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Anthelmintic activity of *Cymbopogon martinii*, *Cymbopogon schoenanthus* and *Mentha piperita* essential oils evaluated in four different *in vitro* tests

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ABSTRACT

Anthelmintic resistance is a worldwide concern in small ruminant industry and new plant-derived compounds are being studied for their potential use against gastrointestinal nematodes. *Mentha piperita*, *Cymbopogon martinii* and *Cymbopogon schoenanthus* essential oils were evaluated against developmental stages of trichostrongylids from sheep naturally infected (95% *Haemonchus contortus* and 5% *Trichostrogylus* spp.) through the egg hatch assay (EHA), larval development assay (LDA), larval feeding inhibition assay (LFIA), and the larval exsheathment assay (LEA). The major constituent of the essential oils, quantified by gas chromatography for *M. piperita* oil was menthol (42.5%), while for *C. martinii* and *C. schoenanthus* the main component was geraniol (81.4% and 62.5%, respectively). In all *in vitro* tests *C. schoenanthus* essential oil had the best activity against ovine trichostrongylids followed by *C. martini*, while *M. piperita* presented the least activity. *Cymbopogon schoenanthus* essential oil had LC_{50} value of 0.045 mg/ml in EHA, 0.063 mg/ml in LDA, 0.009 mg/ml in LFIA, and 24.66 mg/ml in LEA. The anthelmintic activity of essential oils followed the same pattern in all *in vitro* tests, suggesting *C. schoenanthus* essential oil could be an interesting candidate for nematode control, although *in vivo* studies are necessary to validate the anthelmintic properties of this oil.

1. Introduction

Gastrointestinal nematodes in livestock are usually controlled by commercial anthelmintics. However, few commercial anthelmintics are available for veterinary use due to reduced effectiveness caused by emerging drug-resistant parasite strains (Molan et al., 2002). For this reason, losses in weight gain and high morbidity and mortality are a consequence of those parasite infections. *Haemonchus contortus* is the most prevalent and pathogenic nematode found in small ruminants in the tropics. Many alternative strategies to control nematodes have been studied such as adequate nutrition, selection of resistant animals, integrated pasture management, use of nematophagus fungus, and new anthelmintic compounds derived from plants. The search for new solutions to chemical treatments is nowadays a worldwide necessity to achieve more sustainable control. There is increased

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evidence indicating that some bioactive plants might possess anthelmintic properties and, thus, represent a promising alternative to commercially available drugs (Brunet and Hoste, 2006).

Mentha piperita, *Cymbopogon martinii* and *Cymbopogon schoenanthus* essential oils were chosen to be evaluated against nematodes in *in vitro* tests because they have shown some insecticide effect. *In vitro* bioassays have the advantage of providing a simple, rapid, and inexpensive means of primary screening of products with anthelmintic potential. *M. piperita* essential oil was active in killing insects of stored products (Shaaya et al., 1991), and had larvicidal and mosquito-repellency activity (Ansari et al., 2000). *C. martini* essential oil was active against *Meloidogyne incognita*, a soil nematode, (Pandey et al., 2000), and against *Caenorhabditis elegans* (Kumaran et al., 2003). *C. schoenanthus* essential oil was active against termites (Koba et al., 2007) and against the bruchid *Callosobruchus maculatus*, which is a major pest of stored grains (Ketoh et al., 2002).

For this *in vitro* screening, different methods were employed to compare the results among essential oils from different plant species. The evaluation of essential oil emulsions was performed on trichostrongylids immature life stages by the egg hatching assay (EHA), to test egg to L_1, larval development assay (LDA), using larval stages L_1 to L_3, larval feeding inhibition assay (LFIA), using L_1 stage, and larval exsheathment assay (LEA), using L_3 stage. Therefore, the purpose of this study was to evaluate the anthelmintic activity of three essential oils using different *in vitro* assays and different larval stages of trichostrongylids.

2. Materials and methods

2.1. Sheep nematodes

All early life stages of trichostrongylids used in our work were obtained from sheep naturally infected and kept at Embrapa Pecuária Sudeste (fecal culture indicated 95% of *Haemonchus contortus* and 5% *Trichostrogylus* spp.). Once feces were collected, tests were performed, with six replicates, to compare three essential oils at the same concentrations.

2.2. Essential oils

Oils were purchased from WNF Ind. e Com. Ltda (R. Dr. Mario Pinto Serva, 64 – Sao Paulo, SP, Brazil). *M. piperita* oil lot no. 164, density (d) = 0.919, *C. martinii* oil lot no. 081, d = 0.884 and *C. schoenanthus* oil lot no. 10608, d = 0.911. Essential oils were tested in EHA, LDA and LFIA at concentrations ranging from 0.018 mg/ml to 22.75 mg/ml (*C. schoenanthus* and *M. piperita* oil) and from 0.017 mg/ml to 22 mg/ml (*C. martinii*). In LEA, doses ranging from 18.2 mg/ml to 136.5 mg/ml for *C. schoenanthus* and *M. piperita*, and doses ranging from 17.6 mg/ml to 132 mg/ml for *C. martinii* were evaluated. To improve emulsification of essential oils in water, solvents (0.5% DMSO or 2% Tween 80) were added and solutions were mixed in a vortex shaker until oil, solvent, and water became a stable emulsion.

2.2.1. GC–MS analysis

Analysis of the chemical composition of the essential oils were performed by gas chromatography coupled to mass spectrometry using an Agilent 5973N GC–MS system equipped with a HP5MS capillary column (5% diphenyl–95% dimethylsilicone, 30 m × 0.25 mm × 0.25 μm). The injector was set at 250 °C and the oven programmed to go from 60 to 240 °C at 3 °C/min. Mass detector was operated in electron ionization mode, at 70 eV. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. Sample volume was 1.0 μL, and consisted of 1% essential oil in dichloromethane. A split ratio of 1:100 was used. Mass spectra were compared with data from Wiley 6th edition library. The retention indexes were calculated based on data generated by a series of alkenes (C_7–C_{26}) injected in the same column and conditions specified above, and compared to those found in the literature (Adams, 2007). Identification was based on both mass spectrum and retention index. Menthone, menthol, geraniol and geranial were also identified by injection of authentic standards.

2.2.2. GC-FID analysis

For quantification, the oils were analyzed in an Agilent 7890A gas chromatograph equipped with a flame ionization detector and a HP5 capillary column (5% diphenyl–95% dimethylsilicone, 30 m × 0.32 mm × 0.25 μm). Hydrogen was used as the carrier gas at a flow rate of 1.5 ml/min. All other parameters were the same as described above. Results were reported in relative percentage of peak area.

2.3. In vitro tests

2.3.1. Egg hatching assay (EHA)

A pre-established procedure was followed for this assay (Bizimenyera et al., 2006) after some modifications. About 5 g of feces, directly collected from the rectum, were mixed with warm water (37 °C) and filtered through sieves with apertures of 1 mm, 105 μm, 55 μm, and 25 μm, the latter retaining the eggs. Recovered eggs were added to saturated NaCl solution, centrifuged at 3000 rpm for 3 min and the floating eggs were collected using the 25 μm sieve and washed with distilled water. One hundred eggs in 20 μl distilled water were added to the treatments (water, Tween 80 at 2%, or the essential oil tested). All concentrations, positive (water + Tween 80 at 2%), and negative (distilled water) controls had six replicates and were performed in 24-well plates. Plates were incubated at 26 °C for 48 h and read in an inverted microscope to count eggs and L_1 larvae.

2.3.2. Larval development assay (LDA)

Following Bizimenyera et al. (2006), with some modifications, one hundred eggs were added into the wells with distilled water in a total volume of 200 μl, incubated for 24 h at 27 °C to obtain L_1 larvae. To each well containing the treatment (water, dimethyl sulfoxide at 0.5% (DMSO), essential oil), we added nutritive medium (*Escherichia coli*, yeast extract, Amphotericin-B) according to Hubert and Kerboeuf, 1992. All concentrations, the positive control (water + DMSO 0.5%), and negative control were tested in six replicates. They were performed in 24-well plates and

Table 1
CL_{50} (mg/ml) and confidence limits of *Cymbopogon schoenanthus*, *Mentha piperita* and *Cymbopogon martinii* essential oils in egg hatch assay (EHA), larval development assay (LDA), larval exsheathment assay (LEA) and larval feeding inhibition assay (LFIA) against gastrointestinal nematodes of sheep.

	C. schoenanthus	*M. piperita*	*C. martinii*
EHA	0.04 (0.03–0.05)	0.26 (0.24–0.28)	0.13 (0.11–0.14)
LDA	0.06 (0.05–0.07)	0.26 (0.23–0.30)	0.15 (0.14–0.16)
LEA	24.66 (19.44–29.46)	61.93 (55.81–68.58)	28.17(26.28–30.33)
LFIA	0.009 (0.009–0.018)	0.07 (0.06–0.08)	0.03 (0.03–0.04)

incubated at 27 °C for four days, when plates were read in an inverted microscope to count all L_3 and undeveloped larvae.

2.3.3. Larval feeding inhibition assay (LFIA)

Eggs plus distilled water were kept in a Petri dish covered and incubated at 27 °C for 24 h. Active L_1 were recovered by Baermannization using a 25 μm sieve. In a 1500 μl Eppendorf tube, one hundred L_1 larvae were added to the treatments (water, DMSO 0.5% and essential oil). This solution was pre prepared in six replicates. As example, a concentration of 22.75 mg/ml = 225 μl *C. schoenanthus* essential oil + 45 μl DMSO + 8130 μl distilled water were mixed in a vortex shaker and 1400 μl were distributed in six Eppendorf tube and then, 100 μl solution with 100 L1 was added to each tube to complete 1500 μl. Tubes were incubated horizontally at 24 °C for 2 h. The work proceeded in dark from this point to the end of procedure. *E. coli* marked with fluorescein isothiocyanate was added in a volume of 20 μl and incubated horizontally, covered with aluminum foil for 24 h at 24 °C. Tubes were centrifuged at 6000 rpm for 1 min, and 800 μl of supernatant was removed. All larvae from the bottom were examined under a fluorescence microscope, counting all nematodes that had fed on *E. coli* (luminous intestine). Thereafter, counting was performed under an optical microscope. All concentrations, positive (water + DMSO 0.5%), and negative controls were done with six replicates (Álvarez-Sánchez et al., 2005).

2.3.4. Larval exsheathment assay (LEA)

Active L_3 larvae from coproculture were separated using a 25 μm sieve. They were concentrated by centrifugation at 6000 rpm for 2 min to prepare a solution with 100 L_3/100 μl. One hundred L_3 larvae were added to the treatments (water, Tween 80 at 2% and essential oil). The L_3 larvae were exposed to emulsion of essential oils during 3 h at 22 °C, centrifuged at 6000 rpm for 2 min, removed supernatant, and added distilled water to clean the larvae from essential oil. This procedure was repeated twice with 1200 μl with 1200 larvae kept as residual at the bottom of centrifuge tubes. One hundred larvae were added to each well and 1400 μl of bleach solution (150 μl domestic bleach with 6% sodium hypochlorite diluted in 15.625 ml of water) was added into wells containing 100 L_3. At every 10 min, the exsheathment was stopped with iodine solution. The gradual exsheathment along 60 min should be found in control groups. All concentrations, positive (water + Tween 80 at 2%) and negative controls were tested with two replicates (Alonso-Diaz et al., 2008).

2.4. Statistics

The calculation of the extract lethal concentration (LC) in the *in vitro* tests was performed by fitting regression using normal and logistic distribution, with the parameters estimative of these equations obtained by maximum likelihood. The procedure used was the SAS Probit to estimate the LC_{50} and LC_{99} with the independent variables (dose) transformed by natural logarithm (log dose).

3. Results

In the EHA, *C. schoenanthus* essential oil showed the lowest LC_{50} value (0.045 mg/ml) when compared to *C. martinii* and *M. piperita* essential oils, and this result was close to the LC_{50} value obtained for the LDA (0.063 mg/ml). The LFIA indicated that the L_1 were very sensitive to *C. schoenantus* oil and required less essential oil to inhibit their feeding activity. *C. schoenanthus* essential oil had LC_{50} of 0.009 mg/ml, while the LEA demonstrated that L_3 were very resistant and higher concentrations of essential oils were needed. In the LEA, *C. schoenanthus* LC_{50} presented the lowest value, 24.66 mg/ml, while the highest was 61.93 mg/ml for *M. piperita*. In all *in vitro* tests *C. schoenanthus* essential oil had the best activity against ovine trichostrongylids followed by *C. martini*, while *M. piperita* presented the worst results (Table 1). The same tendency in essential oil effectiveness was found for the LC_{99} in EHA, LEA, and LDA (Table 2).

The sensitivity of immature larval stages to solvents was tested (Table 3). In order to make an emulsion of essential oils and water, Tween 80 was used in both EHA and LEA due to the tolerance of eggs and L_3 to this solvent. However, Tween 80 was not used in either LFIA or LDA

Table 2
CL_{99} (mg/ml) and confidence limits of *Cymbopogon schoenanthus*, *Mentha piperita* and *Cymbopogon martinii* in egg hatch assay (EHA), larval development assay (LDA), larval exsheathment assay (LEA) and larval feeding inhibition assay (LFIA) against gastrointestinal nematodes of sheep.

	C. schoenanthus	*M. piperita*	*C. martinii*
EHA	0.27 (0.18–0.36)	1.0 (0.81–1.27)	0.61 (0.45–0.88)
LDA	0.27 (0.18–0.36)	0.91 (0.72–1.27)	0.35 (0.35–0.44)
LEA	54.23 (40.22–151.69)	240.24 (187.55–347.52)	56.32 (48.48–72.77)
LFIA	0.18 (0.18–0.27)	0.18 (0.18–0.27)	0.17 (0.17–0.26)

Table 3
Inhibitory action of solvents DMSO at 0.5% or Tween 80 at 2% expressed by percentages and its (standard deviation) in egg hatch assay (EHA), larval development assay (LDA), larval exsheathment assay (LEA) and larval feeding inhibition assay (LFIA) against gastrointestinal nematodes of sheep.

Test	DMSO (0.5%)	Tween 80 (2%)
EHA		4.79 (1.55)%
LDA	12.35 (3.37)%	
LFIA	14.53 (5.24)%	
LEA		3.23 (0.24)%

because it resulted in high mortality in control groups and, was substituted by a less toxic compound, DMSO.

Oxygenated monoterpenes were the major constituents of the essential oils tested. *M. piperita* oil presented 29 compounds and had 42.5% menthol, followed by 27.4% menthone as major constituents. *C. martinii* oil presented 11 compounds and had 81.4% of geraniol and 10.1% isomenthyl acetate, and *C. schoenanthus* oil presented 28 compounds and had 62.5% geraniol, followed by 12.5% geranial and 8.2% neral and 3.4% beta-caryophyllene (Table 4).

4. Discussion

The objective of this study was to evaluate three essential oils using four different *in vitro* tests. The EHA and LDA are the most widely employed *in vitro* methods for detection of anthelmintic resistance in ovine nematodes under field conditions (Várady et al., 2009). The LFIA was successful to detect anthelmintic resistance to macrocyclic lactones and imidazothiazoles (Álvarez-Sánchez et al., 2005). The LEA was extensively used to confirm effect of tannin rich plant extracts and its inhibitory process on L_3 (Brunet and Hoste, 2006). The LFIA and LDA are not currently employed in *in vitro* tests however those tests can be used as a complement of other *in vitro* methods. All *in vitro* tests are usually interpreted by using LC_{50} values (Várady et al., 2009).

In this study, *C. martinii*, *C. schoenanthus* and *M. piperita* essential oils presented high *in vitro* activity against sheep trichostrongylids. The results obtained *in vitro* here were superior to other oils tested previously. For instance, *Eucalyptus globulus* essential oil inhibited 99.3% egg hatching and 98.7% larval development at concentrations of 21.75 and 43.5 mg/ml (Macedo et al., 2009). *Ocimum gratissimum* essential oil inhibited 100% egg hatching at concentration of 0.5% (Pessoa et al., 2002). *Croton zehntneri* and *Lippia sidoides* essential oils inhibited egg hatching in more than 98% at 1.25 mg/ml and larval development in over 98% at 10 mg/ml (Camurça-Vasconcelos et al., 2007). *Chenopodium ambrosioides* essential oil inhibited 100% egg hatching at 1.33 μl/ml (Ketzis et al., 2002). The LC_{50} of *Eucalyptus staigeriana* essential oil in the EHA was 0.324 mg/ml and LC_{50} in the LDA was 1.702 mg/ml (Macedo et al., 2010). Those values are higher in comparison to our results that showed LC_{50} in EHA of 0.04; 0.26 and 0.13 mg/ml and LC_{50} in LDA of 0.06; 0.26 and 0.15 mg/ml to *C. schoenanthus*, *M. piperita* and *C. martinii* essential oils, respectively.

Terpenes are a chemical class of chemicals found in essential oils. *C. schoenanthus* had approximately 20 constituents, being rich in geraniol, geranial, and neral. Terpenoid compounds are known to be active against a range of organisms and the synergy of several terpenoids can be effective on several targets because they are a

Table 4
Percentage of composition of *Cympobogon martinii*, *Cympobogon schoenanthus* and *Mentha piperita* essential oils obtained by gas chromatography coupled to mass spectrometry.

C. martinii		*C. schoenanthus*		*M. piperita*	
Geraniol	81.4	Geraniol	62.5	Menthol	42.5
Isomenthyl isomenthyl acetate	10.1	Geranial	12.5	Menthone	27.4
Linalool	2.6	Neral	8.2	1.8-Cineole	4.6
Geranial	2.1	Citronelol	3.6	menthyl acetate	4.6
Trans-ocimene	1.7	(E)-beta-caryophyllene	3.4	Limonene	2.0
(E)-beta-caryophyllene	0.8	Geranyl acetate	2.0	Pulegone	1.8
Cis-ocimene	0.4	Linalool	1.3	(E)-beta-caryophyllene	1.6
Myrcene	0.4	Delta-cadinene	0.9	4-Terpineol	1.3
Limonene	0.3	Caryophyllene oxide	0.6	Beta-pinene	0.9
Neral	0.2	Citronelal	0.5	Alpha-pinene	0.6
Nerol	0.1	6-Methyl-5-heptenone	0.5	p-Cymene	0.4
		Alpha-humulene	0.4	Isomenthol	0.4
		Elemol	0.4	Sabinene	0.2
		Alpha-cadinol	0.4	Isopulegol	0.2
		Epi-alpha-cadinol	0.3	Alpha-terpineol	0.2
		Beta-elemene	0.3	Piperitone	0.2
		Decanal	0.3	Neo-menthyl acetate	0.2
		Geranyl formiate	0.2	Caryophyllene oxide	0.2
		Eugenol	0.2	3-Octanol	0.1
		Gamma-humulene	0.2	Sabinene cis-hydrate	0.1
		Alpha-muurolene	0.2	Linalool	0.1
		Not identified	0.7	Isomenthyl acetate	0.1
				Beta-bourbonene	0.1
				Beta-elemene	0.1
				Alpha-humulene	0.1
				Germacrene D	0.1
				Not identified	0.2

complex mixture of compounds that can interact with multiple molecular targets on various developmental stages of the parasite (Marie-Magdeleine et al., 2009). So, it is quite reasonable to consider that the major constituents of each plant species, as detected by gas chromatography, had some biological activity *in vitro* against trichostrongylids in the present study. Because geraniol was the main component in both *Cymbopogon* species, which had better anthelmintic effects than *Mentha* (devoid of geraniol), we can hypothesize that geraniol might be of potential interest for *in vivo* tests. However, the concentration of geraniol does not preclude the potential synergistic effect of geranial and neral, present at higher concentrations in *C. schoenanthus*, the essential oil with the best anthelmintic activity.

The insolubility of essential oils and many of their constituents in aqueous media is likely to impair their performance in susceptibility tests, and attempts to overcome this problem have been made by using tensio-active agents such as Tween 20 and Tween 80 (Juven et al., 1994). Although Tween 80 has low toxicity to nematodes (4.79% inhibition hatchability and 3.23% inhibition exsheathment) compared to Tween 20, it caused toxicity on LFIA and LDA assays. DMSO, which is less toxic to L_1 larvae than Tween 80, was used for both LFIA (14.53% inhibition feeding activity) and LDA (12.35% inhibition development). Those solvents can work as bioenhancers, as they have the ability to increase the bioavailability of drugs by increasing their transport across membranes, increasing anthelmintic effect. Thus, the type and concentration of solvents required to make an emulsion should be considered before tests are performed. Depending on the organism, the solvents can be highly lethal. Nematodes in immature form are very sensitive to a range of compounds, including tap water (von SamsonHimmelstjerna et al., 2009). Because of this sensitivity, it was noticed that is important to keep good control by removing all possible agents that can negatively affect the regular life cycle of trichostrongylids. Regarding water solubility, although desirable, it might not be required in some cases. For instance, tests in dogs using carbon tetrachloride against hookworms showed that the efficacy of the tested compounds increased as solubility in water decreased (Bennet-Jenkins and Bryant, 1996).

Another point of difficulty found in *in vitro* tests was the contamination. Álvarez-Sánchez et al. (2005) used the LFIA to detect anthelmintic resistance to ivermectin and levamisole and reported that such assay offers the advantage of simplicity and rapidity in comparison to the LDA. LDA takes a long time to be performed and problems related to contaminations frequently occur. These problems reflected in fewer laboratories using the assay as the primary screen for activity *in vitro* (Jackson and Hoste, 2010). In our work we dealt with both bacterial and fungal contamination by using sterile techniques as much as possible because development stages could not stand higher quantities of antibiotics than found in nutritive medium (Hubert and Kerboeuf, 1992). In addition, instead of seven days of incubation at 23 °C (Bizimenyera et al., 2006), we increase temperature to 27 °C and decreased incubation time to five days. This modification allowed larval development at higher rates and more reproducible results. It also decreased fungal and bacterial contamination in the plates.

Different LC_{50} values found between assays can be attributed to the sensitivity of each stage. Eggs are more resistant than L_1 due to its hard and resistant shell. On the other hand, L_3 were more resilient due to their double sheath. L_1 was the most sensitive stage due to its pharynx that is more sensitive to the paralysis caused by drugs than the axial muscles (Molan et al., 2002). These facts lead to higher or lesser volumes of active compound to achieve LC_{50} for each test. Várady et al. (2007) used the criterion of LC_{99} concentration to evaluate the sensitivity to thiabendazole under field screening. Those authors found in egg hatch and larval development assay the comparison of LC_{50} values was not significantly different, however the LC_{99} concentration is able to differentiate susceptible group, susceptible heterozygote group and resistant group in test for resistance diagnosis. Our LC_{99} results in EHA, LDA and LEA showed the same pattern of activity found in LC_{50} values.

For trichostrongylids, the L_3 exsheathment is a key process in the life cycle because it is the transition step between the free living and the parasitic stages (Hertzberg et al., 2002). Studies on the kinetics of larvae exsheathment have emphasized that any disturbing factors or toxic compounds might reduce the parasite establishment in the host (Dakkak et al., 1981).

The *in vitro* methods provide means to screen rapidly for potential anthelmintic activities of different plant extracts and to analyze the possible mechanisms involved in the interactions between active compounds and parasites. *C. schoenanthus* showed the best anthelmintic activity *in vitro*. Thus, based on the LC_{50} of 24.66 mg/ml obtained for *C. schoenanthus* in LEA, an approximate dose of 1.18–2.45 g of oil/kg body weight (BW) would provide a 50% reduction in exsheathment and worm reduction considering an animal of 40 kg and 2–4l abomasal volume. However, sometimes the effect *in vitro* or in a different animal system can be lower than when tested in the target host. A recent illustration of this point is the work with orange emulsion oil, where 600 mg of the oil emulsion per kg BW caused 7% and 62.6% worm reduction in gerbils with a single dose or daily for five days, respectively (Squires et al., 2010). However, when these authors tested the emulsion with 600 mg of orange oil per kg BW in sheep infected with *H. contortus*, it resulted in a 97.4% reduction in fecal egg count (adult worm reduction was not evaluated). Although encouraging, these results must be interpreted with caution because of the high doses of the preparation (40% orange terpenes, 20% Valencia orange oil, 4% polysorbate 80, and 1.5% hydrogen peroxide) required for anthelmintic effects. The authors mentioned that few lambs presented toxicity signs such as head shaking and feed aversion. These symptoms may be aggravated if the active component(s) has(ve) a low LD_{50}. In the case of the orange oils used, the authors (Squires et al., 2010) reported that >95% was d-limonene, which has a high LD_{50} (5000 mg/kg).

When a potential compound or plant extract is found, more comprehensive studies are needed to assess its bioavailability. How much is being absorbed and metabolized versus how much is being disposed in gastrointestinal

content, and which metabolites are being generated. Besides nematocidal effect, plant extracts/compounds are tested for their ability to impair egg hatching and larval development from feces of infected animals treated with those plant extracts. Desired effects can result in reduced re-infection and lighter worm loads leading to decreased pasture contamination levels (Ketzis et al., 2002; Max, 2010). *In vivo* tests, problems with absorption through the gastrointestinal tract, and compound solubility and stability after oral intake are the main obstacles in developing herbal formulations with good bioavailability and anthelmintic efficacy. According to Stepek et al. (2007), given the sensitivity to pH, it is not surprising that plant enzymes for instance have lower efficacy against stomach nematodes in situ than against those residing further down the gastrointestinal tract. It is necessary to evaluate additional parameters on ongoing research, such as performance measurements, indicators of immunity, and behavioral observations when considering the potential of such plants (Athanasiadou et al., 2007).

Thus, albeit encouraging, our positive results with *C. schoenanthus* have to be interpreted with caution and tested *in vivo* to confirm or refute our *in vitro* results, and within the realm of host–parasite interactive physiology, biochemistry, compound availability or toxicity.

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