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Effect of essential oils on cattle gastrointestinal nematodes assessed by egg hatch, larval migration and mortality testing

S. Saha[1,2] and S. Lachance[2]

[1]Department of Dairy Science, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet, Bangladesh and [2]Ridgetown Campus, University of Guelph, Ridgetown, Ontario, Canada

Short Communication

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Author for correspondence:
Simon Lachance,
E-mail: slachanc@uoguelph.ca

Abstract

The efficacy of eight essential oils (EOs) (*Solidago canadensis*, *Eucalyptus globulus*, *Pelargonium asperum*, *Ocimum basilicum*, *Thymus vulgaris*, *Mentha piperita*, *Cymbopogon citratus* and *Cymbopogon martinii*) against gastrointestinal nematodes (GINs) was evaluated using eggs collected from naturally infected cattle and cultured infective larvae (L_3). The larvae species cultured from the faecal samples and subjected to two *in vitro* tests were *Haemonchus* spp. (55.5%), *Trichostrongylus* spp. (28.0%), *Cooperia* spp. (15.0%) and *Oesophagostomum* spp. (1.5%). The genus of EO *Cymbopogon* (*C. citratus* and *C. martinii*) showed the highest anthelmintic activity at the dose of 8.75 mg/ml, for the egg hatch, the larval migration and mortality assays. All of the EOs tested reduced egg hatching to rates <19.0%, compared to the controls (water and water + Tween 20) that had rates >92.0%. *Cymbopogon citratus* and *C. martinii* treatments resulted in 11.6 and 8.1% egg hatch, had the lowest migration of larvae through sieves, 60.5 and 54.9%, and the highest mortality rates, 63.3 and 56.3%, respectively. Dose–response tests showed that EO from *C. citratus* had the lowest larval LC_{50} and migration inhibition concentration (IC_{50}) values of 3.89 and 7.19 mg/ml, respectively, compared to two other EOs (*C. martinii* and *O. basilicum*). The results suggest that EOs from the genus *Cymbopogon* can be interesting candidates for nematode control in cattle, although it may prove challenging to deliver concentrations to the gastrointestinal tract sufficient to effectively manage GINs.

Introduction

Gastrointestinal nematode (GIN) infection hampers animal welfare and productivity, resulting in reduced weight gain, carcass quality, milk production and fertility (Charlier *et al.*, 2009). Gastrointestinal parasitism is often characterized by a combination of several species infecting the host (Kaplan & Vidyashankar, 2012). The most common cattle GINs recovered from pastures in Ontario, Canada, are *Cooperia* spp. and *Ostertagia* spp. (Nodtvedt *et al.*, 2002; Scott, 2017). Recently, following an epidemiological study on 306 farms in Canada, the prevalent GIN species present were found to be *Cooperia oncophora* and *Ostertagia ostertagi*, with heifers having access to pasture bearing higher faecal egg counts (FECs) than animals in non-pastured herds (Scott, 2017). Owen *et al.* (1989) recovered *Cooperia* sp. (46%), *Ostertagia* sp. (39%) and *Haemonchus* sp. (15%) larvae in a herd of cow/calf pastured in September in Ontario, Canada. The trichostrongyles group of nematodes, including all of the aforementioned species, have a general life cycle in which cattle become infected by the ingestion of L_3 (Verschave *et al.*, 2015). The control of parasites in dairy cattle relies most commonly on commercially available anthelmintic drugs; however, resistance to anthelmintic drugs by GINs has increased over the last few years (Gasbarre, 2014).

The use of plants or plant-derived products, such as condensed tannins or saponins, for the treatment of GIN infections has increased significantly worldwide (Sandoval-Castro *et al.*, 2012). Most of the work performed with plant-based phytochemicals has focused on the control of parasites in small ruminants (Sandoval-Castro *et al.*, 2012), while few studies have tested the antiparasitic effects of different plants and plant-derived products to control parasite infections in large ruminants (Shepley *et al.*, 2015).

Several of the common major components of essential oils (EOs), such as thymol, menthol, limonene and geraniol, have shown significant antiparasitic effects for the control of animal diseases (Hrckova & Velebny, 2013). EOs are volatile, natural, complex compounds derived from aromatic plants, and have been found to possess antimicrobial, antifungal, antiparasitic, anti-oxidant and anti-inflammatory activities (Bakkali *et al.*, 2008), as well as being repellent for cattle pest flies (Lachance & Grange, 2014). In ruminant nutrition, EOs have been used as feed additives to improve rumen fermentation efficiency (Cobellis *et al.*, 2016). The potential use of EOs from plants is a promising line of research that may give rise to the improved

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treatment of helminth infections (Grando *et al.*, 2016), in addition to the other benefits. To the best of our knowledge, no studies have been carried out to test cattle nematode control using EOs.

In vitro techniques have been developed to investigate and validate the efficacy of plant extracts or plant EOs against GINs of ruminants, such as the egg hatch, larval development, larval migration inhibition (LMI), larval feeding inhibition and larval exsheathment assays. *In vitro* assays seldom reflect *in vivo* bioavailability (Hrckova & Velebny, 2013), but they are highly reproducible, convenient, rapid to perform and constitute low-cost pre-screening tests to evaluate the anthelmintic activity of secondary metabolites of plants (Novobilsky *et al.*, 2011).

Before testing therapeutic plant extracts in effort-intensive *in vivo* trials with dairy cows, it is important to select extracts more likely to have an anthelmintic effect. The present study was, therefore, conducted to evaluate the *in vitro* anthelmintic efficacy of selected EOs on egg hatch, mobility and mortality of GINs collected and cultured from naturally infected cattle.

Materials and methods

EOs

EOs were purchased from Aliksir Inc. (Grondines, QC, Canada). The eight experimental EOs were: *Solidago canadensis* L. (Asteraceae), *Eucalyptus globulus* Labill. (Myrtaceae), *Pelargonium asperum* Willd. cv. Bourbon (Geraniaceae), *Ocimum basilicum* L., *Thymus vulgaris* L., *Mentha* x *piperita* L. (Lamniaceae), *Cymbopogon citratus* (DC.) Stapf and *Cymbopogon martinii* (Roxb.) Wats. (Poaceae). A chemical high-performance gas chromatography analysis was provided by the supplier for each EO tested (supplementary table S1). The EOs were kept at 4°C until use. The EOs were chosen based on the lack of potential adverse effects on animals and efficacy (Bakkali *et al.*, 2008; Sandoval-Castro *et al.*, 2012), cost and availability. The dose of 8.75 mg/ml used for the first assays was selected based on doses used in other studies (Camurca-Vasconcelos *et al.*, 2007; Macedo *et al.*, 2011).

Collection and culture of faecal samples

Faecal samples were collected weekly from mid-September to October 2014 from a 27-head milking animals organic dairy farm in eastern Ontario, Canada, from three highly parasitized dairy cattle, directly from the rectum. The samples were placed in plastic bags, labelled and brought to the Organic Dairy Research Centre, University of Guelph – Campus d'Alfred for processing. Procedures involving animals in this study were approved (Protocol #1641) by the animal care committee at the University of Guelph, which adheres to the Canadian Council on Animal Care guidelines (CCAC, 2009).

Several fresh faecal samples from the three animals containing the highest number of eggs (average 17 eggs per gram; modified Wisconsin technique), over several collection days, were cultured to obtain the infective nematode stages (L_3) for the experiments. About 100 g of faeces were transferred into a 500 ml glass container and mixed with 20 g of vermiculite for culture. Vermiculite was mixed with the manure to provide aeration and to absorb excessive moisture from faeces. A plastic lid with a 2 cm diameter hole covered with mesh was screwed to the glass container. The rearing conditions were 22 ± 2°C and 16:8 L:D. The mixture was stirred twice every day until extractions of L_3 larvae, 8–10 days after the start of the culture.

The third-stage larvae (L_3) were recovered using a modified Baermann technique. Fifteen grams of faecal samples were placed on the Baermann apparatus and allowed to stand in tap water for 24 h. During this time, larvae settled down the tube of the funnel. Five millilitres of the fluid in the tube were then drawn into a 15 ml conical tube and centrifuged at 123 g for 2 min. The supernatant was siphoned out and the 2 ml sediment of several funnels transferred and mixed in a conical flask and kept in the fridge until used for the experiments, up to a maximum of two months.

For genus identification, the third-stage larvae from culture were killed by adding a drop of diluted Lugol's iodine solution to a drop of the larval suspension on a microscope slide and examined under 400× magnification. The larvae were identified to the genus level on the basis of the characteristic tail length according to Van Wyk & Mayhew (2013). The percentage of each genus was calculated on a mixture of 200 larvae cultured from the three experimental cows.

Egg hatch assay (EHA)

The EHA was conducted using a modified method described by Coles *et al.* (1992). Briefly, 20 g of faecal samples were mixed with 30 ml tap water and filtered through 250, 212, 150 and 28 µm sieve sizes, the latter retaining the eggs. The material retained by the 28 µm sieve was processed (Coles *et al.*, 1992) to extract the eggs. The concentration of eggs was estimated by counting the eggs in aliquots of 50 µl at 25× magnification, and concentrations of 50–100 eggs in 100 µl of solution were used for the experiment.

The eight EOs were prepared by dissolving them in water and 2% Tween 20, to improve solubility. The solutions (water, EO and Tween 20) were mixed in a vortex shaker (Mini Vortexer, Fisher Scientific, USA) for 10 min. A solution of 8.75 mg/ml was prepared for each EO. One hundred microlitres of the egg solution was pipetted in each tested well of a 24-well polystyrene tissue culture plate (Corning Incorporated, NY, USA). Then, 1600 µl of EO solution were added in each well containing the eggs. The control consisted of 1600 µl of a solution of 2% Tween 20 and distilled water, and an additional treatment of only distilled water was added. The tissue culture plates were then placed by groups of ten on top of wet paper towels in a sealed polyethylene container, to ensure high relative humidity, and incubated at 26°C. After 48 h, a drop of Lugol's iodine solution was added to each well to stop the egg hatching and to kill hatched larvae. All the larvae (L_1) and unhatched eggs were then counted. Five replicates of each treatment were performed. The percentage of hatched eggs for each treatment was calculated using the following equation:

$$\text{Percentage (\%) of egg hatch} = \left(\frac{L}{(E+L)}\right) \times 100$$

where L = number of larvae in well and E = number of unhatched eggs in well.

LMI assay

The technique from Rabel *et al.* (1994) was used. Briefly, a solution containing 100–150 larvae per 100 µl was prepared from reared L_3 larvae. Then, 100 µl of this solution was added into

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each tested well of a 24-well tissue culture plate and 1600 µl of the 8.75 mg/ml EO solutions were then added to tested wells. The plates were incubated for 3 h at room temperature. After incubation for 3 h at 21 ± 2°C, the content of each well was transferred into a sieve placed in the next corresponding well and incubated for 24 h. The sieves were constructed from translucent acrylic tubing (2 mm in length, 1 mm internal diameter and 1.3 mm external diameter). One end of the sieve was covered with a 28 µm mesh (Sefar Nitex 03-28/17 102 cm, Sefar Inc, Depew, NY, USA) glued with cyanoacrylate adhesive. The 28 µm mesh size was selected to permit active migration of cattle live larvae (Demeler *et al.*, 2010) through the mesh. Each sieve was held in place 3 mm above the bottom of the cell with an acrylic plate holder consisting of holes where the sieves were inserted.

After the 24-h incubation period, the sieves were raised and the content allowed to drain in each well. The outside of the sieves was washed gently with distilled water. The number of dead (not moving when prodded) and live larvae retained in sieve or having migrated and present in the well was counted by using a stereo microscope at 25× magnification. There were five replicates for each treatment. The percent migration of larvae was calculated with the formula:

$$\text{Percentage (\%) of larvae migrating} = \frac{M}{(M+R)} \times 100$$

where R = number of larvae retained in sieve (dead and alive) and M = number of larvae migrated through sieve (dead and alive).

The percentage of dead larvae was also calculated using the formula:

$$\text{Percentage (\%) of dead larvae} = \frac{D}{T} \times 100$$

where T = total number of larvae deposited in sieve and D = number of dead larvae after 24 h (in sieve or having migrated).

Dose–response of three EOs on larvae

Based on the results of migrating larvae of the previous experiment, three EOs were selected for dose–response testing. The EOs were *C. citratus*, *C. martinii* and *O. basilicum*, and doses of 35.00, 17.50, 8.75, 4.38, 1.75, 0.88, 0.09 and 0.04 mg/ml of each EO were chosen for the assays. The LMI assay was performed and we used the same methods as described above. Three replicates were performed for each dose. The percent migration of larvae and the percentage of dead larvae were also calculated using the same methods as described above.

Statistical analysis

Percentage egg hatch, migrating larvae and mortality were analysed in generalized linear mixed models using the GLIMMIX procedure of SAS Software, version 9.4 (SAS Institute Inc., 2012). The residuals were tested for normality, and the normal distribution was used as it was the best fit for the data. The PDIFF option was used in the LSMEANS statement with the Tukey multiple comparison test for analysing differences between treatments (SAS Institute Inc., 2012). Data are reported as LSMEANS ± standard error of mean, and differences among treatments were considered at a significance level of $\alpha = 0.05$.

Mortality and migrating larvae responses vs. concentration were analysed using a chi-square test to measure goodness-of-fit, describing the relationship between dosage levels and observed and expected data. Lethal concentration 50 and 90% (LC_{50} and LC_{90}) for larval mortality, and inhibition concentration (IC) 50 and 90% (IC_{50} and IC_{90}) for larval migration, were calculated using PROC PROBIT. The predicted values were corrected if necessary using the procedure LACKFIT, when the goodness-of-fit statistic test *P*-value was <0.1, where variances and covariances are adjusted by a heterogeneity factor (the goodness-of-fit chi-square divided by its degrees of freedom) and a critical value from the *t* distribution is used to compute the fiducial limits (SAS Institute Inc., 2012). The LC_{50} and IC_{50} values of the three EOs were compared to one another using a ratio test (Robertson *et al.*, 2007) to determine differences in GIN susceptibility to the EOs.

Results and discussion

No significant differences were observed between treatments with distilled water only and distilled water and 2% Tween 20, with hatching rates of 94.5 and 92.0%, respectively (fig. 1). All EOs had a significant direct impact on the hatching rates of nematode eggs (fig. 1). The treatment with *C. martinii* had the lowest percentage of hatched eggs (8.1%), while *S. canadensis* had the highest percentage of hatched eggs (19.0%) of all the EOs tested (fig. 1).

In the control groups (distilled water; distilled water and 2% Tween 20), the percentage of migrating L_3 larvae was higher than 92.6% (fig. 1). All EOs significantly inhibited larval migration, to various degrees, compared to the distilled water control (fig. 1). The EOs from the genus *Cymbopogon* had the most significant impacts, contributing to the lowest migration percentage of larvae (54.9% for *C. citratus* and 60.5% for *C. martinii*) (fig. 1). Although significantly different than the water-only control, larval migration still occurred for 85.9% of larvae with *S. canadensis* EO, and was no different than with *P. asperum* and *T. vulgaris* (fig. 1).

In the larval migration assay, the number of dead larvae were also counted. The percentage of dead larvae was highest for *C. citratus* (63.3%) and *C. martinii* (56.3%), and lowest in *S. canadensis* (13.9%) (fig. 1). All EOs had an effect on the mortality of larvae compared to the distilled water and Tween control treatments (fig. 1).

The mortality rate of larvae (L_3) was dose dependent and the results of the Probit analysis are shown in table 1. *Cymbopogon citratus* showed the lowest estimated LC_{50} at 3.89 mg/ml (table 1) when compared to *C. martinii* and *O. basilicum*. At our higher dosage level of 35 mg/ml, *C. martinii* yielded a 87.1% mortality rate, while *C. citratus* and *O. basilicum* yielded mortality rates of 78.6 and 84.7%, respectively (not shown). In the control groups (distilled water and 2% Tween 20), the percentage of dead larvae was below 5%.

The migration inhibition (IC) increased with dose (table 1). *Cymbopogon citratus* had the lowest estimated IC_{50} (7.19 mg/ml) (table 1). *Ocimum basilicum*, on the other hand, showed the lowest estimated IC_{90} (158.42 mg/ml) of the three EOs (table 1). At our highest dose of 35 mg/ml, the EO from *C. citratus* showed 72.8% inhibition of larval migration, whereas *O. basilicum* and *C. martinii* showed a 60.6 and 56.3% inhibition, respectively (not shown). In the control groups (distilled water and 2% Tween 20), the migration inhibition was less than 9% (not shown).

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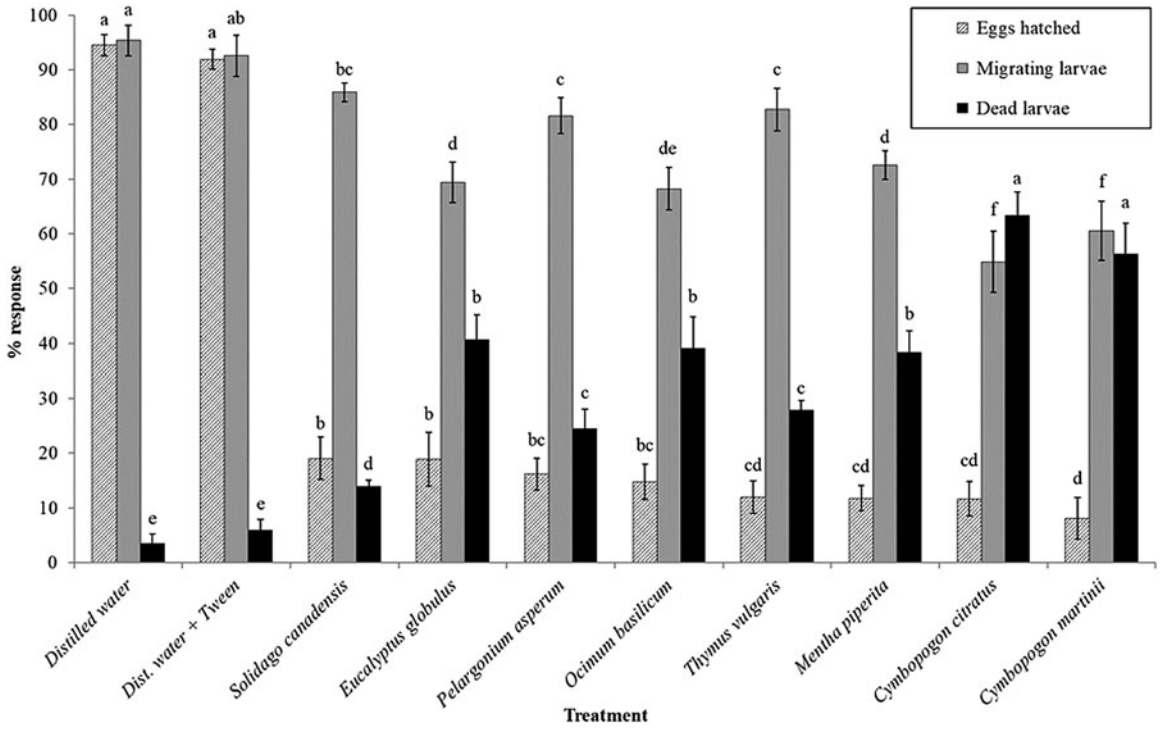


Fig. 1. Percentages of nematode egg hatch, larval migration and dead larvae at 8.75 mg/ml concentration for eight EOs. Data with the same letter for the same test are not significantly different based on Tukey's Honest Significant Difference test at $P < 0.05$ ($n = 5$).

Ratio tests showed that *C. martinii* had a significantly higher LC_{50} and IC_{50} than *C. citratus*, therefore indicating that GIN larvae were more susceptible to the latter (table 2). Further, when compared with *O. basilicum*, *C. citratus* had a significantly lower LC_{50} and IC_{50} (table 2). No significant differences in values between *C. martinii* and *O. basilicum* were found, since the 95% confidence intervals for the ratios included 1 (table 2).

Using both EHA and LMI, the current study showed a significant impact of EOs on egg hatching rates and on the migration and mortality rates of larvae. The relative effect of each EO was somewhat similar for the eggs and the L_3 larvae, with the two plant extracts of *Cymbopogon* being the most efficient as an anthelmintic for all performed bioassays. All of the EOs tested showed an important reduction in egg hatch, as a concentration of 8.75 mg/ml decreased egg hatch to rates lower than 20% for all EOs, compared to controls that had hatching rates above 90%.

Macedo *et al.* (2009) demonstrated that EOs from *E. globulus* at 17.4 mg/ml inhibited *Haemonchus contortus* egg hatching by 87.3%, while, in our study, half of that dose inhibited egg hatching by 81.7%. In our study, *E. globulus* was efficient at decreasing egg hatch, but not to the level reported for *Eucalyptus citriodora* by Macedo *et al.* (2011), which inhibited egg hatching by 99.8% in goat GINs at a dose of 5.3 mg/ml. Different proportions of active ingredients, such as cineole, α-pinene and limonene, in the two plant species (*E. globulus* and *E. citriodora*) may, in part, explain the differences. Katiki *et al.* (2011) reported 99% egg hatch inhibition (LC_{99}) values on sheep nematodes (95% *H. contortus* and 5% *Trichostrongylus* spp.) using 0.27, 0.61 and 1 mg/ml for *Cymbopogon schoenanthus*, *C. martinii* and *Mentha piperita*, respectively, showing lower LC_{99} values for the two *Cymbopogon* spp. than for *M. piperita*. The lower efficiency rates of egg hatch inhibition obtained in the present study (91.9 and 88.8% for *C. martinii* and *M. piperita*, respectively), using 8.75 mg/ml, may be due, in part, to the nematodes tested being a mixture from different genera following isolation from cattle faeces. In fact, the proportion of L_3 larvae identified from cultured eggs collected in September–October were highest for *Haemonchus* spp. (55.5%). Other genera found were *Trichostrongylus* spp. (28.0%), *Cooperia* spp. (15.0%) and *Oesophagostomum* spp. (1.5%). Rossanigo & Gruner (1994) reported that *Haemonchus* spp. egg deposition predominates in the fall season for cattle. It was, however, surprising to find *Haemonchus* as the dominant genus in cattle, as previous studies have mainly found *Ostertagia* and *Cooperia* as the two main genus present in Canada, and specifically Ontario (Nodtvedt *et al.*, 2002; Scott, 2017). The *Haemonchus* genus, and specifically the species *H. contortus*, is generally considered to prefer warmer climates typical of more southern areas (Emery *et al.*, 2016), although *H. contortus* in sheep has been reported to expand to northern cooler climates (Domke *et al.*, 2013).

Although not identified to the species level, it is likely that the species found to be more common in our samples was *Haemonchus placei*, the bovine *Haemonchus* sp., even though calves may be susceptible to *H. contortus* infections (Zajac, 2006). The bioassays performed were not designed to discriminate between the major genera of nematodes found in the samples, as the tests were performed on live reared parasites of naturally infected cattle. *In vitro* tests with a single species would be necessary to determine species-specific sensitivity to the various EOs, as resistance to anthelmintic is a species-specific character (Kaplan & Vidyashankar, 2012).

Significantly fewer larvae were found migrating with the genus *Cymbopogon* compared with the other EOs tested. In addition, the number of dead larvae was the highest with *Cymbopogon* species. Several of the L_3 larvae able to move through the sieve subsequently died during the 24-h period. This suggests that EOs

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Table 1. Larval lethal concentration (LC) and migration inhibitory concentration (IC) values (mg/ml) with fiducial limits for *C. citratus*, *C. martinii* and *O. basilicum* EOs.

Essential oil	*N*	Test	$Concentration_{50}$ mg/ml (95% FL)	$Concentration_{90}$ mg/ml (95% FL)	Intercept ± SE	Slope ± SE	χ^2 (df = 6)	*P*-value
Cymbopogon citratus	5643	LC	3.89 (2.48–5.97)	135.16 (65.90–392.14)	−0.491 ± 0.082	0.832 ± 0.076	43.94	<0.0001
Cymbopogon martinii	3495	LC	11.61 (7.82–16.83)	71.20 (40.67–214.12)	−1.732 ± 0.286	1.627 ± 0.244	46.06	<0.0001
Ocimum basilicum	2983	LC	11.10 (10.12–12.17)	53.75 (45.42–65.68)	−1.955 ± 0.1060	1.870 ± 0.095	3.7968	0.7042
Cymbopogon citratus	5643	IC	7.19 (5.52–9.33)	298.16 (175.42–597.93)	−1.679 ± 0.057	0.792 ± 0.048	12.01	0.0616
Cymbopogon martinii	3495	IC	31.04 (20.89–56.14)	484.53 (190.29–2859)	−1.602 ± 0.175	1.074 ± 0.142	20.00	0.0028
Ocimum basilicum	2983	IC	21.59 (19.00–24.93)	158.42 (118.26–228.28)	−1.976 ± 0.106	1.481 ± 0.005	8.9849	0.1744

Values calculated from Probit analysis.
N, total number of nematodes used in generating the probit regression estimates; FL, 95% fiducial limits; SE, standard error of the estimate; χ^2 values for goodness-of-fit model.

Table 2. Comparisons of LC_{50} (lethal concentration) and IC_{50} (migration inhibitory concentration) values between *C. citratus*, *C. martinii* and *O. basilicum* EOs, using a ratio test.

Essential oil comparison[a]	Ratio for mortality (95% CI)[b]	Ratio for migration (95% CI)[b]
Cymbopogon martinii–Cymbopogon citratus	2.98 (1.24–7.18)*	0.24 (0.10–0.54)*
Cymbopogon martinii–Ocimum basilicum	1.05 (0.63–1.74)	1.44 (0.65–3.16)
Ocimum basilicum–Cymbopogon citratus	2.85 (1.35–6.02)*	0.16 (0.11–0.26)*

*Significant ($P < 0.05$).
[a]The essential oil mentioned first has a higher LC_{50} and IC_{50} value.
[b]When the 95% confidence interval (CI) includes 1, the LC_{50} or IC_{50} is not significantly different.

action on nematodes is gradual. Two major constituents of *C. martinii* and *C. citratus* are geraniol and geranial. Geraniol and citronellol are the main components of *Pelargonium*, and both constituents have shown nematicidal properties in *Caenorhabditis elegans* (Abdel-Rahman *et al.*, 2013). In our study, *P. asperum* showed some anthelmintic activity in regard to egg hatching, migration and mortality of larvae, but not to the extent of the EO of the *Cymbopogon* spp. As single constituents were not tested in the present study, it is difficult to pinpoint the EO constituent(s) with the most active principles against GINs. However, Macedo *et al.* (2015) concluded that citral (geranial and neral) was responsible for the anthelmintic activity of *C. citratus* on *H. contortus in vitro* and showed 38.5% reduction of *H. contortus* when the EO was fed to gerbils using an oral dose of 800 mg/kg. Variations in extraction methods, plant parts and varieties used, as well as geographic location and harvest time, can affect the chemical content of bioactive compounds in plants (Sandoval-Castro *et al.*, 2012).

The L_3 stage is usually more resilient to adverse substances than the first-stage larvae due to the double-sheath exoskeleton, and is also less sensitive to paralysis of its pharynx muscles (Molan *et al.*, 2002). In a development bioassay, Katiki *et al.* (2011) demonstrated that *C. martinii* and *M. piperita* had low LC_{50} values of 0.15 and 0.26 mg/ml, respectively, for *H. contortus* L_1 larvae. Bioassays with L_3, such as in the present study, will likely result in higher lethal concentrations than in those using L_1. Using L_3 for *in vitro* tests may be more biologically relevant if the objective is to use the EO directly as a feed supplement with anthelmintic properties, although results of assays *in vitro* would not be sufficient to suggest direct anthelmintic efficiency in field situations (Sandoval-Castro *et al.*, 2012).

Our test showed a LC_{50} of 3.89 mg/ml using *C. citratus*, significantly better than the two other EOs tested (*C. martinii* and *O. basilicum*) for dose–response. Katiki *et al.* (2011) also had consistently better efficacy using another *Cymbopogon* species, *C. schoenanthus*, than with *C. martinii*. The results seem to confirm that *C. martinii* should not be the preferred *Cymbopogon* species to use for *in vivo* tests. *Cymbopogon citratus* and *C. martinii* contain approximately 20 constituents, being rich in geraniol, geranial, neral, geranyl and myrcene. These are terpenoid compounds, which can also cause insect death (Bakkali *et al.*, 2008) and may likely be involved in the inhibition, retarded growth, reduced reproduction capacity and damage to the mature larvae.

EOs administered alone or in combination to the animals can have a variety of effects, such as killing GINs, reducing the

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establishment or development of nematodes in the host, decreasing contamination by nematode eggs or reducing egg hatching (Athanasiadou & Kyriazakis, 2004). Andre *et al.* (2016) showed that carvacryl acetate, when fed to sheep at 250 mg/kg fresh weight, reduced FEC 16 days post-treatment, but nonetheless concluded that its effectiveness could be increased, possibly by encapsulation. Sandoval-Castro *et al.* (2012), however, identified several challenges that need to be overcome to confirm *in vivo* efficacy of EOs. For instance, the sole use of EOs as anthelmintic might be problematic, given the high concentration that would be necessary in the gastrointestinal tract to directly affect the nematodes, without causing any adverse effect to the animals. The rumen of 12.5-month-old heifers can contain a volume of digesta ranging from 50.1 to 63.6 l, 5 h post-feeding, depending on the diet (Suarez-Mena *et al.*, 2013). Digesta volumes, which can be fairly large, can give indications to the quantity of EOs needed to be delivered to the digestive tract of heifers, if the goal is to reach concentrations directly affecting GINs in the gastrointestinal system.

The effect of the EOs on maturation of infective larvae to adult GINs, establishment in the gastrointestinal tract, and reduction in adult fecundity, has not been evaluated in the present study. Relying solely on assessing the direct impact of EO on mobility and mortality of L_3 is not sufficient to extrapolate to the *in vivo* effect. However, when the findings of *in vitro* assay are conclusive, *in vivo* assays can be used in subsequent investigation in field conditions, to confirm *in vitro* results (Sandoval-Castro *et al.*, 2012). Anthelmintic plant extracts such as EOs can also provide a lower risk of GIN resistance development than synthetic anthelmintic drugs, due to the potential synergistic effect of the combination of secondary metabolites present in each EO. Further research on the practical use of EOs needs to be carried out to standardize the doses needed and to develop practical delivery methods.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X19001081

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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