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Short communication

Synergistic interaction of ten essential oils against *Haemonchus contortus in vitro*

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ABSTRACT

Anthelmintic resistance in sheep gastrointestinal nematodes is a worldwide problem. Multi-drug resistant haemonchosis is the most serious impediment for small ruminant systems, and there are no new drug candidates currently under development. Molecules from natural sources have demonstrated anthelmintic activity against parasites. In this work, the monoterpenoids carvacrol, carvone, cineole, linalool, limonene, and thymol and the phenylpropanoids cinnamaldehyde, anethole, vanillin, and eugenol were assessed individually or in mixtures of ten binary, three ternary, and three quaternary combinations using the *in vitro* egg hatch assay with eggs of a multi-drug resistant strain of *Haemonchus contortus*. The main objective of this study was to identify the most effective interaction among essential oils with the greatest individual anthelmintic efficacy and to determine the most powerful combinations. The essential oils were ranked by their 50% lethal concentration (LC_{50}) as follows (mg/mL): cinamaldehyde (0.018), anethole (0.070), carvone (0.085), carvacrol (0.11), thymol (0.13), linalool (0.29), vanillin (0.57), eugenol (0.57), cineole (4.74), and limonene (207.5). Quantification of synergism, additive effect, and antagonism were calculated for binary, ternary, and quaternary combinations. The best anthelmintic effect resulting from synergistic activity among 16 different combinations was for cinnamaldehyde:carvacrol (CL_{50} 0.012 mg/mL) and anethole:carvone (CL_{50} 0.013 mg/mL). These results indicate that these binary combinations would be promising to be tested in sheep infected with *H. contortus*.

1. Introduction

Gastrointestinal nematodes are one of the main causes of impairment in the productivity of small ruminants, with the aggravating factor that this disease can easily lead to death. Treatment of these parasitic infections is usually based on commercial anthelmintics. However, the presence of strains with multi-drug resistance (Le Jambre et al., 2005; Almeida et al., 2010; Chagas et al., 2013) has decreased the control options over the years, especially for *Haemonchus contortus*, one of the most important parasitic species due to its high prevalence and pathogenicity for small ruminants in the tropics (Almeida et al., 2010).

Several studies with plants essential oils were done to evaluate their (and major components) *in vitro* anthelmintic activity against *H. contortus*. These plants (and major oil components) include *Chenopodium ambrosioides* (ascaridol) and *Ocimum gratissimum* (eugenol) (Pessoa et al., 2002), *Lippia sidoides* (thymol), and *Croton zehntneri* (estragole) (Camurça-Vasconcelos et al., 2007), *Eucalyptus globulus* (cineole) (Macedo et al., 2009), *Eucalyptus staigeriana* (limonene) Macedo et al. (2010), and *Cinnamomum verum* (trans-cinnamaldehyde) (Williams et al., 2015). Essential oils derived from entire plants may contain up to 40 components that can be divided into major bioactive components (according to the purported anthelmintic activity) and other major or minor components that may contribute with additive or synergistic effects.

As an attempt to reproduce what occurs in nature, studies with mixtures of major components of essential oils become promising because synergism can be sought in order to obtain a formulation with standard, constant, and consistent efficacy (Miresmailli and Isman, 2014). However, interactions between these components may lead to synergistic, additive or antagonist effects.

In this work, the terpenoids carvone, carvacrol, cineole, limonene, linalool, and thymol, and the phenylpropanoids trans- anethole, trans-cinnamaldehyde, eugenol, and vanillin were evaluated for their anthelmintic activity as single compounds, as well as in binary, ternary, and quaternary combinations. As the screening method, in vitro egg hatch assay with *H. contortus* was used to access the LC_{50} and for the

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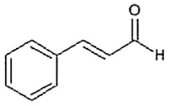
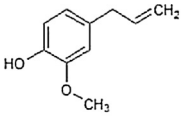
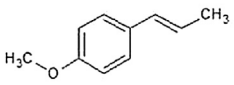
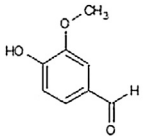
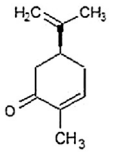
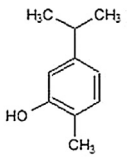
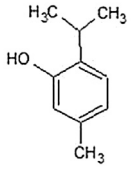
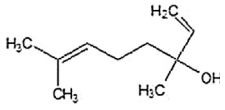
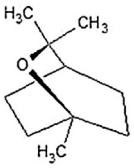
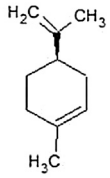
Table 1
Molecular structures and formulas of tested essential oils. Upper four compounds are phenylpropanes while bottom six are terpenoids. Their purities in comparison to analytical standards assessed by gas chromatography.

Molecular structure and formula of essential oils		Purities
		Cinnamaldehyde: 97.23% Anethole: 98.14% Eugenol: 98.35% Vanilin: 100%
Trans-cinnamaldehyde (C_9H_8O)	Eugenol ($C_{10}H_{12}O_2$)	
		
Trans-anethole ($C_{10}H_{12}O$)	Vanillin ($C_8H_8O_3$)	
		Carvone: 99.88% Thymol: 97.84% Cineole: 99.05% Carvacrol: 100% Linalool: 99.63% Limonene: 95.53%
Carvone ($C_{10}H_{14}O$)	Carvacrol ($C_{10}H_{14}O$)	
		
Thymol ($C_{10}H_{14}O$)	Linalool ($C_{10}H_{18}O$)	
		
Cineole ($C_{10}H_{18}O$)	Limonene ($C_{10}H_{16}$)	

Limonene was obtained from natural source (orange industry) whereas carvacrol, thymol, cineole, anethole, cinnamaldehyde, linalool, vanillin, carvone and eugenol were synthetically produced.

determination of synergism or antagonism within the essential oil combinations.

2. Materials and methods

2.1. Essential oils

The oils were provided by Grasp Ind. Com. Ltda. (Curitiba, Paraná, Brazil). Limonene was obtained from citrus peel (orange industry) whereas carvacrol, thymol, cineole, *trans*-anethole (anethole), *trans*-cinnamaldehyde (cinnamaldehyde), linalool, vanillin, carvone and eugenol were synthetically produced. The purity of the major components of each essential oil was established by gas chromatography in comparison with their analytical standards (Sigma-Aldrich, São Paulo, São Paulo, Brazil), and are presented with their molecular structures in Table 1. A gas chromatographer (HP7890A, Agilent Technologies, New Castle, Delaware, USA) equipped with an HP-5 capillary column (0.32 mm i.d., 0.25 μm film, and 30-m long) produced by J & W Agilent Technologies Inc. (Palo Alto, CA). An aliquot of 1 μL was injected using a 50:1 split ratio with H_2 flow at 35 mL/min. Column, injector, and flame ionization detector temperatures were 110 °C, 110 °C, and 250 °C, respectively. Oven heating slope was set to start at 110 °C, increasing to 148 °C (12 °C/min), then to 149.5 °C (0.5 °C/min), raising to a final temperature of 165 °C (40° C/min) with a total analytical time of 6.55 min. An external calibration curve was prepared with pure compounds for the quantification of essential oils.

2.2. Parasitic nematode

The study was approved by the Ethics Committee (CEUA-IZ) and received protocol number: 2012/153. After a successful deworming (negative fecal egg count) with Monepantel at 2.5 mg/Kg (Zolvix®, Novartis- Animal Health), two donor lambs were infected with 4.000 L3 *H. contortus* strain with resistance to multiple anthelmintic drugs (moxidectin, closantel, trichlorfon, levamisole phosphate, albendazole, and ivermectin), according to Almeida et al. (2010). These animals were infected with this strain and kept in a clean pen and fed hay, mineral salt, and water *ad libitum* during all experiment. At the end, animals were dewormed with Monepantel.

2.3. In vitro egg hatch assay

H. contortus eggs were recovered according to the methodology described by Bizimenyera et al. (2006) with some modifications. The feces were homogenized in warm water (37 °C) and filtered through a set of sieves, with the following cross-sections: 1 mm, 106 μm, 53 μm, and 25 μm. Approximately 100 eggs were placed in each well (24-well plate) and incubated with the essential oil solutions (dH_2O + Tween 80 at 2%) in decreasing concentrations (2.0, 1.0, 0.4, 0.16, 0.06, 0.02, and 0.007 mg/mL). Each treatment and control had six replicates. The plates were incubated for 24 h at 27 °C, after which the numbers of larvae L1 and unhatched eggs were counted.

2.4. Evaluation of essential oils and combinations

Initially, carvacrol, thymol, cineole, anethole, cinnamaldehyde, limonene, linalool, vanillin, carvone, and eugenol were tested individually. The five compounds that had the lowest LC_{50} (cinnamaldehyde, anethole, carvone, carvacrol, and thymol) were mixed in equal proportions forming binary combinations (1:1), so that 10 binary formulations were tested. The three best binary combinations (cinnammaldehyde + carvacrol; anethole + carvone; cinnamaldehyde + carvone) were mixed with each other (1:1:1:1), so that three quaternary combinations were formed. Then, the three best binary combinations were mixed with one of the three best pure oils (cinnamaldehyde, anethole and carvone) giving rise to three ternary combinations (1:1:1).

2.5. Statistical analysis

Eight concentrations (2.0, 1.0, 0.4, 0.16, 0.06, 0.02, and 0.007 mg/mL) of single compounds or binary, ternary, and quaternary combinations were tested in order to achieve the maximum and the minimum hatchability of eggs. This procedure allowed the calculation of lethal concentration producing 50% eggs mortality (LC_{50}) with estimated 95% fiducial limits by a regression curve generated by SAS® probit (SAS v. 9.2 ® Cary, NC) and allowed the classification of anthelmintic activity of essential oils. CompuSyn Version 1.0 (Chou and Martin, 2005) was used for analysis of drug combinations. The effects of combinations proposed by Chou (2006) were classified according to their combination index (CI) as: strong synergy (CI < 0.1); synergy (0.1 > CI < 0.9); additive (0.9 > CI < 1.1), antagonism (1.1 > CI < 10) and strong antagonism (CI > 10).

Table 2
Ranking of anthelmintic activity based on LC_{50} (mg/mL) and fiducial limits (95%) of single essential oils obtained by *in vitro* egg hatch assay.

Single compound	LC_{50} (fiducial limits)	LC_{99} (fiducial limits)
Cinnamaldehyde	0.018 (0.017–0.019)	0.085 (0.075–0.099)
Anethole	0.070 (0.068–0.076)	0.475 (0.416–0.554)
Carvone	0.085 (0.081–0.088)	0.366 (0.327–0.416)
Carvacrol	0.11 (0.107–0.125)	5.517 (4.391–7.128)
Thymol	0.13 (0.123–0.140)	5.004 (4.166–6.123)
Linalool	0.29 (0.279–0.319)	17.47 (14.10–22.11)
Eugenol.	0.57 (0.537–0.623)	51.65 (38.74–71.37)
Vanilin	0.57 (0.475–0.716)	815.16 (253.7–3368)
Cineole	4.74 (3.281–9.044)	1787 (328.3–4031)
Limonene	207.56 (80.31–760.69)	–

3. Results

The essential oils were individually evaluated by egg hatch assay and ranked in Table 2 based on their LC_{50} (mg/mL). Cinnamaldehyde (LC_{50} 0.018) and anethole (LC_{50} 0.070) were the most active essential oils and the least were cineole (LC_{50} 4.54) and limonene (LC_{50} 207.56).

The five oils with best activity (Table 2) were mixed in equal portions to perform 10 binary compositions. Results were ranked in Table 3 from the lowest to the highest LC_{50}. Data of combination index (CI) that classifies and quantifies the synergism, additive and antagonism effect also were present in Table 3. According to Chou (2010), synergism is more than additive effect and antagonism is less than an additive effect. Carvacrol + cinnamaldehyde (LC_{50} 0.012) and anethole + carvone (LC_{50} 0.013) presented the best activity. These two formulations, when compared, presented synergism. However, the combination anethole + carvone had a lower combination index (CI = 0.14) than cinnamaldehyde + carvacrol (CI = 0.2), and therefore was considered as more synergistic. According to Chou (2010), the CI offers quantitative definition for additive (CI > 1.1), synergistic (0.1 > CI < 0.9), and antagonistic (1.1 > CI < 10) effects in drug combinations. Anethole + carvone also had CL_{99} = 0.081 mg/mL, which was lower than cinnamaldehyde + carvacrol (CL_{99} = 0.099), suggesting a better effect.

The combination cinnamaldehyde + carvone + anethole + carvone presented a LC_{50} of 0.020 and it presented a synergistic effect (CI = 1.69) compared to the other quarternary mixtures. The combination cinnamaldehye + carvacrol + cinnamladehyde + carvone (CI = 2.37) and cinnamaldehyde + carvacrol + anethole + carvone (CI = 5.84) had antagonistic effects (Table 4). For all quarternary combinations, the anthelmintic activity was weaker than for single compounds and/or for binary combinations.

Another type of combination involved the mixture of three essential oils, such as cinnamaldehyde + carvacrol + anethole (LC_{50} 0.037 and CI 1.77), cinnamaldehyde + carvacrol + carvone (LC_{50} 0.050 and CI 0.92), and anethole + carvone + carvacrol (LC_{50} 0.205 and CI 10.74). No synergistic effect was observed for any of these combinations and anethole + carvone + carvacrol actually had a strong antagonistic effect (CI > 10) as shown in Table 5.

Table 4
Ranking of anthelmintic activity based on LC_{50} of quaternary mixtures of essential oils obtained by egg hatch assay. Combination index (CI) categories: strong synergy (CI < 0.1); synergy (CI 0.1 − 0.9); additive (CI 0.9–1.1), antagonism (CI 1.10–10) and strong antagonism (CI > 10).

Quaternary compositions (1 : 1 : 1 : 1)	LC_{50} (fiducial limit)	CI	description
Anethole + Carvone + Cinnamaldehyde + Carvone	0.020 (0.019–0.021)	1.69	Synergy
Cinnamaldehyde + Carvacrol + Cinnamaldehyde + Carvone	0.028 (0.027–0.029)	2.37	Antagonism
Cinnamaldehyde + Carvacrol + Anethole + Carvone	0.045 (0.043–0.048)	5.84	Antagonism

Table 5
Ranking of anthelmintic activity based on LC_{50} of ternary combinations of essential oils obtained by egg hatch assay. Combination index (CI) categories: strong synergy (CI < 0.1); synergy (CI 0.1 − 0.9); additive (CI 0.9–1.1), antagonism (CI 1.10–10) and strong antagonism (CI > 10).

Ternary composition (1:1:1)	LC_{50} (mg/mL)	CI	description
Cinamaldehyde + Carvacrol + Anethole	0.037(0.03–0.045)	1.77	Antagonism
Cinamaldehyde + Carvone + Carvacrol	0.050 (0.039–0.064)	0.92	Additive
Anethole + Carvone + Carvacrol	0.205 (0.180–0.232)	10.74	Strong antagonism

4. Discussion

In the present work, limonene, linalool, carvone, cineole, thymol, carvacrol, anethole, vanillin, eugenol, and cinnamaldehyde were evaluated for their anthelmintic activity *in vitro*. Several *in vitro* tests have been used to screen natural and synthetic compounds or plant extracts with potential anthelmintic activity. However, some assays are specific to evaluate anthelmintic mechanisms due to the mode of action, like egg hatch assay (EHA) for benzimidazoles (Várady and Corba, 1999) or larval development assay for avermectins, for example (Kotze et al., 2014). Some other assays are more sensitive for the screening of phytochemicals, such as tannins and phenolic compounds, in which case larval exsheathment assay is the choice (Klongsiriwet et al., 2015). We chose EHA as it is sensitive, simple, and fast. In addition, results for the EHA are similar to the larval development assay, larval feeding assay, and larval exsheathment assay when screening aromatic compounds (Katiki et al., 2011; Zhu et al., 2013; Ferreira et al., 2016; Grando et al., 2016).

Our first goal was to determine the individual activity of each essential oil belonging to the chemical groups of phenylpropanoids (cinnamaldehyde, anethole, eugenol, and vanillin) and terpenoids

Table 3
Ranking of anthelmintic activity based on LC_{50} (mg/mL) and LC_{99} from binary composition of essential oils obtained by *in vitro* egg hatch assay. Combination index (CI) categories: strong synergy (CI < 0.1); synergy (CI 0.1 − 0.9); additive (CI 0.9 – 1.1), antagonism (CI 1.10 – 10) and strong antagonism (CI > 10).

Binary composition (1:1)	LC_{50} (fiducial limit)	LC_{99} (fiducial limit)	CI	category
Cinnamaldehyde:Carvacrol	0.012 (0.011–0.013)	0.099 (0.853–0.119)	0.2	Synergy
Anethole:Carvone	0.013 (0.012–0.014)	0.081 (0.070–0.096)	0.14	Synergy
Cinnamaldehyde:Carvone	0.028 (0.027–0.030)	0.162 (0.853–0.192)	0.94	Additive
Cinnamaldehyde:Anethole	0.029 (0.027–0.031)	0.372 (0.314–0.452)	1.01	Additive
Cinnamaldehyde:Thymol	0.031 (0.029–0.032)	0.149 (0.131–0.452)	0.87	Synergy
Carvacrol:Thymol	0.037 (0.034–0.041)	2.718 (2.080–3.686)	0.35	Synergy
Anethole:Thymol	0.057 (0.053–0.061)	0.817 (0.688–0.994)	0.94	Additive
Carvacrol:Anethole	0.085 (0.081–0.089)	0.813 (0.707–0.751)	0.99	Additive
Carvone:Carvacrol	0.10 (0.093–0.10)	0.751 (0.665–0.860)	1.15	Antagonism
Carvone:Thymol	0.10 (0.090–0.10)	1.382 (1.164–1.178)	1.22	Antagonism

(carvone, carvacrol, thymol, linalool, cineole, and limonene). Limonene had the least ovicidal effect (LC_{50} = 207.56 mg/mL) against *H. contortus in vitro*. Although the literature is scarce on reports of ovicidal effects, or mode of action, of essential oil components on *H. contortus*, it is thought that these oils exert their effect on acetylcholinesterase (AChE) receptors found in vertebrates and invertebrates producing neurotoxic damage similar to organophosphates (López and Pascual-Villalobos, 2010). These authors reported that fenchone, carvone, and linalool had the strongest AChE inhibition *in vitro.* One study reported that *Eucalyptus staigeriana* essential oil (73% limonene, 9.5% cineole, 4.5% cimene) had an 99.96% efficacy in the EHT, at 1.0 mg/mL, and over 95% in the LDT, at 5.8–8.0 mg/mL and in a nematode load reduction of 46.4% when tested in artificially-infected gerbils (Ribeiro et al., 2013).

A recent review on insecticidal effect of essential oils (Dambolena et al., 2016) cited several authors who evaluated essential oils for their ovicidal activity against head lice (*Pediculus humanus*). These authors reported that linalool, thymol, carvone, anethole, cinnmaldehyde, among others, had high ovicidal activity. However, literature reports are sometimes contradictory depending on the species, developmental stage and technique used for each bioassay (Dambolena et al., 2016).

In our study, cinnamaldehyde, anethole (both phenylpropanes), and carvone (a monoterpenoid) were the most effective against *Haemonchus* in the EHA. Cinnamaldehyde and anethole are very similar in structure, but cinnamaldehyde is soluble in water while anethole is not. While this contrasting solubility may favor cinnamaldehyde *in vitro*, anethole could have better *in vivo* effects as its non-polar nature may favor its interaction with cell membranes of the parasite. The chemical compounds from the phenylpropanoids class tested in our work presented the first and second best anthelmintic activity: cinnamaldehyde (LC_{50} 0.018) and anethole (LC_{50} 0.070). Cinnamaldehyde is the major component of cinnamon bark essential oil and was effective when tested alone or in mixtures against animal parasites. Lee et al. (2011) evaluated cinnamaldehyde against avian coccidiosis *in vitro* tests (effective at 10 μg/mL) and obtained significant results *in vivo* (at the dose of 144 mg/kg) against *Eimeria tenella.* Bang et al. (2000) reported that the mode of action of cinnamaldehyde in fungi is related to the interference on cell division and in the cell wall, acting as an inhibitor of ß-glucan synthase and chitin synthase. This could be one of the modes of action of cynnamaldehyde in *H. contortus,* because its egg external layer is rich in chitin (Mansfield et al., 1992). Cinnamaldehyde was also very effective in vitro against Ascaris suum and concentrations of 200 μM (25.5 μg/mL) caused larval death within three hours, but had no effect in vivo (Williams et al., 2015). These authors used microscopy to postulate that the mode of action appeared to involve the destruction of the intestinal tissue of the parasite and explained that the fast absorption of cinnamaldehyde from the stomach may prevent its *in vivo* activity.

After cinnamaldehyde and anethole, the terpenoids carvacrol and thymol had very similar LC_{50}. These compounds have identical formulas, only differing in the position of their hydroxyl group. Carvacrol and thymol interact with tyramine receptors of *Drosophyla* sp. With the same activity and the presence of the hydroxyl group on their benzene ring is critical for good insecticidal activity (Dambolena et al., 2016). Macedo et al. (2009) performed EHA with the essential oil of *Eucalyptus globulus* (containing 83.89% of Cineole) and obtained LC_{50} = 8.3 mg/mL. *Lippia sidoides* (59.65% Thymol) was studied by Camurça-Vasconcelos et al. (2007) who found 95% inhibition of hatching of *H. contortus* eggs at a concentration of 0.62 mg/mL and Carvalho et al. (2012) obtained LC_{50} of 0.4 mg/mL (*L. sidoides* essential oil containing 76.6% Thymol). Thymol, also presented in the ethanolic extract of *Timus capitatus* (71.22%), had ovicidal activity on *H. contortus* (LC_{50} 0.368 mg/mL) as observed by Elandalousi et al. (2013).

The phenylpropanes eugenol and vanillin also have similar structures and equal LC_{50} in our EHA. The terpenoid cineole had the second lowest activity and limonene had, by far, the lowest LC_{50}, over 40 times less effective than cineole. The lowest activity of limonene *in vitro* contrasts with its high anthelmintic activity (97.5%) against *Haemonchus* in artificially-infected sheep (Squires et al., 2010). However, these authors tested an orange essential oil emulsion (40% orange terpenes, 20% orange oil, tween 80, hydrogen peroxide). These results indicate that limonene may have a synergistic effect with orange terpenes.

Our work with single pure molecules offers an unique view on the anthelmintic effect of compounds from the same class, but with small differences in their molecular structures that, not surprisingly, provided similar results, such as cinnamaldehyde and anethole (phenylpropanes), carvacrol and thymol (terpenoids), and eugenol and vanillin (phenylpropanes). However, the phenylpropanes eugenol and vanillin were 8–28 fold less effective than phenylpropanes cinnamaldehyde and anethole. After testing single compounds, we tested the mixtures of the best five aromatic compounds to evaluate their possible synergistic, additive, or antagonistic interactions (Miresmailli and Isman, 2014). The synergism of essential oils was reported for binary or ternary combinations in several antimicrobial tests (Hyldgaard et al., 2012). Ntalli et al. (2011) associated nine essential oils in binary combinations to evaluate nematicidal activity against *Meloidogyne incognita* (soil nematode) and observed synergism of the following compounds in descending order: anethole + geraniol, anethole + eugenol, carvacrol + eugenol, and geraniol + carvacrol.

Cinnamaldehyde + carvacrol presented the lowest LC_{50} found (LC_{50} 0.012). The second was anethole + carvone (LC_{50} 0.013). Both presented synergistic interactions. Zhou et al. (2007) found the same synergistic effect for cinnamaldehyde + carvacrol against *Salmonella.* The authors explained that carvacrol could increase the permeability of cytoplasmic membrane and, probably, allow cinnamaldehyde to be better transported into the cell. Another mode of action of carvacrol is that it could increase the number, size, or duration of existence of the pores created by binding of cinnamaldehyde to proteins in the *Salmonella* cell membrane, supporting the synergistic effect when these two compounds were used in combination (Zhou et al., 2007). Cinnamaldehyde, used as a single compound had similar LC_{50} to binary combinations of cinnamaldehyde + carvacrol and anethole + carvone. With the exception of an amino group, cinnamaldehyde is strikingly similar in structure to both of the essential aminoacids phenylalanine and tyrosine suggesting its interference with parasite metabolism if cinnamaldehyde can lead to a competitive inhibition of the essential enzymes that use phenylalanine and tyrosine for essential biological functions.

Carvacrol and carvacryl acetate (acetylated carvacrol) inhibited *H. contortus* egg hatching by 97.7% and 89.3% at 1 and 8 mg/mL, respectively. At 2 mg/mL, both compounds inhibited larval motility in 100%, and at 200 μg/mL both inhibited adult motility in 58 and 100% 24 h after exposure (Andre et al., 2016). These authors also reported that carvacryl acetate (at 250 mg/kg LBW) resulted in a fecal egg reduction of 66% 16 days after treatment, although the animals had only 500 FEC or higher at the beginning of treatment.

Antagonism occurs when the blend of oils has no better effect than when used individually (Hyldgaard et al., 2012). Chou (2010) used the combination index (CI) to depict quantitative synergism, additive effect, and antagonism. In this context, the synergism is a superior effect observed through the combination of substances in relation to the effect of the isolated compounds with the aim of improving the therapeutic effect, reducing doses and side effects (Williamson, 2001).

The efficacy observed in ternary composition and in quaternary compositions was lower than the binary associations. The mixture of three or more compounds decreased the anthelmintic activity either due to the dilution of their active components or due to antagonism. Cinnamaldehyde, carvacrol and anethole were combined to three most potent binary compositions in order to form ternary mixtures. Antagonism, additive or strong antagonism occurred and no synergism was observed. The mechanisms responsible for the antagonism are, however, less known and further investigation is required.

5. Conclusion

This study allowed us to evaluate the potential of 10 essential oils and combinations of best results in order to explore synergism. The results allowed us to perceive peculiar occurrences when different oils were mixed with each other. We concluded that binary combinations of cinnamaldehyde + carvacrol and anethole + carvone could become promising formulations for parasite control and deserve further investigations, which should include their validation in experimental animals, efficacy, and toxicity to the host, providing a clinical study for this new formulation with anthelmintic activity.

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