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Ethanolic extracts of different fruit trees and their activity against *Strongyloides venezuelensis*

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ABSTRACT

***Strongyloides venezuelensis* and *Strongyloides ratti* are both rodents' parasites that are important models both in an immunologic and biologic perspective, for the development of new drugs and diagnostic tests. This study was aimed at studying the anthelminthic *in vitro* effect of ethanolic extracts, obtained from several species of Brazilian fruit trees, against *S. venezuelensis* parasitic females in an attempt to search for new therapeutic alternatives. Plant leaves were collected in the Multidisciplinary Center of Chemical, Biological and Agricultural Research (CPQBA) at Campinas State University (UNICAMP), in Paulínia, Brazil. Ethanolic extracts were obtained by mixing dried powdered plant leaves (10 g) with 150 mL of ethanol for 10 min/16.000 rpm in a mechanical disperser (Ultra Turrax T50, IKA Works Inc., Wilmington, NC, USA), followed by filtration. The residue was re-extracted with 100 mL of ethanol. The extracts were pooled and evaporated under vacuum until dry, resulting in the final dried ethanolic extracts. Inhibitory concentration was determined using Origin 7 program. Statistical analysis was performed using SAS program. Significant differences between groups were calculated using one-way Analysis of Variance (ANOVA) ($p<0,0001$). The correlation between time, motility, oviposition, and mortality with the extract concentration was accessed using Duncan's Multiple Range Test. *Spondias mombin* ethanolic extract and aqueous fraction showed the most promising results, with anti-Strongyloides activity and 100% mortality rate after 72 h, for all tested concentrations. Overall, most of the extracts showed a satisfactory anthelmintic effect for at least one of the tested concentrations.**

INTRODUCTION

Strongyloidiasis is an intestinal neglected disease caused by the nematode *Strongyloides stercoralis* with over 100 million estimate cases worldwide (Bisoffi et al., 2013). The risk of infection is greater in regions with hot and humid climate, in people who work with soil and/or belonging to less favored socioeconomic groups (Olsen et al., 2009).

Although most infected individuals are either asymptomatic or have few symptoms, an important characteristic of *S. stercoralis* lies on the ability to replicate inside the host and auto-infect it. Autoinfection may lead to persistent chronichyper infections, with a wide variation of chronic hyper manifestations, which

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could evolve to a disseminated strongyloidiasis (Gryseels et al., 2006) with an 87% mortality rate (Olsen et al., 2009).

Strongyloides venezuelensis and *Strongyloides ratti*, both rodents' parasites, are important models both in an immunologic and biologic perspective, for the development of new drugs and diagnostic tests (Nolan et al., 1993; Olson and Schiller, 1978; Sato and Toma, 1990).

Nowadays, strongyloidiasis treatment is performed with either albendazole (400 mg/kg) or ivermectin (200 µg/kg) (Olsen et al., 2009; WHO, 2012). Thiabendazole, mebendazole and cambendazole, have been used for strongyloidiasis treatment, however their use has been dropped due to their toxicity and adverse effects, frequent treatment fail and drug resistance (Bisoffi et al., 2013; Legarda-Ceballos et al., 2016; Olsen et al., 2009).

Due to high prevalence and low therapeutic efficiency of synthetic drugs currently available for strongyloidiasis treatment, there is a need for new therapeutic alternatives. Medicinal plants and their extracts have been used in the treatment of several diseases, including parasite infections, making them a viable alternative in the search for new drugs against *S. stercoralis* (Anthony et al., 2005).

In this report, the anthelminthic *in vitro* effect of ethanolic extracts, obtained from several species of Brazilian fruit trees, against *S. venezuelensis* parasitic females is described; in an attempt to search for new therapeutic alternatives.

MATERIALS AND METHODS

Vegetable material

Plant leaves were collected in the Multidisciplinary Center of Chemical Biological and Agricultural Research (CPQBA) at Campinas State University (UNICAMP), in Paulínia, SP. 24 plants were used (Table 1). The criteria for selection of plant was to use only plants that bear fruit and were easy to cultivate.

Ethanolic extracts and fractions

The ethanolic extracts were obtained by mixing dried powdered plant leaves (10 g) with 150 mL of ethanol for 10 min/16.000 rpm in a mechanical disperser (Ultra Turrax T50, IKA Works Inc., Wilmington, NC, USA), followed by filtration. The residue was re-extracted with 100 mL of ethanol. The extracts were pooled and evaporated under vacuum until dry, resulting in the final dried ethanolic extracts.

The extracts with better results were then fractioned. Briefly, 1.33 g of dry extract was dissolved in 50 mL of distilled water with help of a sonicator, transferred to a separation funnel and then, 50 mL of acetone were added. The process was repeated and instead of acetone, 50 mL of dichloromethane was added. The organic and aqueous fractions were separated and dried in a rotary evaporator.

Drug control

For the control group three synthetic compounds were used, albendazole (generic drug EMS, 400 mg), cambendazole (Cambem®, UCI-farma, 180 mg) and ivermectine (Ivermec®, UCI-farma, 6 mg).

Biological models

Strongyloides venezuelensis

In the research, a *S. venezuelensis* strain isolated from wild rodents (*Bolomys lasiurus)*; that has been maintained for several years in our lab at UNICAMP was used, through successive infections in *Rattus norvegicus,* Wistar lineage. The experiments were approved by the Ethics Commission for Animal Use (CEUA/UNICAMP, protocol 2174-1), as they were in accordance with the ethical principles of animal experimentation adopted by CEUA.

Parasite female recovery

Fifteen days after infection, the rats were euthanized and 15 cm of the small intestine was removed and slice longitudinally. The intestine was washed with sterile saline solution (0.15 mol/L) and placed in a Petri dish containing RPMI 1640 (Nutricell®) medium (supplemented with 0.05 g/L of streptomycin, 10.000 UI/ml of penicillin, 0.3 g/L of L-Glutamine, 2.0 g/L of D-Glucose, 2.0 g/L of $NaHCO_3$ and 5,958 g/L of Hepes), and were kept at 37°C for 1 h. Afterwards, female parasites were collect and washed three times in RPMI medium to avoid later contamination during the *in vitro* assays. Only parasitic females (pathenogenetic) were used since they are the main parasitic form living in the vertebrate host and for their ability to reproduce inside the host which may lead to a disseminated infection. Males only exist as a free-living form in the soil.

In vitro assay

Anthelminthic evaluation

Both the extracts and synthetic drugs were diluted in 2% Phosphate buffered saline (PBS) solution and tested for

Table 1. List of plants used and ethanolic extract yield.

Family	Species	CPQBA plant code	Yield (%)
Anacardiaceae	*Spondias dulcis*	50	21
	Spondias mombin	1	17.3
Arecaceae	*Butia capitata*	132	12.4
Celastraceae	*Salacia elliptica*	104	22.4
Fabaceae	*Inga cylindrica*	80	9.9
Jungladaceae	*Carya illinoensis*	31	12.1
Malpighiaceae	*Byrsonima crassifolia*	15	7.6
Myrtaceae	*Aceima smeithii*	8	12.7
	Eugenia brasiliensis	38	29
	Eugenia involucrata	28	8.3
	Eugenia uniflora	60	10.8
	Eugenia pyriformis	4	18.7
	Hexachlamys edulis	51	19.6
	Myrcianthes pungens	124	10.4
	Psidium cattleianum	53	17.8
Proteaceae	*Macadamia integrifolia*	103	7
Sapindaceae	*Dimocarpus longan*	17	12.5
	Litchi chinensis	44	14.7
Sapotaceae	*Labramia bojeri*	115	21.5
	Lucum acaimito	66	22.8
	Manilkara zapota	16	15.3
	Pouteria campechiana	81	13
Tiliaceae	*Muntingia calabura*	36	10.3
Urticaceae	*Pourouma cecropiifolia*	69	7.8

three concentrations, 0.05, 0.1 and 0.2 mg/mL. The *in vitro* assay was performed in 24 wells plates with RPMI (Nutricell®) medium.

Two worms were kept in each well and each concentration was tested three times (n = 6). A control group of RPMI medium and 2% PBS. The plates were incubated at 37°C in a 5% CO_2 atmosphere and observed at 2, 4, 6, 24, 48 and 72 h, using an inverted microscope (DM-500-Leica®). Motility (Absence, low, moderate and high), oviposition and mortality were observed.

IC_{50} determination and statistical analysis

Inhibitory concentration was determined using Origin 7 program. Statistical analysis was performed using SAS program. Significant differences between groups were calculated using one-way Analysis of Variance (ANOVA) ($p<0.0001$). The correlation between time, motility, oviposition, and mortality with the extract concentration was accessed using Duncan's Multiple Range Test.

RESULTS

Plant yield

The ethanolic extracts yield, from an initial 10 g mass, varied between 1.3 to 29% (*P. campechiana* and *E. brasiliensis*, respectively) and its represented in Table 1.

Effects on worm motility

Worm motility was compared between the average of each tested sample for its corresponding time. In Table 2, the averages of the extract that show significant effect ($p<0.0001$) against *S. venezuelensis* is highlighted namely:

S. mombin, Psidium cattleianum, Inga cylindrica, Manilkara zapota, Eugenia pyriformis, Labramia bojeri, Myrcianthes pungens, Byrsonima crassifolia, Eugenia brasiliensis, Muntingia calabura, Carya illinoensis, Hexachlamys edulis, Eugenia uniflora and *Pourouma cecropiifolia.*

Table 2. Duncan's Multiple Range Test – worms mean motility at different observation times: 1-6 h (T1), 12 h (T2), 24 h (T3), 48 h (T4), e 72 h (T5). Calculated according with the scale: 0, Absence; 1, low; 2, moderate; 3, high.

Worm motility X observation time						
Extract	**Species**	**Group mean**				
		T1	**T2**	**T3**	**T4**	**T5**
EE	*Spondias mombin*	3.00 (a)	2.38 (d,c)	0.83 (l, k)	0.00 (l)	0.00 (m)
FA	*Spondias mombin*	3.00 (a)	3.00 (a)	2.11 (f, h, e, g)	1.00 (i, g, h)	0.61 (k, j, i, h)
FO	*Spondias mombin*	3.00 (a)	2.77 (a, b)	1.83 (h, g)	0.00 (l)	0.00 (m)
EE	*Psidium cattleianum*	3.00 (a)	1.55 (h, i)	0.83 (l, k)	0.55 (j, k)	0.44 (k, j, l)
EE	*Inga cylindrica*	1.83 (d)	1.27 (i)	1.00 (j, k)	0.72 (i, j)	0.16 (m, l)
EE	*Manilkara zapota*	2.16 (c)	2.22 (e, d, f)	1.22 (j, k)	0.66 (i, j, k)	0.61 (k, j, i, h)
EE	*Eugenia pyriformis*	2.50 (b)	2.44 (b, d, c)	1.77 (h)	0.77 (i, j)	0.55 (k, j, i, l)
EE	*Labramia bojeri*	2.50 (b)	1.88 (h, g, f)	1.72 (h, i)	0.61 (i, j, k)	0.44 (k, j, l)
EE	*Myrcianthes pungens*	3.00 (a)	2.72 (b, a, c)	2.22 (f, d, e, g)	0.83 (i, j, h)	0.33 (k, m, l)
EE	*Byrsonima crassifólia*	3.00 (a)	2.00 (e, g, f)	1.22 (j, k)	0.55 (j, k)	0.27 (k, m, l)
EE	*Eugenia brasiliensis*	2.50 (b)	2.00 (e, g, f)	1.38 (j, i)	0.61 (i, j, k)	0.50 (k, j, l)
EE	*Muntingia calabura*	3.00 (a)	2.88 (a)	2.27 (f, d, e)	1.27 (f, g)	0.55 (k, j, i, l)
EE	*Carya illinoensis*	2.50 (b)	2.27 (e, d)	2.00 (f, h, g)	1.22 (f, g)	0.77 (g, j, i, h)
EE	*Hexachlamys edulis*	3.00 (a)	2.00 (e, g, f)	1.83 (h, g)	1.16 (f, g, h)	0.77 (g, j, i, h)
EE	*Eugenia uniflora*	3.00 (a)	2.33 (e, d)	2.11 (f, h, e, g)	1.27 (f, g)	0.44 (k, j, l)
EE	*Lucum acaimito*	3.00 (a)	3.00 (a)	2.61 (b, d, a, c)	1.94 (c, b, d)	1.00 (g, f, h)
EE	*Pourouma cecropiifolia*	2.55 (b)	2.38 (d, c)	2.38 (f, d, e, c)	2.27 (b)	0.94 (g, i, h)
EE	*Pouteria campechiana*	3.00 (a)	3.00 (a)	3.00 (a)	2.00 (c, b, d)	1.50 (c, e, b, d)
EE	*Salacia elliptica*	3.00 (a)	2.77 (b, a)	2.50 (d, e, c)	2.00 (c, b, d)	1.16 (g, e, f)
EE	*Macadamia integrifólia*	3.00 (a)	3.00 (a)	2.94 (b, a)	2.22 (c, b)	1.77 (c, b)
EE	*Spondias dulcis*	2.94 (a)	2.94 (a)	2.94 (b, a)	2.94 (a)	1.83 (b)
EE	*Dimocarpus longan*	3.00 (a)	3.00 (a)	3.00 (a)	1.77 (e, d)	1.38 (c, e, d)
EE	*Litchi chinensis*	3.00 (a)	3.00 (a)	2.77 (b, a, c)	1.83 (c, d)	1.55 (c, e, b, d)
EE	*Aceima smeithii*	3.00 (a)	3.00 (a)	2.50 (d, e, c)	2.00 (c, b, d)	1.61 (c, b, d)
EE	*Eugenia involucrata*	3.00 (a)	3.00 (a)	3.00 (a)	3.00 (a)	3.00 (a)
EE	*Butia capitata*	3.00 (a)	3.00 (a)	3.00 (a)	3.00 (a)	3.00 (a)
DC	*Cambendazole*	3.00 (a)	3.00 (a)	2.55 (b, d, c)	1.44 (f, e)	1.33 (e, f, d)
DC	*Albendazole*	3.00 (a)	3.00 (a)	3.00 (a)	3.00 (a)	3.00 (a)
DC	*Ivermectin*	3.00 (a)	3.00 (a)	3.00 (a)	1.94 (c, b, d)	1.77 (c, b)
CONT	*Control (RPMI)*	3.00 (a)	3.00 (a)	3.00 (a)	3.00 (a)	3.00 (a)

EE, Ethanolic extract; FA, aquous fraction; FO, organic fraction; DC, drug control; Cont, RPMI control.
Means with the same letters do not have significant differences between each other.

Worm mortality

S. mombin ethanolic extract and aqueous fraction showed the most promising results, with anti-*Strongyloides* activity and 100% mortality rate after 72 h, for all tested concentrations. Overall most of the extracts showed a satisfactory anthelmintic effect for at least one of the tested concentrations (Table 3): *P. cattleianum*, *M. zapota*, *L. bojeri, M. pungens*, *B. crassifolia*, *E. brasiliensis* and *S. mombin* aqueous fraction showed 100% mortality at 0.2 e 0.1 mg/mL. *Litchi chinensis, Aceima smeithii, Eugenia involucrata, Butia capitata,* ethanolic extracts did not show activity.

With the purpose of isolating and identifying the chemical compounds that act as antheminthics, *S. mombin* extract, was submitted to a liquid-liquid extraction, resulting in an organic and an aqueous fraction. The results showed a higher efficiency for the organic fraction killing 100% of all females at all tested concentrations, whilst the aqueous fraction only showed activity in the two highest concentrations (0.2 mg/mL e 0.1 mg/mL).

Cambendazole, albendazole and ivermectin did not kill any female worm during the observation period for the tested concentrations. No correlation was found between the plant family and their anthelminthic activity.

Table 3. Total worm mortality at each of the tested concentrations for each extract: C1 (0.2 mg/mL); C2 (0.1 mg/mL); C3 (0.05 mg/mL).

Extract	Species	Mortality (%)			IC50 (mg/kg)
		C1	C2	C3	
EE	*Spondias mombin*	100	100	100	<0.05
FA	*Spondias mombin*	100	100	0	0.07
FO	*Spondias mombin*	100	100	100	<0.05
EE	*Psidium cattleianum*	100	100	0	0.07
EE	*Inga cylindrica*	100	83.33	66.66	<0.05
EE	*Manilkara zapota*	100	100	0	0.07
EE	*Eugenia pyriformis*	100	66.66	0	0.09
EE	*Labramia bojeri*	100	100	0	0.07
EE	*Myrcianthes pungens*	100	100	0	0.07
EE	*Byrsonima crassifólia*	100	100	16.66	0.07
EE	*Eugenia brasiliensis*	100	100	0	0.07
EE	*Muntingia calabura*	100	83.33	0	0.08
EE	*Carya illinoensis*	100	33.33	33.33	0.12
EE	*Hexachlamys edulis*	100	16.66	0	0.13
EE	*Eugenia uniflora*	100	83.33	0	0.08
EE	*Lucum acaimito*	66.66	33.33	0	0.15
EE	*Pourouma cecropiifolia*	100	50	0	>0.2
EE	*Pouteria campechiana*	100	0	0	0.15
EE	*Salacia elliptica*	100	0	0	0.15
EE	*Macadamia integrifólia*	33.33	0	0	>0.2
EE	*Spondias dulcis*	0	16.66	0	>0.2
EE	*Dimocarpus longan*	50	16.66	0	0.2
EE	*Litchi chinensis*	0	0	0	>0.2
EE	*Aceima smeithii*	0	0	0	>0.2
EE	*Eugenia involucrata*	0	0	0	>0.2
EE	*Butia capitata*	0	0	0	>0.2
DC	*Cambendazole*	0	0	0	>0.2
DC	*Albendazole*	0	0	0	>0.2
DC	*Ivermectin*	0	0	0	>0.2

EE, Ethanolic extract; FA, aquous fraction; FO, organic fraction; DC, drug control.

DISCUSSION

Strongyloidiasis is a silent, underdiagnosed and the most neglected helminthic disease (Bisoffi et al., 2013). Currently, there is no satisfactory drug in the treatment of this parasite, since its efficiency varies between different patients (Bisoffi et al., 2013). Both efficient and inefficient treatment are often reported in the same regions with the same treatment scheme (Panic et al., 2014), there is, therefore a need to search for new drugs. The use of medicinal plants in the search of new drugs is increasing, and several plants have shown their anthelminthic activity (Muthee et al., 2011). Plants active compounds identification has been increasing, contributing for a higher variability and availability of drugs and bringing highly accepted therapeutic alternatives (de Oliveira et al., 2014). The majority of published studies test the *in vitro* activity against *Strongyloides* larvae (Keiser et al., 2008; Kotze et al., 2004), to our knowledge, this is the first *in vitro* study investigating the anti-*Strongyloides* activity against adult parasitic females. Our methodology was derived from the one published by de Oliveira et al. (2012), where *Schistosoma mansoni* was used as an experimental model, and provided an efficient way to perform *in vitro* tests with *S. venezuelensis*.

Promising results of some plant species anti-parasite effect against parasites such as *S. mansoni* (de Oliveira et al., 2014; de Oliveira et al., 2012) and *Giardia duodenalis* have been described (Machado et al., 2011; Muthee et al., 2011). Their use as also been researched against other organisms, such as fungus (Wianowska et al., 2016), virus (Gonçalves et al., 2005) and tumors

(Atjanasuppat et al., 2009). Little is known regarding the pharmacologic action of most plants used in this paper. Among the tested extracts, *S. mombin* showed the most promisingresults. Several other authors have been exploring the activity of *S. mombin* ranging from their use against the diarrhea rotavirus (Gonçalves et al., 2005), against larvae and adult mosquitos (Ajaegbu et al., 2016; Eze et al., 2014), *Candida albicans* (Okwuosa et al., 2012), *Leishmania chagasi* (although it has shown low activity against *L. amazonensis* amastigote) (Accioly et al., 2012; Estevez et al., 2007), anti-bacterial activity (da Silva et al., 2012), and activity against *Eudrilus eugeniae* (annelida, Oligochaeta) (Gbolade and Adeyemi, 2008). *S. mombin* anthelminthic activity has also been reported against sheep nematode (Ademola et al., 2005) and against small ruminants gastro-intestinal parasites in Benin (Attindéhou et al., 2012). It is also reported that *S. mombin* as anti-anemic activity in rats (Ayoka et al., 2006), showing a wide variety of potential uses for this plant. Several other extracts showed promising results, *I. cylindrica* showed the best results after *S. mombin* and may represent a potential drug candidate.

Ivermectin and albendazole present irregular cure rates (55 - 100% and 38 - 87%, respectively) as well as several side effects, showing a need for new drugs to be developed. However very few papers have been published, and even fewer with promising results (Boonmars et al., 2005; de Oliveira et al., 2014; Keiser et al., 2008; Kotze et al., 2004; Legarda-Ceballos et al., 2016; Olounlade et al., 2012). In this paper, we show that the majority of the extracts had a higher *in vitro* efficiency than the drugs currently used for the treatment of strongyloidiasis, with emphasis on *S. mombin* and *I. cylindrica* that showed the best results. *S. mombin* organic fraction showed great potential and should be further studied to isolate and identify their active compounds, which are responsible for the anthelminthic activity so that, hopefully, a new drug can be developed to fight against strongyloidiasis.

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