Short Communication

In vitro anthelmintic activity of *Siparuna guianensis* extract and essential oil against *Strongyloides venezuelensis*

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Abstract

New therapeutic approaches are necessary to control strongyloidiasis due to the side effects of, and resistance to, currently available drugs thiabendazole, albendazole, and ivermectin. This study examined the anthelmintic properties of extracts and isolated compounds from *Siparuna guianensis* against *Strongyloides venezuelensis* eggs and larvae, using the egg hatching test (EHT) and larval motility test (LMT). Albendazole (0.025 mg/ml) and ivermectin (0.316 mg/ml) were used as the positive controls for the EHT and LMT assays, respectively. *Strongyloides venezuelensis* eggs or larvae (±50 specimens) were treated with ethanol extract (0.05–1.0 mg/ml), ethyl acetate and aqueous fractions (0.05–0.8 mg/ml), essential oil (0.2–1.0 mg/ml) and α-bisabolol (0.2–1.0 mg/ml) from *S. guianensis*, and analysed by optical microscopy after 48 h (EHT), or after 24, 48 and 72 h (LMT). All the tested compounds exhibited ovicidal activity equivalent to the positive control and changed the morphology of the eggs. The *S. guianensis* ethanol extract and aqueous fraction were as effective as the positive control. Phytochemical analysis of the ethanol extract and fractions revealed the presence of phenolic compounds, tannins and flavonoids. Therefore, *S. guianensis* is effective against *S. venezuelensis* eggs and larvae *in vitro*, and can be considered as a potential alternative treatment for strongyloidiasis.

Introduction

Strongyloidiasis is a globally distributed, but neglected disease caused by *Strongyloides stercoralis*, which is more common in tropical and subtropical regions of Africa, South-East Asia and Latin America in conditions of poor sanitation (Levenhagen & Costa-Cruz, 2014). This disease is especially dangerous for immunosuppressed hosts due to their high risk of developing hyperinfection syndrome, a condition in which proliferation of larvae in various organs culminates in death (Greaves *et al.*, 2013). *Strongyloides stercoralis* larvae can also infect sites that are not part of their usual life cycle and cause disseminated strongyloidiasis (Puthiyakunnon *et al.*, 2014). Treatment of these severe forms of strongyloidiasis in patients who are immunocompromised and/or have reduced intestinal absorption of oral antiparasitic drugs is still controversial (Moura *et al.*, 2012).

The indiscriminate use of anthelmintic drugs has favoured the development of parasite resistance (Carvalho *et al.*, 2012), which reduces the effectiveness of drugs therapies (Grove, 1996) and prompts the continuous search for complementary anthelmintic drugs (Kumarasingha *et al.*, 2016). In this context, medicinal plant research is a rich source of knowledge on the potential action of plant extracts in treating human and animal diseases (Carvalho *et al.*, 2012), including the control of intestinal nematodes through their anthelmintic properties (Wangchuk *et al.*, 2016).

Siparuna guianensis, which belongs to the Siparunaceae family, is a typical bush of the Brazilian Cerrado known as 'negramina', though this popular name varies according to the region or country where it occurs (Valentini *et al.*, 2010). *Siparuna guianensis* leaves and flowers are used in Brazilian folk medicine due to their aromatic, stimulatory, anti-inflammatory, diuretic, anti-dyspeptic, anti-rheumatic and carminative properties (Aguiar *et al.*, 2015).

As there are no reports on the anthelmintic activity of *S. guianensis*, the present study examined the action of ethanol extracts, fractions, essential oil and major constituent α-bisabolol derived from it against *Strongyloides venezuelensis* eggs and larvae *in vitro*.

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Materials and methods

Strongyloides venezuelensis eggs and larvae

The strain of *S. venezuelensis* was maintained by serial passage of infective larvae (L3) in gerbils (*Meriones unguiculatus*). Faeces from infected animals were used for egg collection and vermiculite culture (72 h at 28°C) to obtain infective (L3) larvae (Rugai *et al.*, 1954).

Plant material

Aerial parts of *S. guianensis* were collected in May 2014 at Jataí, Goiás, Brazil, within the Cerrado. The voucher specimen (Herbário Jataiense 1997) was deposited at the herbarium of the Universidade Federal de Jataí, Goiás, Brazil.

Preparation of extracts and fractions

Leaves of *S. guianensis* were washed with distilled water and dried for three days in an oven at 40–45°C. The dried material was powdered, submitted to ultrasonic extractions with ethanol for five times and filtered. The filtrate was rotary evaporated at 50–55°C to yield the ethanol extract. This extract was dissolved in an ethanol/water (3:7) solution and partitioned by liquid–liquid extraction with hexane and ethyl acetate. The resulting hexane, ethyl acetate and aqueous fractions were concentrated and stored at −20°C until used.

Extraction of S. guianensis essential oil

Leaves of *S. guianensis* were crushed and hydrodistilled in a Clevenger-type apparatus for 4 h. The oil was collected with the hydrolate and extracted with dichloromethane (3×10 ml) in a separatory funnel. The organic phases were dried with anhydrous magnesium sulphate and rotary evaporated at 40°C, under low pressure. The resulting essential oil was weighed in an analytical balance and the extraction yield was expressed as mg/g plant dry mass. Dry matter was determined by drying three aliquots of approximately 10 g in an oven at 103 ± 2°C to constant mass. The essential oil was stored in sealed tubes at −20°C until used.

Isolation of α-bisabolol

The *S. guianensis* essential oil was fractionated on a silica gel chromatographic column (36 cm × 18 mm) according to the method reported by Diniz (2014). Briefly, 0.2 g of essential oil was applied onto the top of the column and eluted with a hexane/ethyl ether gradient in the following ratios: 10:0, 9:1, 8:2, 7:3, 6:4 and 5:5. The fractions were analysed by thin-layer chromatography, using hexane/ethyl ether (7:3) as the mobile phase and phosphomolybdic acid as the developer. α-bisabolol was identified by gas chromatography–tandem mass spectrometry, and compared with an α-bisabolol standard previously characterized by the Laboratory of Analysis and Synthesis of Agrochemicals at the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil (Diniz, 2014). The α-bisabolol fraction was stored in a stoppered sample vial at −20°C until used.

Dilution of S. guianensis samples

The *S. guianensis* extract and fractions were dissolved in 1% dimethyl sulfoxide (DMSO) to achieve final concentrations of 0.2–1.0 mg/ml and 0.05–0.8 mg/ml, respectively. The essential oil and isolated α-bisabolol were diluted in 1% DMSO to provide 0.2–1.0 mg/ml solutions. All the solutions were stored under refrigeration until used.

Phytochemical analysis

Qualitative phytochemical characterization and analysis by thin-layer chromatography were performed according to the protocol reported by Barbosa *et al.* (2004). The ethanol extract and ethyl acetate and aqueous fractions were screened for the presence of tannins, phenolic compounds, flavonoids and saponins. Aliquots of the extract and fractions were applied to silica gel plates, eluted with a mixture of solvents with different polarities (hexane, ethyl acetate, acetone and methanol) and revealed using ultraviolet light (254–360 nm) and acid vanillin.

Egg hatching test

The egg hatching test was performed using the procedure reported by Coles *et al.* (1992), with some modifications. Faeces from *S. venezuelensis*-infected gerbils were homogenized in water and filtered through a 50-μm sieve. Fifty microliters of the filtrate containing 50 eggs were added to 50 μl of each *S. guianensis* test-sample (extract, fractions, essential oil and isolated compound). After 48 h of incubation at 28°C, egg hatching and larvae from hatchings were quantified using an optical microscope. Albendazole solution (0.025 mg/ml) was used as the positive control (Carvalho *et al.*, 2012), and water and 1% DMSO were used as the negative controls.

Larval motility test

The larval motility test was performed according to the method described by Cordeiro *et al.* (2010), with some modifications. Fifty microliters of a solution containing 50 infective larvae (L3) were mixed with 50 μl of each *S. guianensis* test-sample (extract, fractions, essential oil and isolated compound). After 24, 48 and 72 h of incubation at 28°C, larval motility was analysed and quantified using an optical microscope. Ivermectin solution (0.316 mg/ml) was used as the positive control (Rebollo *et al.*, 2003), and water and 1% DMSO were used as the negative controls. All the tests were performed in triplicate for each concentration.

Data analysis

The percentage of inhibition of eggs hatching (IEH) was calculated as reported by Wood *et al.* (1995), who followed the Guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP):

$$IEH(\%) = \left[\frac{\text{number of eggs}}{\text{number of eggs} + \text{number of larvae}} \right] \times 100$$

The percentage of inhibition of larval motility (ILM) was calculated using the formula reported by Al-Rofaai *et al.* (2013):

$$ILM(\%) = \left[\frac{(\%)\text{motility in the treated group} - (\%)\text{motility in negative control}}{100 - (\%)\text{motility in negative control}} \right] \times 100$$

Data were analysed using the GraphPad Prism 5.0 software. The half-maximal inhibitory concentration of each test-sample (IC_{50}) was calculated. The experimental, positive and negative control groups were compared using Fisher's exact test. Comparison of mobile and immobile larvae at the different times of analysis and between the different concentrations tested was performed by one-way analysis of variance (ANOVA) and the Kruskal–Wallis test. Data from the extracts and fractions at the same dilution were compared using the Mann–Whitney test. $P < 0.05$ was considered statistically significant.

Results and discussion

The *S. guianensis* samples differed in their ability to inhibit *S. venezuelensis* egg hatching (table 1; see supplementary table S1). None of the ethanol extract concentrations tested suppressed egg hatching as effectively as the positive control; however, those from 0.6 to 1.0 mg/ml suppressed eggs hatching more strongly than the negative controls ($P < 0.05$). The inhibitory effect of the ethyl acetate and aqueous fractions, essential oil and isolated α-bisabolol on egg hatching only differed from the negative controls at the highest concentrations tested (1.0 mg/ml; $P < 0.05$). The ethyl acetate and aqueous fractions suppressed egg hatching in a concentration-dependent manner, their inhibitory effect increasing from 71.2% and 70.7% at 0.05 mg/ml, respectively.

There are few studies on the *in vitro* anthelmintic activity of plants against *Strongyloides* spp., and most of these are related to other nematodes, such as *Haemonchus contortus* (Macedo *et al.*, 2009, 2012).The *S. guianensis* samples were effective against *S. venezuelensis* at concentrations lower than plant extracts from *Eucalyptos globulus* (Macedo *et al.* 2009), *Alpinia zerumbet*, *Coriandrum sativum*, *Lantana camara* and *Tagetes minuta* (Macedo *et al.*, 2012) were against *H. contortus*.

In this study, we demonstrated that the negative controls (water and DMSO 1%) showed 62.7% and 69.4% inhibition of *S. venezuelensis* egg hatching, respectively; however, a higher number of larvae were observed in relation to other tested compounds of *S. guianensis*. These results were similar to the findings of negative controls in other IEH tests of the same species (Moraes *et al.*, 2017). This study suggested that IEH caused by negative controls was due to the isolation of the eggs from another environment that was not faeces. A previous study showed that the viability of *S. venezuelensis* eggs was lower when isolated from saline solution compared to storage in faeces, and when exposed to different environmental conditions (Baek *et al.*, 1998). Compared with the negative controls, all the *S. guianensis* samples tested, ethanol extract, aqueous and ethyl acetate fractions, essential oil and isolated α-bisabolol, reduced the number of embryonated eggs and caused degradation of L1 larvae inside the egg. These larvae resembled a condensed mass, suggesting that the *S. guianensis* samples reached the interior of the egg and interfered with the blastomeration phase. All the samples caused morphological changes in *S. venezuelensis* eggs within the concentrations tested (fig. 1). They all suppressed egg hatching as effectively as the positive control, albendazole, at the highest concentrations tested, and caused morphological alterations in the eggs with degradation of L1. This was consistent with previous reports that the anthelmintic efficiency of a given compound relies on its ability to inhibit egg development and hatching during the first larval stage (Krychak-Furtado *et al.*, 2005).

Table 1. Inhibition of *S. venezuelensis* egg hatching after 48 h of treatment with *S. guianensis* samples.

Sample	Inhibition of eggs hatching (%)
Ethanol extract (mg/ml)	
1.0	93.5 ± 0.0[a,c]
0.8	89.6 ± 0.3[a,c]
0.6	87.4 ± 1.9[a,c]
0.4	84.9 ± 0.6[a]
0.2	84.4 ± 2.7[a]
Essential oil (mg/ml)	
1.0	90.1 ± 0.0[a,c]
0.8	84.7 ± 2.7[a]
0.6	81.9 ± 3.9[a]
0.4	81.0 ± 1.0[a]
0.2	80.0 ± 1.9[a]
Alpha-bisabolol (mg/ml)	
1.0	90.6 ± 1.4[a,c]
0.8	87.6 ± 0.4[a]
0.6	87.4 ± 0.2[a]
0.4	83.2 ± 0.1[a]
0.2	83.2 ± 1.8[a]
Water	62.8 ± 1.9
DMSO (1%)	69.4 ± 1.2
Albendazole (0.025 mg/ml)	94.4 ± 0.1

Letters indicate significant difference ($P < 0.05$) from the controls, [a]water and [c]dimethyl sulfoxide (DMSO), for the Fisher's exact test.

The inhibitory effect of the five *S. guianensis* samples on *S. venezuelensis* larval motility did not significantly differ across the treatment periods (24, 48 and 72 h; data not shown); however, they significantly differed from the negative controls (water and DMSO) and showed a concentration-dependent manner, except for α-bisabolol (table 2; see supplementary table S2). The ethanol extract suppressed larval motility by 88.4% at 0.2 mg/ml and by 100% at concentrations >0.8 mg/ml (table 2). At concentrations of 0.8 mg/ml, the ethyl acetate and aqueous fractions suppressed larval motility by 82.2% and 99.4%, respectively (see supplementary table S2). Essential oil and α-bisabolol at the range of concentrations tested, as well as ethyl acetate and aqueous fractions at concentrations <0.4 mg/ml, only weakly inhibited larval motility (table 2; see supplementary table S2). The ethanol extract was the *S. guianensis* sample that suppressed larval motility the most strongly, inhibiting larval motility by 88.4% at the lowest concentration tested. These findings were consistent with the inhibitory effects of *Spondias lutea* ethanol extract and fractions against *S. venezuelensis* adult females (Medeiros, 2014), and *Carica papaya* latex and purified papain against L3 larvae (Moraes *et al.*, 2017). WAAVP classifies an anthelmintic compound as highly and moderately effective when it inhibits >90.0% and 80.0–90.0% of parasite activity, respectively. Anthelmintic compounds kill worms by either starving or paralysing them, causing death in up to 24 h. The efficacy of *S. guianensis* ethanol extract against
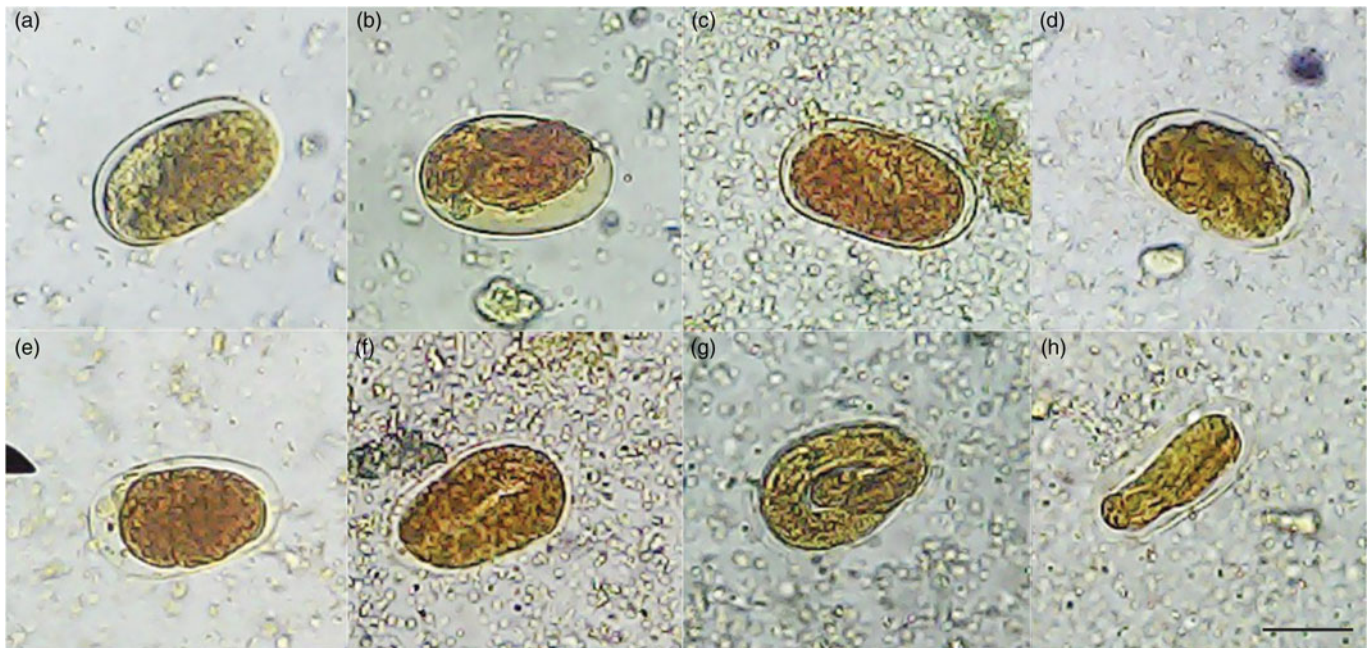
Fig. 1. Morphological changes in *S. venezuelensis* eggs after treatment with (a) an ethanol extract, (b) essential oil, (c) α-bisabolol, (d) ethyl acetate and (e) an aqueous fraction of *S. guianensis* leaves. Negative controls: (f) water and (g) 1% DMSO. Positive control: (h) 0.025 mg/ml albendazole. Note: there was no development of L1 larvae inside the eggs and only a condensed mass was visualized for treatments (a)–(e) and (h). Negative controls: (f) water and (g) 1% DMSO showed L1 larvae inside the eggs. Images were captured at 40× magnification. Scale bar 40 μm.

Table 2. Inhibition of *S. venezuelensis* larval motility after 24 h of treatment with *S. guianensis* samples.

Sample	Inhibition of larval motility (%)
Ethanol extract (mg/ml)	
1.0	100.0 ± 0.0[a,c]
0.8	100.0 ± 0.0[a,c]
0.6	98.9 ± 0.0[a,c]
0.4	94.5 ± 0.6[a,c]
0.2	88.4 ± 2.2[a,b,c]
Essential oil (mg/ml)	
1.0	38.6 ± 0.7[a,b,c]
0.8	23.0 ± 0.6[a,b,c]
0.6	21.3 ± 2.4[a,b,c]
0.4	16.4 ± 0.8[a,b,c]
0.2	15.9 ± 1.1[a,b,c]
Alpha-bisabolol (mg/ml)	
1.0	18.5 ± 0.4[a,b,c]
0.8	16.4 ± 0.2[a,b,c]
0.6	11.1 ± 1.5[b]
0.4	8.9 ± 1.4[b]
0.2	6.4 ± 3.2[b]
Water	0.0 ± 0.5
DMSO (1%)	0.0 ± 0.6
Ivermectin (0.316 mg/ml)	100.0 ± 0.0

Letters indicate significant difference ($P < 0.05$) from the controls, [a]water, [b]ivermectin and [c]dimethyl sulfoxide (DMSO), for the Fisher's exact test.

S. venezuelensis after only a 24-h treatment indicated its anthelmintic potential.

As the chemical components of *S. guianensis* grown in Jataí, Goiás, Brazil, had not previously been described, we qualitatively analysed the ethanol extract and ethyl acetate and aqueous fractions. The major classes of secondary metabolites identified were tannins, phenolic compounds and flavonoids (see supplementary table S3). Although flavonoids, including quercetin (Leitão *et al.*, 2005) and vicenin-2 (Negri *et al.*, 2012), have been identified in *S. guianensis* leaf extracts from other Brazilian regions, chemical components of leaves, stem and fruits can vary according to geographic location and season (Valentini *et al.*, 2010). The combined use of phytochemical screening and thin-layer chromatography analysis increases the probability of selecting bioactive secondary metabolites (Macedo *et al.*, 2012).

We hypothesize that the ovicidal and larvicidal activity of the ethanol extract and fractions of *S. guianensis* was mediated by one or more classes of metabolites identified here. Previous studies have reported the presence of condensed tannins and different subclasses of flavonoids in plants with anthelminthic potential (Kozan *et al.*, 2013; Femex *et al.*, 2016). The mechanism of action of flavonoids is related to their ability to change the parasite's metabolic processes and/or enzymatic activities (Kerboeuf *et al.*, 2008), while the action of tannins depends on the place where they bind to cause structural changes, such as the external cuticle and the digestive or reproductive tracts (Hoste *et al.*, 2006).

Taken together, the findings reported herein demonstrate the anthelmintic potential of *S. guianensis*, especially the strong efficacy of its ethanol extract and fractions when compared with the other samples tested. Such fractions show promise for the development of new drugs. Notably, the ethanol extract and fractions were not cytotoxic towards NIH/3T3 murine fibroblasts and Vero cells, at least at the concentrations that exhibited significant anthelmintic activity (data not shown). However, additional

studies are required to isolate and identify the chemical constituents responsible for the anthelmintic activity of *S. guianensis* as well as to establish their effectiveness in *in vivo* tests.

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