The anti-giardial assays, based on cell growth inhibition of *G. lamblia* trophozoites, revealed that the samples EB, FE, Sn, Sg, and mixture Sn + Sg (1:1), decreased the number of *G. lamblia* trophozoites as a function of concentration (not shown). Table 2

Table 1: Concentration of glycoalkaloids in the samples from *Solanum lycocarpum*

Samples	Sn (%)	Sg (%)
EB	4.6	4.4
EF	15.3	35.7
Sn	71.5	2.8
Sg	1.9	63.1

EB: 96% ethanolic extract; EF: 40% ethanolic fraction; Sn: Solasonine; Sg: Solamargine

Table 2: Anti-Giardia activity and cytotoxicity on macrophages of samples from *Solanum lycocarpum*

Samples*	IC ₅₀ (µg/mL)		SI**
	<i>Giardia lamblia</i>	Macrophages	
EB	105.40	31.25	0.29
EF	95.05	1000.00	10.5
Sn	103.70	62.50	0.61
Sg	120.30	>2000.00	16.6
Sn + Sg (1:1)	13.23	250.00	18.9
Metronidazole	0.50	-	-

*EB: 96% ethanolic extract; EF: 40% ethanolic fraction; Sn: Solasonine; Sg: Solamargine; Sn + Sg (1:1): Mixture Sn + Sg (1:1). **SI selective index (IC₅₀ macrophages/IC₅₀ Giardia). IC₅₀: Inhibitory concentration 50

summarizes results of anti-*Giardia* activity and cytotoxicity on macrophages. The cytotoxicity of *S. lycocarpum* samples was compared with the activity against *G. lamblia* using the selective index (SI; IC_{50} (macrophage cells)/ IC_{50} (protozoa)) [Table 2]. A value greater than 1.0 is considered that the sample is more selective for the parasite.^[24] According to the classification of giardicidal activity established by Amaral *et al.* (2006),^[25] all samples can be considered active ($100 < IC_{50} \leq 250.00 \mu\text{g/mL}$) or in the case of EF and the mixture as highly actives ($IC_{50} \leq 100.00 \mu\text{g/mL}$). However, EB as well as Sn were cytotoxic for macrophages showing a low index of selectivity (SI of 0.29 and 0.61, respectively) [Table 2].

Surprisingly, although the lower concentrations of glycoalkaloids, the activity of EF (IC_{50} values of $95.00 \mu\text{g/mL}$) is better than those registered for each one of isolated glycoalkaloids (Sn: $IC_{50} = 103.70 \mu\text{g/mL}$; Sg: $IC_{50} = 120.30 \mu\text{g/mL}$). This finding raised the hypothesis that the activity of fraction EF (and also extract EB) could depend from synergistic effects resulting from the presence of both glycoalkaloids.

In fact, when testing the mixture of Sn and Sg (Sn + Sg) activity raises considerably (IC_{50} value of $13.20 \mu\text{g/mL}$) with a high index of selectivity ($SI = 18.9$) [Table 2]. Synergistic effects of these glycoalkaloids were already reported concerning the activity on tumor cells,^[26] fungi,^[5] *L. amazonensis*,^[9] *L. infantum*,^[7] *T. cruzi*,^[8] and fibroblast cell line (LLCMK2 cells).^[9]

Previously were demonstrated that extracts of *S. lycocarpum* and its glycoalkaloids, Sg and Sn, are active against *T. cruzi* (IC_{50} values of $194.7-15.3 \mu\text{g/mL}$),^[7,8] *L. infantum* (IC_{50} value of $16.2 \mu\text{g/mL}$)^[7] and *L. amazonensis* (IC_{50} values of $238.4-6.6 \mu\text{M}$).^[9] Comparing these with the present data, we see that *S. lycocarpum* and its glycoalkaloids shows anti-giardial activity that is similar or higher than that described for the other flagellated protozoa.

The report of the literature suggests that steroidal glycoalkaloids containing a chacotriose moiety (rhamnose-glucose-rhamnose) are usually more biologically active than those containing a solatriose moiety (rhamnose-galactose-glucose). Sg, containing chacotriose moiety, was more active on nematodes.^[27] *T. cruzi*,^[8] and *L. amazonensis*,^[9] than Sn that contains solatriose moiety. The cell membranes recognize the rhamnose moieties and absorb the compound as a whole.^[9,28] In the present study, Sg was not more active on *G. lamblia* trophozoites. In this case, it seems that the type of the sugar moiety do not influences how Sg and Sn interacts with a cell membrane of *Giardia*.

Although having in mind that glycoalkaloids are recognized as potentially toxic, in particular, fetal toxicity and phytoestrogenic effects after long-term consumption,^[29-32] our results are optimistic concerning the usefulness of the mixture of Sn and Sg for development of new therapeutic strategies for treatment of giardiasis.

CONCLUSION

The present study describes for the first time the anti-*Giardia* activity of Sn and Sg, proving the synergic effects of these compounds. Therefore, we propose further *in vitro* and *in vivo* studies to consolidate the usefulness of the mixture of Sn + Sg in view of a new alternative therapeutic strategy for giardiasis.

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