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Short communication

Ovicidal and larvicidal activity of extracts of *Opuntia ficus-indica* against gastrointestinal nematodes of naturally infected sheep

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ABSTRACT

This study describes the *in vitro* anthelmintic activity of extracts from *Opuntia ficus indica* against gastrointestinal nematodes of sheep. The anthelmintic activity was evaluated by inhibition of egg hatching, larval development and larval migration assays. The residual aqueous fractions from cladodes and fruits showed higher ovicidal activity with EC_{50} values of 7.2 mg/mL and 1.5 mg/mL, respectively. The aqueous, hexane, and ethyl acetate fractions from fruits and the aqueous fraction from cladodes inhibited 100% of larval development at the lowest concentration tested (1.56 mg/mL). The crude cladode and fruit ethanolic extracts inhibited larval migration and showed EC_{50} values of 0.74 mg/mL and 0.27 mg/mL, respectively. Phytochemical screening detected high concentrations of alkaloids, tannins, flavonoids, and saponins in the fruits and cladodes. The results demonstrated that *O. ficus* exhibits anthelmintic activity *in vitro*, suggesting that, beyond its nutritional potential, this plant can also be an ally for parasite control in sheep.

1. Introduction

The indiscriminate use of synthetic anthelmintics has selected resistant parasites, further aggravating the problem of parasitosis in small ruminants (Botura et al., 2013).

Medicinal plants have emerged as an alternative to the use of synthetic anthelmintics worldwide, but there is a lack of scientific evidence regarding their effectiveness and their excess ingestion may pose a risk to animals (Rajeswari, 2014). Therefore, it is mandatory to evaluate whether medicinal plants are safe to use for the control of parasitosis in small ruminants.

Opuntia ficus-indica, belonging to the family Cactaceae, is a common plant in the Cerrado and Caatinga biomes (Griffith, 2004). This plant displays anti-inflammatory, analgesic, antioxidant and antiviral activities (Stintzing and Carle, 2005). Furthermore, a review on folk veterinary medicine demonstrated the use of *O. ficus* as a plant for ethnoveterinary medicine in Italy (Viegi et al., 2003), and in Brazil this species is widely used in feeding sheep (Veras et al., 2002). Considering that sheep feed on *O. ficus* in several places, and that finding alternative ways to control parasitosis is necessary, this study evaluated the ovicidal and larvicidal activity of *O. ficus* by means of *in vitro* assays.

2. Materials and methods

2.1. Plant materials

Plant materials were collected in the municipality of Ilha Solteira in the state of São Paulo, Brazil in September 2014. Cladodes were dried in a circulating air oven (50 °C). The pulp was removed from ripe fruits and frozen. The crude cladode ethanolic extract (CCE) was prepared from 28 g of powdered cladodes in 200 mL of ethanol (70%) by the exhaustive maceration method for seven days. The crude fruit ethanolic extract (CFE) was obtained by centrifuging the pulp (2054g) with 10 mL of ethanol (70%) for 15 min. The supernatant was filtered and evaporated. The partition of the extracts was performed by diluting 1.5 g of the crude extract with 20 mL of ethanol (70%) and successively extracting with hexane, dichloromethane, and ethyl acetate. The extraction process was repeated three times for each solvent. All extracts were frozen.

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Table 1
Percentages of inhibition of egg hatching (mean ± SD) of sheep gastrointestinal nematodes (95% *Haemonchus contortus*) by the *O. ficus* extracts and EC_{50} of the *O. ficus* extracts.

Concentration(mg/mL)	Fruit extract					Cladodes extract				
	Ethanol	Hexane	Dichloro-methane	Ethyl acetate	Residualaqueous	Ethanol	Hexane	Dichloro-methane	Ethylacetate	Residualaqueous
100	100.0 ± 0.0^{Aa}	–	–	–	–	93.0 ± 2.2^{Bb}	–	–	–	–
50	98.2 ± 0.5^{Aa}	–	–	–	–	90.5 ± 1.5^{Bb}	–	–	–	–
25	58.5 ± 2.6^{Bc}	47.0 ± 2.2^{Bd}	32.2 ± 2.3^{Be}	98.7 ± 1.5^{Aa}	100.0 ± 0.0^{Aa}	77.5 ± 2.1^{Cb}	14.2 ± 1.7^{Bf}	32.7 ± 1.7^{Be}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}
12.5	24.7 ± 1.2^{Ce}	26.5 ± 1.0^{Ce}	22.5 ± 2.1^{Ce}	58.5 ± 1.9^{Bc}	90.0 ± 0.8^{Bb}	58.7 ± 2.3^{Dc}	6.7 ± 0.9^{Cf}	24.5 ± 2.6^{Ce}	51.5 ± 1.3^{Bd}	100.0 ± 0.0^{Aa}
6.25	14.0 ± 0.8^{Ce}	28.2 ± 2.3^{Cd}	15.0 ± 0.8^{De}	32.7 ± 1.9^{Cc}	84.2 ± 0.9^{Ca}	37.0 ± 2.2^{Eb}	7.5 ± 0.9^{Cf}	11.0 ± 0.9^{De}	15.0 ± 0.8^{Ce}	30.7 ± 0.9^{Bc}
3.12	13.0 ± 0.8^{Cc}	7.5 ± 0.3^{Dd}	6.7 ± 0.9^{Ed}	19.2 ± 0.9^{Db}	69.0 ± 0.8^{Da}	13.7 ± 0.9^{Fc}	7.5 ± 0.5^{Cd}	6.2 ± 1.3^{Ed}	7.0 ± 0.8^{Dd}	$16.5 \pm 0.6^{Cb,c}$
Albendazole 0.025	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}
5% DMSO	6.0 ± 1.4^{D}	6.0 ± 1.4^{D}	6.0 ± 1.4^{E}	6.0 ± 1.4^{E}	6.0 ± 1.4^{E}	6.0 ± 1.4^{G}	6.0 ± 1.4^{C}	6.0 ± 1.4^{E}	6.0 ± 1.4^{D}	6.0 ± 1.4^{D}
EC_{50}	9.90	27.3	8.90	55.7	1.50	9.90	71.5	11.9	50.7	7.12

Small letters compare mean between lines and capital letters between columns ($p < 0.05$). – Not tested.

Table 2
Percentages of inhibition of larval development (mean ± SD) of sheep gastrointestinal nematodes (95% *Haemonchus contortus*) by the *O. ficus* extracts and EC_{50} of the *O. ficus* extracts.

Concentration (mg/mL)	Fruits extracts					Cladodes extracts				
	Ethanol	Hexane	Dichloro-methane	Ethylacetate	Residualaqueous	Ethanol	Hexane	Dichloro-methane	Ethylacetate	Residualaqueous
100	100.0 ± 0.0^{Aa}	–	–	–	–	100.0 ± 0.0^{Aa}	–	–	–	–
50	100.0 ± 0.0^{Aa}	–	–	–	–	100.0 ± 0.0^{Aa}	–	–	–	–
25	96.2 ± 0.9^{Aa}	100.0 ± 0.0^{Aa}	85.0 ± 0.8^{Bb}	85.7 ± 1.7^{Bb}	100.0 ± 0.0^{Aa}	99.7 ± 0.5^{Aa}	6.1 ± 0.5^{Bc}	5.6 ± 1.5^{Bc}	100.0 ± 0.0^{Aa}	100.0 ± 0.00^{Aa}
12.5	86.7 ± 2.2^{Bb}	100.0 ± 0.0^{Aa}	74.5 ± 1.7^{Cc}	83.2 ± 0.7^{Bb}	100.0 ± 0.0^{Aa}	98.7 ± 1.0^{Aa}	5.1 ± 0.8^{Bd}	5.9 ± 0.5^{Bd}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}
6.25	68.5 ± 2.1^{Cc}	100.0 ± 0.0^{Aa}	68.3 ± 1.3^{Dc}	77.1 ± 1.1^{Cc}	100.0 ± 0.0^{Aa}	73.7 ± 1.7^{Bb}	5.8 ± 0.3^{Bd}	4.9 ± 1.3^{Bd}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}
3.12	49.2 ± 0.9^{Dc}	100.0 ± 0.0^{Aa}	63.2 ± 0.9^{Db}	67.0 ± 2.1^{Db}	100.0 ± 0.0^{Aa}	51.2 ± 0.96^{Cc}	6.2 ± 1.0^{Bd}	5.2 ± 0.8^{Bd}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}
1.56	12.5 ± 0.7^{De}	86.0 ± 1.4^{Bb}	53.2 ± 1.7^{Ec}	44.2 ± 1.7^{Ed}	100.0 ± 0.0^{Aa}	50.3 ± 1.9^{Cc}	5.9 ± 0.7^{Bf}	5.0 ± 0.7^{Bf}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}
Ivermectin 0.010	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}
5% DMSO	5.1 ± 1.4^{E}	5.1 ± 1.4^{C}	5.1 ± 1.4^{F}	5.1 ± 1.4^{F}	5.1 ± 1.4^{B}	5.1 ± 1.4^{D}	5.1 ± 1.4^{B}	5.1 ± 1.4^{B}	5.1 ± 1.4^{B}	5.1 ± 1.4^{B}
EC_{50}	3.70	<1.56	1.70	1.20	<1.56	3.40	>25	>25	<1.56	<1.56

Small letters compare mean between lines and capital letters between columns ($p < 0.05$). – Not tested.

2.2. Phytochemical analysis

Phytochemical assays were performed to detect flavonoids, alkaloids, anthraquinones, saponins, coumarins and tannins in the fruit and cladode extracts according to Harborne (1998).

2.3. Bromatological composition

The moisture content, mineral matter, crude fat, dry matter, crude protein and crude fibre were determined according to Silva and Queiroz (2006).

2.4. Egg hatch assay (EHA)

Eggs were collected from the donor sheep (95% *Haemonchus contortus* and 5% *Trichostrongylus* sp.) and washed repeatedly with distilled water according to Coles et al. (1992). The egg suspension (100 μL/100 eggs), and 100 μL of extract at different concentrations (3.12, 6.25, 12.5, 25.0, 50.0 and 100 mg/mL) were incubated for 48 h at room temperature. The eggs and L1 were counted under a microscope. The controls were performed in parallel. Five replicates for each concentration of the extracts and for each control were performed.

2.5. Larval development assays (LDA)

The egg suspension (100 μL/100 eggs), 90 μL of nutritive media (1 g of yeast in 90 mL of normal saline and 10 mL of Earle's balanced salt) and 10 μL of amphotericin (25 μg/mL) were added to each well of a 24-well plate. The plates were incubated at 25 °C for 48 h. Then, 200 μL of extract was added at the same concentrations used for the EHA. Ivermectin (10 μg/mL) and distilled water were used as positive and negative controls, respectively. Five replicates were carried out for each concentration and the controls. The plates were incubated for five days. The number of L1 and L3 in each well was counted under a microscope (Costa et al., 2008).

2.6. Larval migration inhibition assays (LMIA)

One thousand live L3 were added to Falcon® tubes containing the negative control (PBS; pH 7.2), an anthelmintic control (levamisole at 1.25 mg/mL) or the extract at different concentrations (3.12, 6.25, 12.5, 25.0, 50.0 and 100 mg/mL) to be tested. After incubation for 3 h at 28 °C, the L3 in each tube were washed with PBS and centrifuged (2054*g*) three times. The tubes were capped with a 25 μm steel mesh and placed on a Petri dish. After 3 h of incubation, the number of larvae that migrated through the mesh was counted at 40× magnification by means of the 15% aliquot technique (Rabel et al., 1994).

2.7. Statistical analysis

The data obtained during the *in vitro* assays were analyzed by ANOVA and compared by the Dunnet test (5%). The EC_{50} values (at a concentration that yielded a response of 50%) were calculated by means of nonlinear regression analysis conducted with the aid of the GraphPad Prism version 5.0.

3. Results

3.1. Yield of extracts

After the extraction with ethanol (70%), 28 g of the cladodes furnished 3.0 g of CCE. The extraction of 30 g of fruit with ethanol (70%) produced 3.8 g of CFE. The partition of 1.5 g of CCE in solvents of increasing polarity furnished 0.27 g, 0.28 g, 0.30 g and 0.31 g,

Table 3
Percentages of inhibition of larval migration (mean ± SD) of sheep gastrointestinal nematodes (95% *Haemonchus contortus*) by the crude extracts from cladodes (CCE) and fruits (CFE) of *O. ficus*.

Extracts	Concentrations (mg/mL)								Controls		EC_{50}
	100	50	25	12,5	6.25	3.12	1.56	0.78	0.39	Levamisole	PBS	
CCE	95.6 ± 0.42^{Aa}	89.9 ± 0.48^{Ba}	86.2 ± 0.18^{Ca}	83.7 ± 0.47^{Da}	82.4 ± 0.6^{Db}	79.9 ± 0.49^{Eb}	66.3 ± 0.34^{Fb}	44.9 ± 1.8^{Gb}	38.1 ± 0.19^{H}	96.2 ± 0.62^{A}	2.34 ± 0.67^{I}	0.74
CFE	95.5 ± 0.9^{Aa}	90.2 ± 0.55^{Ba}	85.6 ± 0.42^{Ca}	84.6 ± 0.54^{Da}	83.8 ± 0.26^{Da}	83.2 ± 0.24^{Da}	74.5 ± 0.45^{Ea}	64.9 ± 0.53^{Fa}	48.9 ± 1.27^{Ga}	96.2 ± 0.91^{A}	3.2 ± 0.94^{H}	0.27

Small letters compare mean between columns and capital letters lines columns ($P < 0.05$). Levamisole (0.025 mg/mL) is a positive control; PBS is a negative control.

respectively, of the fraction in hexane (HFC), dichloromethane (DFC), ethyl acetate (EAFC) and residual aqueous solution (AFC). The same procedure was repeated with 1.5 g of CFE to obtain 0.60 g, 0.63 g, 0.24 g and 0.27 g, respectively, of the fraction in hexane (HFF), dichloromethane (DFF), ethyl acetate (EAFF) and residual aqueous (AFF) solution.

3.2. Phytochemical analysis

The phytochemical analysis of CCE and CFE revealed the presence of alkaloids, flavonoids, coumarins, tannins, phenolic compounds and saponins in high quantity.

3.3. Bromatological composition

For the fruits, the values for crude fibre, crude protein, ether extract, dry matter and moisture were 17.06%, 5.85%, 3.92%, 7.75% and 92.24%, respectively. For cladodes, these values were 9.91%, 3.79%, 1.51%, 5.28% and 94.72, respectively, with the SD lower than 1% for all parameters. These values are within an acceptable range of variation (Galvão Junior et al., 2014).

3.4. Egg hatch assay

The percentages of egg hatch inhibition by the extracts are presented in Table 1. *Opuntia ficus* extracts and fractions significantly ($P < 0.05$) inhibited egg hatching with a dose-dependent effect.

3.5. Larval development assay

The CCE, CFE and fractions, with the exception of HFC and DFC (Table 2), were effective at inhibiting larval development, even at the lowest evaluated concentration.

3.6. Larval migration inhibition assay

The treatment of infective gastrointestinal nematode larvae with CCE and CFE, as well as levamisole, led to a significant reduction in the number of larvae recovered by the migration test compared to the negative control ($P < 0.05$). The assays revealed a dose-dependent behavior for the crude extracts (Table 3).

4. Discussion

The use of plants and their extracts has been an adopted alternative for the treatment of ovine gastrointestinal parasites in several places around the world (Rajeswari, 2014). Our study clearly showed that *O. ficus* extracts were effective against eggs and larvae of *H. contortus* based on the assay results. The crude extracts CCE and CFE possess several classes of phytoconstituents that were responsible for the anthelmintic activity demonstrated.

The AFC and AFF-containing flavonoids, saponins and tannins were the fractions with the greatest activity in all of the *in vitro* assays. These substances are known for anthelmintic properties that act synergistically to inhibit eggs from hatching (Botura et al., 2013). The less polar fractions were the least active with the exception of HFF, whose action can be related to the presence of terpenoid pigments in the fruits.

The CCE and CFE showed significant inhibition of larval migration with a dose-dependent response. Saponins and tannins in these extracts may act in conjunction; the saponins interact with cell membranes, resulting in destabilization and subsequent increased cell permeability that eases the action of tannins on intracellular proteins of the parasite (D'Addabbo et al., 2011).

Numerous biological properties of the *O. ficus* fruit has been associated with the presence of betalains (Stintzing and Carle, 2005; Gonçalves et al., 2015); however, the literature does not contain any mention of the anthelmintic action of these substances. The results of the phytochemical screening for cladodes and fruits are in accordance with Chiteva and Wairagu (2013), however minor variations can occur due to age, environment conditions and maturation stage of the fruits. Nutritional parameters are also affected by the age of the plant as demonstrated by Galvão Junior et al. (2014) in that younger plants present higher crude protein levels than used in this study.

Many studies have shown the anthelmintic action of plants (Rajeswari, 2014); however, our study is the first to analyses and test *O. ficus* for its anthelmintic activity. We demonstrated that *O. ficus* showed ovicidal and larvicidal activity. Furthermore, studies conducted by Bispo et al. (2007) showed that the inclusion of up to 56% of *O. ficus* in ovine feed does not cause toxicity or deleterious effects on the animals' performance, which further supports the use of the *O. ficus*.

In conclusion, our results provide support for conducting *in vivo* assays to verify the anthelmintic activity of this plant and for using edible plants in parasite control.

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