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In vitro effects of *Coriandrum sativum*, *Tagetes minuta*, *Alpinia zerumbet* and *Lantana camara* essential oils on *Haemonchus contortus*

Efeitos *in vitro* dos óleos essenciais de *Coriandrum sativum*, *Tagetes minuta*, *Alpinia zerumbet* e *Lantana camara* sobre *Haemonchus contortus*

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Abstract

Phytotherapy can be an alternative for the control of gastrointestinal parasites of small ruminants. This study evaluated the efficacy of *Alpinia zerumbet*, *Coriandrum sativum*, *Tagetes minuta* and *Lantana camara* essential oils by two *in vitro* assays on *Haemonchus contortus*, an egg hatch test (EHT) and larval development test (LDT). No effect was observed for *L. camara* in the EHT. *A. zerumbet*, *C. sativum* and *T. minuta* essential oils exhibited a dose-dependent effect in the EHT, inhibiting 81.2, 99 and 98.1% of *H. contortus* larvae hatching, respectively, at a concentration of 2.5 mg mL^{-1}. The effective concentration to inhibit 50% (EC50) of egg hatching was 0.94, 0.63 and 0.53 mg mL^{-1} for *A. zerumbet*, *C. sativum* and *T. minuta* essential oils, respectively. In LDT, *L. camara*, *A. zerumbet*, *C. sativum* and *T. minuta* at concentration of 10 mg mL^{-1} inhibited 54.9, 94.2, 97.8 and 99.5% of *H. contortus* larval development, presenting EC50 values of 6.32, 3.88, 2.89 and 1.67 mg mL^{-1}, respectively. Based on the promising results presented in this *in vitro* model, it may be possible use of these essential oils to control gastrointestinal nematodes. However, their anthelmintic activity should be confirmed *in vivo.*

Keywords: Phytotherapy, anthelmintic, gastrointestinal nematodes.

Resumo

Fitoterapia pode ser uma alternativa para o controle de parasitas gastrintestinais de pequenos ruminantes. Este estudo avaliou a eficácia dos óleos essenciais de *Alpinia zerumbet*, *Coriandrum sativum*, *Tagetes minuta* e *Lantana camara* sobre *Haemonchus contortus* através de dois testes *in vitro*, teste de eclosão dos ovos (TEO) e teste de desenvolvimento larval (TDL). Nenhum efeito foi observado para *L. camara* no TEO. Os óleos essenciais de *A. zerumbet, C. sativum* e *T. minuta* exibiram um efeito dose dependente no TEO inibindo a eclosão das larvas de *H. contortus* em 81,2, 99 e 98,1%, respectivamente, na concentração de 2,5 mg mL^{-1}. A concentração efetiva para inibir 50% (CE50) da eclosão dos ovos foi de 0,94, 0,63 e 0,53 mg mL^{-1} para os óleos essenciais de *A. zerumbet*, *C. sativum* e *T. minuta*, respectivamente. No TDL, 10 mg mL^{-1} de *L. camara, A. zerumbet, C. sativum* e *T. minuta* inibiram em 54,9, 94,2, 97,8 e 99,5% do desenvolvimento larvar, apresentando valores de CE50 de 6,32, 3,88, 2,89 e 1,67 mg mL^{-1}, respectivamente. Com base nos resultados promissores apresentados neste modelo *in vitro*, pode ser possível a utilização destes óleos essenciais para controlar os nematoides gastrintestinais. No entanto, a sua atividade anti-helmíntica deve ser confirmada *in vivo*.

Palavras-chave: Fitoterapia, anti-helmínticos, nematoides gastrintestinais.

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Introduction

Parasitic nematodes are a major threat to livestock production worldwide leading to huge economic losses (ADEMOLA; ELLOF, 2010), because of the support treatments and increased manpower that the infected animals require and because of the high mortality rate within flocks, especially in small ruminants (DIEHL et al., 2004). Anthelmintics have been used to minimize the losses caused by helminth infections. However, the efficacy of anthelmintics is increasingly endangered by development of resistance in nematode populations (MILLER et al., 2012). Control by anthelmintics has presented problems, such as the development of resistance in nematodes, the poor availability and high cost of commercial products, especially to low income farmers in developing countries, and increasing concern over the risk of environmental contamination because the presence of drug residues in animal products when pure compounds are administered (WALLER, 2006). These concerns have led to the search for and evaluation of alternative control methods (ATHANASIADOU et al., 2001). One promising alternative investigated for the control of gastrointestinal parasites has been the use of phytotherapics (MAPHOSA et al., 2010). Efforts are being made to evaluate several medicinal plants for their anthelmintic potential in different parts of the world. *Haemonchus contortus*, a highly pathogenic and one of the most prevalent nematode parasite, has been used by researchers to evaluate the anthelmintic effects of plants in small ruminants (EGUALE et al., 2011).

Among the criteria used to select plant species are their availability and the existence of studies researching their biological activity on nematodes. Of the plant species that have been documented as having medicinal effects against helminths, *Lantana camara, Tagetes minuta* (ALBUQUERQUE et al., 2007), *Alpinia zerumbet* (ALMEIDA, 1993) and *Coriandrum sativum* (HUSSAIN et al., 2008) are commonly cited.

L. camara, belonging to the Verbenaceae family, exhibits antimicrobial activity (DEENA; THOPPIL, 2000), efficacy against termite workers (VERMA; VERMA, 2006) and has a nematostatic effect on the phytonematode *Meloidogyne incognita* (DIAS et al., 2000). *T. minuta*, from the Asteraceae family, has fungitoxic effects (ROZWALKA et al., 2008), activity against *Anopheles stephensi* larvae (HADJIAKHOONDI et al., 2005), antibacterial activity (SOUZA et al., 2000) and effect on *H. contortus* egg and larvae (MACEDO et al., 2012). *A. zerumbet*, from the Zingiberaceae family, exhibits antioxidant (ELZAAWELY et al., 2007), antibacterial and antifungal activities (VICTÓRIO et al., 2009). *C. sativum*, belonging to the Umbelliferae family, has antifungal activity (FURLETTI et al., 2011), nematicidal activity against the phytonematode *Bursaphelenchus xylophilus* (KIM et al., 2008), and anthelmintic activity on gastrointestinal nematodes (EGUALE et al., 2007).

The objective of this study was to evaluate the effects of essential oils of *L. camara*, *A. zerumbet*, *C. sativum* and *T. minuta* on the egg hatching and larval development of *H. contortus*.

Materials and Methods

Plants and essential oils

L. camara, *A. zerumbet* and *T. minuta* were collected in Fortaleza, State of Ceará, Brazil, in campus of Universidade Estadual do Ceará in January of 2010. These plants were authenticated in the Herbarium Prisco Bezerra of the Universidade Federal do Ceará and voucher specimens were deposited under the numbers 46017, 49659, and 49676, respectively. The seeds of *C. sativum* were purchased commercially from the local market of Fortaleza.

To prepare essential oils, fresh aerial parts of 2.0 kg of *L. camara*, 2.7 kg of *A. zerumbet*, and 0.8 kg of *T. minuta* and 1.0 kg of seeds of *C. sativum* were separately subjected to hydrodistillation for 3 hours in a Clevenger-type apparatus. The calculation of yield was done and the oils were stored at 4 °C until use.

Egg hatch test

The egg hatch test (EHT) was performed based on the methodology described by Coles et al. (1992). Sheep experimentally infected with *H. contortus* were used as a source of fresh eggs. *H. contortus* eggs were recovered according to Hubert and Kerboeuf (1992) and Oliveira et al. (2009). Briefly, 10 g of feces, collected directly from the rectum, were mixed with distilled water and filtered through 590, 149, 101 and 30 µm mesh sieves. To increase the aqueous solubility, the oils were diluted in 3% Tween 80. An egg suspension (250 µL) containing approximately 100 fresh eggs was incubated with 250 µL essential oils at concentrations from 0.15 to 10 mg mL^{-1} for 48 h at 25 °C. Drops of Lugol were added. The eggs and first larval stage (L1) were counted under a microscope. This test had two controls: a negative control containing the diluent (3% Tween 80) and a positive control, 0.025 mg mL^{-1} of thiabendazole. Three repetitions with five replicates for each essential oil concentration and for each control were performed.

Larval development test

A larval development test (LDT) was performed according Camurça-Vasconcelos et al. (2007). For this, *H. contortus* eggs were incubated for 24 h at 28 °C to obtain the L1. Next, 1 mL of larval suspension containing approximately 250 L1 and 1 mL of essential oils at concentrations of 0.62 to 20 mg mL^{-1} were incubated with 2 g feces from a nematode-free sheep for 6 days at room temperature. Then, the third-stage larvae (L3) were recovered according to Roberts and O'Sullivan (1950) and counted under a microscope. This test had two controls, a negative with 3% Tween 80 and a positive with 0.008 mg mL^{-1} ivermectin. Three repetitions with five replicates for each oil concentration and for each control were conducted.

Chemical analysis

The chemical composition of the essential oils used in this study was determined by gas chromatography (GC) and mass spectrometry

(MS). The oil was analyzed on a Hewlett-Packard 5971 instrument using the following experimental conditions: DB-1–coated fused silica capillary column (30m × 0.25 mm) – carrier gas – helio; injector temperature – 220 °C; detector temperature – 200 °C; column temperature program – 35-180 °C at 48 °C /min, then 180-250 °C at 10 °C/min. For MS, the electron impact was 70 eV.

Compounds were identified by their GC retention time, expressed by Kovat's index, which was calculated by the Van den Dool and Kratz equation using a hydrocarbon homologous series and by comparison of test compound mass spectra with those present in the National Institute for Standard Technology computer data bank (NIST; 62,235 compounds) and published spectra (ADAMS, 2001).

Statistical analysis

The effectiveness of each treatment on the EHT was determined based on the percentage of larvae, using the following formula: number of larvae/(number larvae + number eggs) × 100.

The inhibition percentage of the LDT was calculated based on the percentage reduction in the L3 recovered compared to the negative control group (number of L3 in the control group - number of L3 in the treated group)/number of L3 in control group × 100.

The results of the *in vitro* tests are expressed as the percentage of egg hatching or larval development inhibition. Data were analyzed using ANOVA and compared by the Tukey test (P<0.05) using the GraphPad Prism program 5.0. The effective concentrations that inhibited 50% (EC50) of egg hatching and larval development were determined by the probit method using SPSS 8.0 for Windows.

Results

The yields of the essential oil were 0.05% to *L. camara*, 0.14% to *T. minuta*, 0.24% from *C. sativum* and 0.38% to *A. zerumbet*.

The mean percentages of the effectiveness of the essential oils obtained in the EHT are presented in Table 1. *L. camara* was not effective against egg hatching at the concentrations tested. However, the oils of *A. zerumbet, T. minuta* and *C. sativum* present at concentrations ≥ 1.25 mg mL^{-1} egg hatch inhibition superior to negative control (P<0.05).

Table 2 shows the mean efficacy of essential oils by the LDT. The effective concentration of *L. camara* was 20 mg mL^{-1}, did not differ from positive control (P>0.05). However, at a concentration of 10 mg mL^{-1}, the *A. zerumbet* and *C. sativum* oils had similar efficacy to the positive control (P>0.05). Moreover, the effectiveness of 5 mg mL^{-1} *T. minuta* was not significantly different from the positive control (P>0.05).

The EC50 values for the EHT and LDT are shown in Table 3. The oils tested on the larvae and eggs of *H. contortus* showed a dose-dependent effect.

The constituents of the essential oils are shown in Table 4. The major constituents of *A. zerumbet* oil were 1,8-cineole (24.69%), p-cimene (22.56%) and 4-terpineol (17.43%); *L. camara* contained caryophyllene oxide (50.26%); *T. minuta* contained piperitone (86.27%) and *C. sativum* contained beta linalool (73.21%).

Table 1. Mean efficacy ± standard error of *Lantana camara*, *Alpinia zerumbet*, *Coriandrum sativum* and *Tagetes minuta* essential oils on *Haemonchus contortus* egg hatching.

Concentrations (mg.mL^{-1})	*L. camara*	*A. zerumbet*	*C. sativum*	*T. minuta*
0.15	-	-	14.8 ± 1.9Aa	18.1 ± 1.0Aa
0.31	-	7.5 ± 0.8Aa	28.6 ± 1.9Bb	28.0 ± 2.0Bb
0.62	18.1 ± 2.2Aa	34.8 ± 1.9Bb	36.8 ± 1.8Cb	49.6 ± 2.4Cc
1.25	20.8 ± 1.8ABa	65.1 ± 2.5Cb	64.1 ± 1.5Db	74.1 ± 1.9Dc
2.5	26.1 ± 1.7BCa	81.2 ± 2.4Db	99.0 ± 0.2Ec	98.1 ± 0.5Ec
5	26.8 ± 1.7BCa	100.0 ± 0.0Eb	-	100.0 ± 0.0Eb
10	30.5 ± 1.2C	-	-	-
Tween 80 (3%)	12.8 ± 0.8Aa	14.3 ± 1.1Aa	11.7 ± 1.1Aa	11.2 ± 0.8Fa
Thiabendazole (0.025 mg.mL^{-1})	96.2 ± 0.6Da	96.2 ± 0.6Ea	96.2 ± 0.6Ea	96.2 ± 0.6Ea

Capital letters compare mean in the columns and small letters compare mean in the lines. Different letters indicate significantly different values (P < 0.05).

Table 2. Mean percentage efficacy ± standard error of *Alpinia zerumbet*, *Coriandrum sativum*, *Tagetes minuta* and *Lantana camara* essential oils on *Haemonchus contortus* larval development.

Concentrations (mg.mL^{-1})	*L. camara*	*A. zerumbet*	*C. sativum*	*T. minuta*
0.62	-	-	-	12.1 ± 4.2A
1.25	10.5 ± 3.7Aa	4.0 ± 1.4Aa	10.1 ± 3.1Aa	44.5 ± 2.6Bb
2.5	21.3 ± 6.5ABa	31.7 ± 4.5Bab	42.6 ± 2.8Bbc	55.1 ± 3.6Bc
5	31.9 ± 4.4Ba	55.2 ± 3.4Cb	77.8 ± 2.3Cc	92.9 ± 2.3Cc
10	54.9 ± 3.4Ca	94.2 ± 1.4Db	97.8 ± 1.1Db	99.5 ± 0.2Cb
20	97.7 ± 0.5Da	100 ± 0.0Da	99.5 ± 0.3Da	-
Tween 80 (3%)	7.2 ± 3.4Aa	8.2 ± 2.1Aa	6.6 ± 2.0Aa	4.8 ± 1.6Aa
Ivermectin (0.008 mg.mL^{-1})	99.9 ± 0.0Da	99.9± 0.0Da	99.9 ± 0.0Da	99.9 ± 0.0Ca

Capital letters compare mean in the columns and small letters compare mean in the lines. Different letters indicate significantly different values (P < 0.05).

Table 3. Effective concentration (mg.mL^{-1}) to inhibit 50% (EC50) of egg hatch test (EHT) and larval development test (LDT) of *Alpinia zerumbet*, *Coriandrum sativum*, *Tagetes minuta* and *Lantana camara* essential oils on *Haemonchus contortus*.

Essentials oils	EHT		LDT	
	EC50	Confidence Limits	EC50	Confidence Limits
L. camara	-	-	6.32	2.93-19.25
A. zerumbet	0.94	0.67-1.28	3.88	2.94-5.09
C. sativum	0.63	0.27-1.84	2.89	2.60-3.20
T. minuta	0.53	0.31-0.85	1.67	1.02-2.53

Table 4. Percentage relative composition of *Alpinia zerumbet*, *Lantana camara*, *Tagetes minuta* and *Coriandrum sativum* essential oils.

Constituents	KI	*A. zerumbet*	*L. camara*	*T. minuta*	*C. sativum*
Alpha Tujene	931	0.91	-	-	-
Alpha Pinene	932	-	-	-	4.2
Camphene	944	-	-	-	0.56
Beta Pinene	972	-	-	-	0.42
Sabinene	976	4.46	-	-	-
Beta Myrcene	995	-	-	-	0.65
Alpha Feladrene	1005	2.51	-	-	-
4-Carene	1011	7.33	-	-	-
o-Cimene	1020	-	-	-	0.94
Silvestrene	1027	4.55	-	-	-
Limonene	1031	-	-	13.73	1.86
1,8-Cineole	1033	**24.69**	-	-	-
Gamma-Terpinene	1062	11.56	-	-	-
Terpinene	1064	-	-	-	3.10
Tertpinolene	1088	1.84	-	-	0.50
P-Cimene	1089	**22.56**	-	-	-
Beta linalool	1091	-	-	-	**73.21**
Camphor	1139	-	-	-	4.25
Borneol	1162	-	-	-	0.98
4-terpineol	1177	**17.43**	-	-	-
Piperitone	1252	-	-	**86.27**	-
Copaene	1376	-	10.58	-	-
Beta-elemene	1391	-	2.74	-	-
Beta-Caryophyllene	1418	-	7.51	-	-
Beta-gurjunene	1432	-	1.88	-	-
Alpha murolene	1499	-	1.31	-	-
Nerolidol	1564	-	13.1	-	-
Espalenol	1576	-	3.18	-	-
Caryophyllene Oxide	1581	-	**50.26**	-	-
Total identified		97.84	90.56	100	90.67

KI – Kovats index; (–) means not detected. The values in bold are to highlight the chemical constituents found in higher percentage in the essential oil.

Discussion

This study verified the presence of biologically active compounds that had ovicidal effect on *H. contortus* in the essential oils of *A. zerumbet*, *C. sativum* and *T. minuta.* The oils inhibited egg hatching at a low concentration compared to other plants that have been studied previously. *Ocimum gratissimum* essential oil inhibited 100% of egg hatching at a concentration of 50 mg mL^{-1} (PESSOA et al., 2002). The maximum effectiveness of the essential oil of *Eucalyptus globulus* on eggs was 99.3% at a concentration of 21.75 mg mL^{-1} (MACEDO et al., 2009). In other study, the decoction of *L. camara* also did not act on eggs and decoctions of *A. zerumbet* and *T. minuta* exhibited inferior efficacy, inhibiting 97.5 and 96.8% of egg hatching at a concentration 5 and 2.5 mg mL^{-1}, respectively (MACEDO et al., 2012). However, the crude aqueous and hydro-alcoholic extracts of the seeds of *C. sativum* completely inhibited the hatching of egg at a concentration of less than 0.5 mg mL^{-1}. Phytochemical screening of these extracts revealed the presence of secondary metabolites such as alkaloids and flavonoids, which are considered to be the chemical components that are responsible for the wide therapeutic activities of several medicinal plants (EGUALE et al., 2007). Variation in activity

between the extract types and essential oil might be due to differences in the proportion of the active components responsible for the tested anthelmintic activity.

The most potent oil tested in LDT was *T. minuta*, which exhibited superior efficacy to other oils tested previously with an EC50 of 1.67 mg mL^{-1}. The essential oil of *Eucalyptus citriodora* had an EC50 of 2.71 mg mL^{-1} (MACEDO et al., 2011). The EC50 of *Lippia sidoides* essential oil was 2.97 mg mL^{-1} (CAMURÇA-VASCONCELOS et al., 2007). However, *T. minuta* essential oil was similar to *Eucalyptus staigeriana* essential oil, which had an EC50 of 1.70 mg mL^{-1} (MACEDO et al., 2010).

Transcuticular diffusion is a common method of entry into in helminth parasites for non-nutrient and non-electrolyte substances (EGUALE et al., 2007). It has also been shown that this route, rather than oral ingestion, predominates for the uptake of major broad-spectrum anthelmintics. It is easier for lipophilic anthelmintics to cross the external surface of helminths than it is for hydrophilic compounds (GEARY et al., 1999). The low density of plant oils and their rapid diffusion across cell membranes can enhance the targeting of the active components of essential oils into endoparasites (ANTHONY et al., 2005).

Essential oils are composed of a mixture of chemical substances whose interaction can result in compounds that can interfere with nematode metabolism, inhibiting or disorganizing vital functions from the initial stages of development onward, and can furthermore interfere with drive mechanisms due to possible destructuring of the nervous system (OKA et al., 2000). Essential oils can interact with the cytoplasmic membrane and may disrupt the structure of polysaccharides, lipids and phospholipids, promoting membrane depolarization and the consequent alteration of its permeability (BAKKALI et al., 2008), as reported for fungi exposed to essential oils (HAMMER et al., 2004).

Chemical analysis identified components with high concentrations in essential oils that may be responsible for their anthelmintic activity. The main component of *A. zerumbet* was 1,8-cineol, which has previously been shown to have antibacterial activity (CHA et al., 2007), antifungal activity (TERZI et al., 2007) and activity against the nematode *Anisakis simplex* (NAVARRO et al., 2008). The main component of *L. camara* was caryophyllene oxide, an antifungal agent (YANG et al., 1999) that has analgesic and anti-inflammatory activity (CHAVAN et al., 2010). The main component of *C. sativum* was linalool, which has antibacterial activity (FISHER et al., 2007) and insecticidal activity against *Tribolium castaneum* (STAMOPOULOS et al., 2007), *Oryzaephilus surinamensis*, *Musca domestica* and *Blattella germanica* (LEE et al., 2003), and nematicidal activity against juveniles of the root-knot nematode, *Meloidogyne incognita* (LEELA et al., 1992). The major constituent of *T. minuta,* piperitone was an antioxidant (DAR et al., 2011), which has insecticidal activity against larvae of *Spodoptera littoralis* and fungicidal activity (ABDELGALEIL et al., 2008).

The plants evaluated in the current study had already been reported as anthelmintic agents. Therefore, the current finding is the first step to justify their use in folk medicine. Further investigation of isolated fractions at different dose levels should be pursued. In addition, tests with animals can be performed with *A. zerumbet*, *C. sativum* and *T. minuta* essential oils to evaluate the toxicity and to confirm their anthelmintic activity in target species.

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