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Author for correspondence:
L.M. Costa-Júnior,
E-mail: livio.martins@ufma.br

Essential oils from *Ocimum basilicum* cultivars: analysis of their composition and determination of the effect of the major compounds on *Haemonchus contortus* eggs

A.I.P. Sousa[1], C.R. Silva[2], H.N. Costa-Júnior[2], N.C.S. Silva[2], J.A.O. Pinto[3], A.F. Blank[3], A.M.S. Soares[1] and L.M. Costa-Júnior[2]

[1]Laboratory of Plant Biochemistry, Federal University of Maranhão, São Luís, MA, Brazil; [2]Laboratory of Parasite Control, Federal University of Maranhão, São Luís, MA, Brazil and [3]Agronomy Department, Federal University of Sergipe, São Cristóvão, SE, Brazil

Abstract

The continuous use of synthetic anthelmintics against gastrointestinal nematodes (GINs) has resulted in the increased resistance, which is why alternative methods are being sought, such as the use of natural products. Plant essential oils (EOs) have been considered as potential products for the control of GINs. However, the chemical composition and, consequently, the biological activity of EOs vary in different plant cultivars. The aim of this study was to evaluate the anthelmintic activity of EOs from cultivars of *Ocimum basilicum* L. and that of their major constituents against *Haemonchus contortus*. The EOs from 16 cultivars as well the pure compound linalool, methyl chavicol, citral and eugenol were used in the assessment of the inhibition of *H. contortus* egg hatch. In addition, the composition of three cultivars was simulated using a combination of the two major compounds from each. The EOs from different cultivars showed mean Inhibition Concentration (IC_{50}) varying from 0.56 to 2.22 mg/mL. The cultivar with the highest egg-hatch inhibition, Napoletano, is constituted mainly of linalool and methyl chavicol. Among the individual compounds tested, citral was the most effective (IC_{50} 0.30 mg/mL). The best combination of compounds was obtained with 11% eugenol plus 64% linalool (IC_{50} 0.44 mg/mL), simulating the Italian Large Leaf (Richters) cultivar. We conclude that different cultivars of *O. basilicum* show different anthelmintic potential, with cultivars containing linalool and methyl chavicol being the most promising; and that citral or methyl chavicol isolated should also be considered for the development of new anthelmintic formulations.

Introduction

Gastrointestinal nematodes (GINs) have very important economically negative effects on several animal production systems (Nieuwhof & Bishop, 2005; Lane *et al.*, 2015). The nematode *Haemonchus contortus* is one of the most relevant GINs that infects small ruminants around the world (Rodríguez *et al.*, 2015). The control of this nematode is performed mainly with synthetic anthelmintics. However, the increased resistance of this parasite to anthelmintics has major economic impacts on livestock worldwide (Kotze *et al.*, 2014; Albuquerque *et al.*, 2017). The development of natural-based formulations is being considered as an alternative.

Natural products can be used to control strains of *H. contortus* that are resistant to synthetic compounds (Andre *et al.*, 2016; Garcia-Bustos *et al.*, 2019). Among these products, plant essential oils (EOs) and their major compounds, terpenoid and phenylpropanoid, have shown promising anthelmintic effects (Katiki *et al.*, 2012; Castilho *et al.*, 2017; Ferreira *et al.*, 2018). However, the yield and composition in terms of bioactive volatile compounds depend on genetic, environmental and agronomic factors (Yang *et al.*, 2018).

The plant *Ocimum basilicum* L., popularly known as basil, is native to Asia and grows spontaneously in tropical and sub-tropical regions (Khair *et al.*, 2012). The *O. basilicum* EOs present compounds of interest to the food, cosmetic and also pharmaceutical industries, with a production higher than 40 tons annually (Lawrence, 1992; Telci *et al.*, 2006). The *O. basilicum* EOs have been shown to exhibit several biological activities (Govindarajan *et al.*, 2013; El-Soud *et al.*, 2015; Silva *et al.*, 2015; Güez *et al.*, 2017), including action against *H. contortus* (Castro *et al.*, 2017).

The distinction among numerous basil varieties is largely based on their EO composition, which is of the utmost importance to biological activities and consumers' preference (Kiferle *et al.*, 2019). Several cultivars of *O. basilicum* present EOs with linalool, methyl chavicol (estragol), citral and eugenol as its main constituents, in variable concentrations (Vieira & Simon, 2000; Pascual-Villalobos & Ballesta-Acosta, 2003; Sajjadi, 2006; Martins *et al.*, 2010; Ottai *et al.*, 2012). These compounds have been shown to have anthelmintic activity, isolated or in a mixture, and they are also present, in different concentrations, in several other EOs (Katiki *et al.*, 2017; Ferreira *et al.*, 2018; Macedo *et al.*, 2019). The standardization of efficient cultivars or the combination of natural compounds is extremally important to human and veterinary pharmaceutical industries.

Considering that different cultivars of the same plant species may have different EO compositions, with different bioactivity, the objective of this study was to evaluate the action of EOs obtained from different cultivars of *O. basilicum*, as well as combinations of their major constituents, on *H. contortus*.

Materials and methods

Plant material and EOs

EOs from 15 commercial cultivars and one experimental hybrid from the Basil Genetic Breeding Program of Universidade Federal de Sergipe were evaluated. The following 15 commercial cultivars were used: Anise, Napoletano, Genovese, Ararat, Edwina, Dark Opal, Italian Large Leaf (Richters), Magical Michael, Mrs Burns, Nufar F1, Purple Ruffles (Richters Herbs, Goodwood, ON, Canada), Italian Large Leaf (Isla), Italian Large Red Leaf, Limoncino (Isla Sementes, Porto Alegre, RS, Brazil) and Maria Bonita (Blank *et al.*, 2007), and the experimental hybrid Genovese × Maria Bonita. All EOs used were obtained from the study of Pinto *et al.* (2019). The cultivars were planted and collected simultaneously during the rainy season (April–June 2016), and EOs were extracted and analysed according to Pinto *et al.* (2019).

Parasitological procedures

The *H. contortus* strain used in the present study was isolated from a goat naturally infected, as described in Silva *et al.* (2021). Third larvae stage (L3) of *Haemonchus contortus* (n = 2000 L3/animal) was used to experimentally infect a donor sheep confirmed to be parasite-free, with five successive negative faecal egg counts (Robert & O'sullivan, 1950) performed in three-day intervals. After 30 days, the infection was confirmed by faecal egg count, faecal culture and L3 identification (Robert & O'sullivan, 1950; Van Wyk & Mayhew, 2013). Through previous *in vitro* tests, the *H. contortus* strain used was confirmed to be resistant to benzimidazoles and susceptible to levamisole.

The nematode eggs were recovered from faeces, according to Silva *et al.* (2021), and stored in a 15 mL conical tube (eggs primary solution). The total number of eggs collected was estimated in three samples of 20 mL of the primary solution, and then a solution of 1000 eggs/mL was prepared. The experimental procedures were performed according to the guidelines of the Animal Ethics Committee (CEUA) of the Federal University of Maranhão, and were approved under the protocol number 23115018061/2011-01.

Egg-hatching assay

The eggs were added to a saturated sodium chloride solution and centrifuged (1350 g) for three minutes. The floating eggs were collected (Coles *et al.*, 1992), washed three times and re-suspended in distilled water. A suspension of 100 eggs/well was placed in a 96-well sterile plate.

The EOs from all cultivars and commercial samples of their major constituents linalool, methyl chavicol, citral and eugenol purchased from Sigma-Aldrich (St Louis, MO, US), were individually diluted in 3% Tween in different concentrations (7.0, 4.9, 3.4, 2.4, 1.7, 1.2, 0.8, 0.6, 0.4 and 0.3 mg/mL). Each samples test was performed in quadruplicate ($n = 4$), using at least six concentrations. The negative control was performed with 3% Tween. The eggs were incubated for 48 h at 27°C. Eggs and first-stage larvae were counted under an inverted microscope at 40× magnification.

Compound combinations

Linalool, methyl chavicol, citral and eugenol (Sigma-Aldrich) were used to simulate the composition of three cultivars using the two major compounds of each. Cultivars with low and intermediate IC_{50} (concentration required to inhibit 50% of hatching) and different major compounds, were selected. The efficacy of compounds in combination to simulate Genovese (57% linalool and 27% methyl chavicol), Mrs Burns (38% linalool and 49% citral) and Italian Large Leaf (Richters) cultivars (64% linalool and 11% eugenol) was assessed in an egg-hatching assay. To complete each mixture to 100% of composition, olive oil was used.

The isolated compounds and their mixtures were diluted in 3% Tween in decreasing concentrations (3.4, 2.4, 1.7, 1.2, 0.8, 0.6, 0.4 and 0.3 mg/mL). The tests of each compound were performed in quadruplicate using at least six of the above-described concentrations. The negative control was performed with 3% Tween in olive oil, at 25 mg/mL. The egg-hatching assays were performed as described above.

Statistical analysis

The results were used to determine the IC_{50} with respective 95% confidence intervals using GraphPad Prism 8.0 software (GraphPad Inc, San Diego, CA, US). The data were initially transformed into Log (X), normalized and then non-linear regression was applied to obtain the IC_{50} values. The differences among the IC_{50} were assessed using the F test (GraphPad Inc). Linear regression was applied to compare the IC_{50} values from isolated compounds, their combinations and cultivars, for which the percentages of the four major constituents are listed (GraphPad Inc).

Results

The EOs from different cultivars showed differences in the IC_{50} (table 1). This difference reached up to 3.96-fold, between the Napoletano cultivar, which presented the highest efficacy (IC_{50} 0.56 mg/mL), and the cultivars with the lowest efficacy such as Purple Ruffles and Italian Large Red Leaf (IC_{50} 2.22 mg/mL) (table 1).

The anthelmintic activity of the major EO constituents – linalool, methyl chavicol, eugenol and citral – was also assessed. Citral was the most effective compound (IC_{50} 0.30 mg/mL) (table 2). Two of the three assessed combinations – eugenol + linalool and methyl chavicol + linalool – showed higher efficacy than their isolated compounds. However, the combination of citral + linalool is less effective than citral alone, and more effective than only linalool.

The best result was obtained with the combination of 11% eugenol plus 64% linalool (IC_{50} 0.30 mg/mL), simulating the Italian Large Leaf (Richters) cultivar (table 2). This compound

Table 1. Major compounds (%) from essential oils of cultivars and hybrid of *Ocimum basilicum* and concentrations required for achieving 50% inhibition of egg hatching in *Haemonchus contortus* (IC_{50}) with respective 95% confidence intervals (95% CI).

	Major compound (%)						
Cultivar	Linalool	Methyl chavicol	Eugenol	Citral	IC_{50}	95% CI	R^2
Napoletano	26	54	0	0	0.56[a]	0.49–0.63	0.86
Genovese	57	27	0	0	0.62[b]	0.60–0.63	0.98
Ararat	16	68	0	0	0.86[c]	0.82–0.92	0.96
Mrs Burns	38	0	0	49	0.97[d]	0.91–1.05	0.95
Dark Opal	55	0	0	0	1.09[e]	1.05–1.14	0.97
Limoncino	9	0	0	50	1.18[f]	1.13–1.22	0.95
Italian Large Leaf (Richters)	64	0	11	0	1.19[g]	1.13–1.26	0.96
Magical Michael	64	0	20	0	1.54[h]	1.48–1.68	0.95
Edwina	73	0	6	0	1.66[h,i]	1.57–1.75	0.97
Italian Large Leaf (Isla)	61	0	8	0	1.69[i]	1.56–1.85	0.92
Genovese x Maria Bonita	68	0	0	20	1.72[j]	1.69–1.77	0.99
Anise	0	81	0	0	1.77[i]	1.65–1.89	0.94
Nufar F1	66	12	0	0	2.11[k]	2.02–2.22	0.97
Maria Bonita	78	0	0	0	2.13[k]	2.05–2.22	0.98
Purple Ruffles	18	57	0	0	2.22[k]	2.08–2.36	0.95
Italian Large Red Leaf	64	0	13	0	2.22[k]	2.11–2.34	0.97

Chemical composition from Pinto *et al.* (2019).
R^2, regression coefficient. The R^2 value quantifies goodness-of-fit at the non-linear regression curve performed to estimate the IC_{50}.
Different superscript letters in the IC_{50} column indicate significant differences ($P < 0.05$).

Table 2. Inhibition concentrations required for achieving 50% of egg hatching in *Haemonchus contortus* (IC_{50}) with respective 95% confidence intervals (95% CI) from major compounds and their combinations simulating cultivars of *Ocimum basilicum*.

Compounds	IC_{50}	95% CI	R^2
Citral	0.30[a]	0.29–0.32	0.98
Methyl chavicol	0.66[c]	0.65–0.68	0.98
Eugenol	1.39[e]	1.33–1.45	0.97
Linalool	1.75[e]	1.65–1.86	0.94
Eugenol + linalool[1]	0.44[b]	0.37–0.54	0.71
Methyl chavicol + linalool[2]	0.65[d]	0.63–0.68	0.97
Citral + linalool[3]	0.69[d]	0.65–0.75	0.94

R^2, regression coefficient. The R^2 value quantifies goodness-of-fit at the non-linear regression curve performed to estimate the IC50; all combinations were used with olive oil to complete 100% composition; among the different treatments, IC_{50} values with the same superscript letter are statistically equivalent ($P < 0.05$).
[1]11% eugenol and 64% linalool, simulating the EO from Italian Large Leaf (Richters) cultivar.
[2]27% methyl chavicol and 57% linalool, simulating the EO from Genovese cultivar.
[3]49% citral and 38% linalool, simulating the EO from Mrs Burns cultivar.

combination was 2.7 times more effective than EOs from the cultivar. On the other hand, the combination of 38% linalool and 49% citral was 1.4 times more effective than EOs from the cultivar Mrs Burns. The other combination used in the present study – 57% linalool and 27% methyl chavicol – did not differ statistically from the EO of the Genovese cultivar.

A negative correlation was observed at increase the concentration of citral in cultivars, compounds isolated and its combinations decreasing the IC_{50} value ($P = 0.03$). No other correlation was found.

Discussion

The EO of *O. basilicum* has several biological activities, such as antifungal (El-Soud *et al.*, 2015), antimicrobial (Lang & Buchbauer, 2012), antiprotozoal (Almeida *et al.*, 2007; Santoro *et al.*, 2007), insecticidal (Rodríguez-González *et al.*, 2019), acaricidal (Martinez-Velazquez *et al.*, 2011) and anthelmintic (Castro *et al.*, 2017). However, there are several basil cultivars with considerably different EO composition (Sharopov *et al.*, 2016). This is the first study to show a statistical difference in the inhibition of *H. contortus* egg hatch – up to 3.96 times – among EOs from cultivars of the same plant species (table 1).

The egg-hatch test used in the present study has been developed as a phenotypic diagnostic of resistant nematodes for the benzimidazoles, looking at the eggs that fail to hatch (Lacey *et al.*, 1987; FAO, 2004). The benzimidazoles inhibit embryonation and hatching by interfering with microtubules' formation (Mandelkow & Mandelkow, 1990; Coles *et al.*, 1992). Additionally, natural compounds altered the egg's surface and increased benzimidazole activity (Silva *et al.*, 2021). Therefore, the rationale for using egg-hatch assay in the present study was to use it as a model to search for new compounds against nematode infection, and not to target specific use in nematode eggs.

Inhibition of *H. contortus* egg hatch was previously demonstrated by the EO of one *O. basilicum* cultivar and associated with methyl chavicol and linalool as major compounds of the EO tested (Castro *et al.*, 2017). In the present study, the EOs from Napoletano,

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Genovese and Ararat cultivars showed the highest anthelmintic activity, and they also contain methyl chavicol and linalool, as major compounds (table 1). However, the EOs from Nufar F1 and Purple Ruffles cultivars exhibited low efficacy against *H. contortus* while having a similar chemical composition with methyl chavicol and linalool as major compounds. Despite methyl chavicol showing a relatively good efficacy in inhibiting *H. contortus* egg hatch, Anise cultivar, which possesses 81% methyl chavicol, does not present good efficacy when compared to other cultivars with low amounts of this compound. Interestingly, the hybrid cultivar Genovese + Maria Bonita presented an intermediate anthelmintic effect when compared with separate Genovese and Maria Bonita cultivars.

Citral, a natural combination of the isomers neral and geranial, has been shown to be effective against several nematodes, including *H. contortus*, both isolated and as the major compound of EO (Hierro *et al.*, 2006; Macedo *et al.*, 2019). A negative correlation between the citral concentration and efficacy was found when the results of all EO cultivars were analysed ($P = 0.03$), whereas isolated citral showed the best activity when tested alone (table 2).

The composition of EOs extracted from basil varies considerably. It can be classified into four, five or seven chemical groups or chemotypes according to the main components and the statistical analysis performed (Martins *et al.*, 2010; Liber *et al.*, 2011; Giachino *et al.*, 2014; Pinto *et al.*, 2019). The variability of chemical composition from different chemotypes has been found in diverse regions of the world (Hassanpouraghdam *et al.*, 2010). Differences in EO efficacy from the same vegetal species with different chemical compositions against parasites have been reported (Peixoto *et al.*, 2015; Costa-Júnior *et al.*, 2016; Lima *et al.*, 2016). However, the efficacy could not be correlated with the chemotype or the EO's main compound, and seems to be associated with a blend of compounds (Cruz *et al.*, 2013; Soares *et al.*, 2016).

The combinations of the components eugenol + linalool and methyl chavicol + linalool showed more efficacy than the isolated compounds (table 2), demonstrating that the combined compounds potentialized egg-hatch inhibition. Linalool represents the main component of many species of *Ocimum*, and is considered responsible for biological activities, representing reasons for its relevance (Ravid *et al.*, 1997).

Despite the benefits of using *O. basilicum* EOs in human and animal health, the present study has considerable importance for the bioprospection of pure or combinations of natural compounds to control ruminant nematodes. Our results clearly show differences in the bioactivity of EOs from different *O. basilicum* cultivars, related to the citral concentration. Additionally, the combinations using linalool and other compounds showed higher inhibition of *H. contortus* eggs than linalool alone, demonstrating the potential use of these compounds for the development of products for nematode control.

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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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