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DAUANA MESQUITA SOUSA

COMBINAÇÃO DE COMPOSTOS NATURAIS E SINTÉTICOS COMO ALTERNATIVA NO CONTROLE DE NEMATÓDEOS GASTRINTESTINAIS EM PEQUENOS RUMINANTES

São Luís - MA
2023

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Combinação de compostos naturais e sintéticos como alternativa no controle de nematódeos gastrintestinais em pequenos ruminantes

Versão da Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de biotecnologia – RENORBIO, ponto focal Maranhão.

Área de concentração: Biotecnologia em Agropecuária

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RESUMO

A carga parasitária de nematoides gastrointestinais pode causar perdas econômicas na produção de ovinos. Na rotina, essa infecção é controlada por compostos anti-helmínticos sintéticos, como o albendazol (ABZ). No entanto, a aplicação frequente desses compostos acelera a seleção de organismos resistentes, impedindo o controle desses parasitos pela dose anteriormente administrada. Assim, passa a existir a necessidade de alternativas, como os óleos essenciais e seus terpenoides para controlar os nematoides gastrointestinais. Estes se apresentam como novos compostos que podem ser administrados em combinações com anti-helmínticos sintéticos. Contudo, são necessários conhecimentos sobre o mecanismo de ação desses compostos para que possamos elaborar estratégias mais eficientes. Diante disso buscamos descrever a base farmacológica das combinações de terpenos e anti-helmínticos sintéticos como uma alternativa para aumentar a eficácia antiparasitária. E reunir em um único estudo as informações sobre as propriedades físico-químicas, as características farmacocinéticas, o metabolismo e o nível de transporte dos monoterpenos que podem ser relevantes para a obtenção de concentrações efetivas contra diferentes nematoides. Também, avaliamos a atividade *in vitro* e *in vivo* de óleo essencial de *Citrus aurantium* var. Dulcis (CaEO) combinado com ABZ contra *Haemonchus contortus* resistente a benzimidazol. Por meio de testes de eclosão de ovos (TEO) *in vitro* usando CaEO e ABZ para estimar a concentração efetiva para atingir os valores de 50% de inibição de eclosão do ovo (EC50) e calcular as combinações entre os compostos usando um desenho de mistura simplex-centroide. Para avaliar a redução da contagem de ovos nas fezes foram necessários um grupo por tratamento, com oito ovinos em cada. Os tratamentos foram ABZ e a combinação de CaEO com ABZ. No TEO, o CaEO e o ABZ apresentaram valores de EC50 de 0,57 e 0,0048 mg/ml, respectivamente. O ABZ reduziu a quantidade de ovos nas fezes em 78%, no entanto, sua a combinação com CaEO reduziu a contagem em apenas 9%. É importante destacar que apesar da eficiência *in vitro* ainda temos que verificar as possíveis interferências causadas pelos produtos naturais na metabolização dos anti-helmínticos, e consequentemente, em sua eficácia.

Palavras-chave: Óleo essencial; Terpenoides; Resistência; Pequenos ruminantes;

ABSTRACT

The parasitic load of gastrointestinal nematodes can cause financial losses in sheep and goat production. In the routine, this infection is controlled by synthetic anthelmintic compounds, such as albendazole (ABZ). However, the frequent application of these compounds accelerates the selection of resistant organisms, preventing the control of these parasites by the dose previously administered. Thus, there is a need for alternatives, such as essential oils and their terpenoids, to control gastrointestinal nematodes. These present themselves as new compounds that can be administered in combination with synthetic anthelmintics. However, knowledge about the mechanism of action of these compounds is required to develop more efficient strategies. In this context, we sought to describe the pharmacological basis of terpenes and synthetic anthelmintics combinations as an alternative to increase antiparasitic efficacy. We also gathered in a single study information on the physicochemical properties, pharmacokinetic characteristics, metabolism, and transport level of monoterpenes that may be relevant for achieving effective concentrations against different nematodes. Additionally, we evaluated the *in vitro* and *in vivo* activity of *Citrus aurantium* var. Dulcis essential oil (CaEO) combined with ABZ against benzimidazole-resistant *Haemonchus contortus*. Through *in vitro* egg hatch assays (EHA) using CaEO and ABZ to estimate the effective concentration to achieve 50% egg hatch inhibition (EC50) and calculate the compound combinations using a simplex-centroid mixture design. To evaluate the reduction in egg counts in feces, one group per treatment was required, with eight sheep in each. The treatments were ABZ and the combination of CaEO with ABZ. In the EHA, CaEO and ABZ presented EC50 values of 0.57 and 0.0048 mg/mL, respectively. ABZ reduced the number of eggs in the feces by 78%, however, its combination with CaEO only reduced the count by 9%. It is important to highlight that despite the *in vitro* efficiency, we still need to verify the possible interference caused by natural products in the metabolism of anthelmintics, and consequently, their efficacy.

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Keywords: Essential oil; Terpenoids; Resistance; Small ruminants.

SUMÁRIO

1. INTRODUÇÃO

A ovinocultura é um importante setor da pecuária, seu sucesso depende, principalmente, de rebanhos saudáveis e com bom ganho de peso. O cumprimento destas exigências é afetado pelo aumento da carga parasitária nos animais (FITZPATRICK, 2013). Isto promove uma série de sintomas, como, anemia, diarreia, redução no escore corporal, culminando na morte do animal e, consequentemente, em perdas econômicas (SARGISON, 2020; SELEMON, 2018).

Estima-se que são gastos 686 milhões de euros anualmente com nematoides gastrointestinais, contudo, os prejuízos causados pelas doenças parasitárias em ovinos são difíceis de quantificar (CHARLIER *et al.*, 2014, 2020). Na Austrália, um dos países com maior produção de ovinos, os prejuízos pelos parasitos ultrapassam a soma de 436 milhões de dólares australianos por ano (SHEPHARD *et al.*, 2022). Dentre os gastos estão a compra de anti-helmínticos. Estes são utilizados como forma de controlar os parasitos e minimizar os prejuízos (OSÓRIO *et al.*, 2020).

As principais classes de anti-helmínticos utilizadas são benzimidazois, imidazotiazois, derivados do amino-acetonitrilo e lactonas macrocíclicas (HERATH *et al.*, 2021). Cada classe possui diferente mecanismo de ação, por exemplo, os bezimidazois agem diretamente sobre as β-tubulinas impedindo a formação dos dímeros, e inibindo a formação dos microtúbulos (SON; LEE; ADUNYAH, 2020). Enquanto, as lactonas macrocíclicas bloqueiam a transmissão química entre os nervos pré-sinápticos e motor, utilizando os canais iônicos dependente de glutamato (MORENO; GEARY; TRITTEN, 2021). Contudo, uso frequente destes compostos e as aplicações irregulares aceleraram a seleção de parasitos resistentes (POTÂRNICHE *et al.*, 2021).

A resistência é caracterizada pela perda da sensibilidade ao anti-helmíntico em uma população anteriormente suscetível (FISSIHA; KINDE, 2021). A pecuária perde anualmente com a resistência parasitaria cerca de 38 milhões de euros. A parcela que corresponde a ovinocultura é equivalente a 10,5 milhões de euros (CHARLIER *et al.*, 2020). Diante deste cenário, busca-se novas alternativas para lidar com os organismos resistentes, dentre elas, destacamos, a utilização de compostos oriundos de plantas, como, os óleos essenciais e seus terpenoides (NEHME *et al.*, 2021; ŠTRBAC *et al.*, 2022).

Os óleos essenciais são, comumente, utilizados na indústria de alimentos, cosméticos e fármacos (SHARMEEN *et al.*, 2021). A atividade anti-helmíntica destes óleos se correlaciona aos seus componentes, sendo ação promovida por seus majoritários ou pela combinação de

compostos que estão em menores quantidades (DE ARAÚJO-FILHO *et al.*, 2018). Os óleos são compostos, em sua maioria, por monoterpenos (C10) e sesquiterpenos (C15), conhecidos como, terpenos ou terpenoides. Os terpenos têm diversas propriedades descritas, sendo elas, acaricidas, repelentes e anti-helmínticas (PENHA *et al.*, 2021; TABARI *et al.*, 2017). Assim, este trabalho avaliou a atividade da associação óleo essencial de *Citrus aurantium* var Dulcis e albendazol, sobre parasitos de pequenos ruminantes, além de reunir informações disponíveis na literatura a cerca da ação dos terpenoides.

2. REFERENCIAL TEÓRICO

2.1. Ovinocultura

A ovinocultura é uma atividade praticada em todos os continentes do mundo. O Brasil possui um rebanho superior a 20 milhões de cabeças. A região nordeste do país detém 70% do rebanho, seguida pela região sul, com, aproximadamente, 3 milhões de animais (MAGALHÃES; HOLANDA-FILHO; MARTINS, 2021). O maior rebanho coincide com as áreas de semiárido, devido o nordeste brasileiro possuir dificuldades em produzir proteína animal por meio da criação de outras espécies, como, os bovinos e a facilidade que esses rebanhos têm em se adaptar às condições adversas (CARVALHO *et al.*, 2020).

No Nordeste, a ovinocultura foi dada como uma atividade de subsistência, com influência direta na economia local. A produção comercial está concentrada, no leite, no couro e, principalmente, na carne (SILVA *et al.*, 2020). Estes animais tem a habilidade de transformar o material fibroso, de baixo valor nutricional, encontrado para alimentação, em carne, de alto valor proteico (CARVALHO *et al.*, 2020; MAGALHÃES; HOLANDA-FILHO; MARTINS, 2021). É possível destacar outros subprodutos dessa cultura de acordo com a raça produzida, por exemplo: o couro, com as raças santa inês e morada nova; a lã, com as raças Texel e Suffolk; e o leite com a raça lacaune (COSTA *et al.*, 2019; MELO *et al.*, 2020; MONTEIRO; BRISOLA; FILHO, 2021).

Um fator importante para o desenvolvimento desta cultura é a estrutura de criação. Contudo, a ovinocultura pode apresentar variações dependendo da região observada (JESUS-JUNIOR; RODRIGUES; DE MORAES, 2010). A região ao sul do Brasil possui boas instalações e equipamentos, resultando em uma linha de produção organizada. Enquanto isso, na região nordeste, existem pequenos rebanhos mantidos no sistema extensivo, abatedouros

informais e sem primazia no controle sanitário (MONTEIRO; BRISOLA; FILHO, 2021).

O Brasil possui grande potencial para geração de renda para ovinocultores. Contudo, é necessário que se façam ajustes na produção, como as condições higiênicas de manejo, a implementação de abatedouros legalizados, a tecnificação do produto final, dentre outros. Os maiores investimentos devem ser na cadeia da produtiva nordestina, com melhorias no manejo de criação, nutrição e melhoramento genético do rebanho (MAGALHAES *et al.*, 2020; NASCIMENTO *et al.*, 2022). Também, é necessário reforçar e destacar as ações de programas e projetos, como o Dom Helder Câmara II (Governo Federal); Projeto de Desenvolvimento Sustentável do Cariri, Seridó e Curimataú (Procase), na Paraíba; Viva o Semiárido, no Piauí; Dom Távora, em Sergipe; Paulo Freire, no Ceará; e Pró-Semiárido, na Bahia (MONTEIRO; BRISOLA; FILHO, 2021).

2.2. Anti-helmínticos e resistência

Os anti-helmínticos sintéticos são os medicamentos mais utilizados no controle de parasitos gastrointestinais (CALVETE *et al.*, 2020). As classes mais utilizadas são: benzimidazóis (BZs), imidazotiazóis e lactonas macrocíclicas (LMs), contendo alvos específicos no parasito (HERATH *et al.*, 2021). BZs exercem seus efeitos bloqueando a polimerização de microtúbulos celulares por ligação seletiva a β-tubulina do nematódeo (SON; LEE; ADUNYAH, 2020). Microtúbulos celulares são formados pela polimerização de monômeros α e β-tubulinas em um processo dinâmico. Com a inibição deste processo, as funções celulares como, divisão celular, estrutura citoesquelética, movimento intracelular de partículas, ficam totalmente desordenadas, levando à morte da célula (BARRÓN-BRAVO *et al.*, 2020).

O grupo das LMs é representado pelas avermectinas e milbemicinas. Estas agem por meio da interrupção da transmissão neuronal via potenciação dos canais de cloro mediados por glutamato (MORENO; GEARY; TRITTEN, 2021). As LMs, como a ivermectina, causam paralisia flácida, inibição de bombeamento faríngeo levando à morte por inanição, e, consequentemente, a expulsão do parasito do trato gastrointestinal (MARTIN; ROBERTSON; CHOUDHARY, 2021).

O uso frequente e aplicações irregulares de compostos anti-helmínticos, selecionaram indivíduos resistentes (POTÂRNICHE *et al.*, 2021). Os mecanismos de resistência-helmíntica incluem a regulação positiva dos mecanismos de efluxo celular, uma redução na disponibilidade do composto no interior das células ou uma mudança nos sítios de ligação ao fármaco

(GIGLIOTI *et al.*, 2022). A resistência aos BZs está associada aos polimorfismos de nucleotídeo único (*SNP*) localizados no gene codificante para o isotipo 1 da β-tubulina (DILKS *et al.*, 2021). Estes polimorfismos (F200Y, F167Y e E198A) impedem a ligação dos BZ por meio de modificações estruturais na proteína (ALI *et al.*, 2019).

Para a ivermectina, o problema é mais complexo. Acredita-se que possa estar associada a polimorfismos em alelos das subunidades glc-5 e lgc-37 dos canais de cloro-glutamato e cloro-GABA, respectivamente (KOTZE *et al.*, 2014). Contudo, também, é possível correlacionar a resistência as glicoproteínas-P. Estas são proteínas transmembranares pertencentes à família dos transportadores ABC, um extenso grupo de proteínas de efluxo responsáveis pela desintoxicação celular (GODOY *et al.*, 2015).

2.3. Óleos essenciais

Os óleos essenciais (OEs) se caracterizam por ser líquidos oleosos extraídos de várias partes das plantas (folhas, sementes, madeiras, cascas, raízes, flores, frutos e rizomas) (AUMEERUDDY-ELALFI *et al.*, 2018). Os principais constituintes dos OEs podem ser categorizados de acordo com o número de hidrocarbonetos, sendo eles: monoterpenos, diterpenos, triterpenos e sesquiterpenos (NIETO, 2017). E se destacam por apresentarem diferentes tipos de atividades biológicas incluindo antibacteriana, antifúngica, anticancerígena e anti-helmíntica (ÁLVAREZ-MARTÍNEZ *et al.*, 2021; SHARMA *et al.*, 2022). Ademais, é possível observar os efeitos dos medicamentos sintéticos quando em associação com OEs (ABIRI *et al.*, 2022).

Essa estratégia visa uma interação entre os princípios ativos e os diferentes sítios de ligação no alvo, além de melhorar a eficiência dos compostos sintéticos utilizando baixas doses de OE (LANUSSE *et al.*, 2018). Estas doses reduzidas são fundamentais para evitar reações tóxicas, como, quando o óleo de laranja foi administrado em doses de 200, 300 e 600 mg/kg de peso vivo a ovinos infetados por *Haemonchus contortus*, e de imediato, foi possível observar reações adversas. Após sete dias as menores concentrações não tiveram eficiência sobre nematoides gastrointestinais (NGI) (SILVA *et al.*, 2021). Contudo outros óleos, como o de *Eucalyptus citriodora* e *Eucalyptus staigeriana,* foram avaliados sobre NGI e resultaram em 69,5% e 76,5% de eficiência após 15 dias da aplicação (DE ARAÚJO-FILHO *et al.*, 2018; MACEDO *et al.*, 2010).

Estas eficiências podem estar relacionadas ao componente majoritário ou a interação

deste com os demais terpenoides que integram os OE. A interação positiva entre duas ou mais moléculas é considerada sinérgica (SUÁREZ; ALCÁNTARA; SALINAS, 2022). Combinações entre componentes de OEs foram avaliadas sobre a eclosão de ovos de *H. contortus* e interações sinérgicas foram formadas (KATIKI *et al.*, 2017). Assim, acredita-se que haja combinações e efeitos semelhante a estes quando o OE é adicionado a um anti-helmíntico.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o potencial anti-helmíntico dos terpenoides e do óleo essencial de *Citrus aurantium* var Dulcis associado a anti-helmíntico sintético no controle dos parasitos gastrointestinais de pequenos ruminantes, principalmente, *Haemonchus contortus*.

3.2 OBJETIVOS ESPECÍFICOS

- Fazer uma revisão de terpenoides utilizado no controle de nematoides gatrointestinais de pequenos ruminantes;
- Avaliar *in vitro* a eficiência da associação de óleo essencial de *Citrus aurantium* var. Dulcis e albendazol sobre a eclodibilidade de ovos de *H. contortus;*
- Avaliar *in vivo* a eficiência da associação de óleo essencial de *Citrus aurantium* var. Dulcis e albendazol sobre nematoides gastrointestinais de pequenos ruminantes*;*

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5. RESULTADOS

Este trabalho de tese resultou em dois produtos acadêmicos, um artigo de revisão e um artigo técnico. O artigo de revisão, capítulo 1, teve como objetivo identificar os estudos prévios que investigaram a ação anti-helmíntica de terpenoides, sozinhos e em associações, além de seus possíveis mecanismos de ação. O segundo capítulo buscou avaliar a atividade da associação de óleo essencial de *Citrus aurantium* var Dulcis e benzimidazóis sobre ovos de *Haemonchus contortus, in vitro,* e sobre os nematoides gastrointestinais de ovinos, *in vivo*.

CAPÍTULO 1

Use of Terpenoids to Control Helminths in Small Ruminants

Capítulo do livro - Terpenoids: Recent Advances in Extraction, Biochemistry and Biotechnology

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CHAPTER 7

Use of Terpenoids to Control Helminths in Small Ruminants

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Abstract: Gastrointestinal nematodes affect the animal's health and cause economic losses in meat, milk, and wool production. Essential oils and their terpenoids have been shown to effectively control gastrointestinal nematodes and may be an alternative to control gastrointestinal nematodes. The great advantage of terpenoids is the possibility of acting on the parasite in a multidirectional way on the neuromuscular system and body structures of nematodes. The current chapter describes the pharmacological basis of the combination of terpenes and synthetic anthelmintics as an alternative for increasing antiparasitic efficacy. It is necessary to evaluate if these combinations show antagonist, additive or synergic effects at the pharmacokinetic and pharmacodynamic levels. The physicochemical properties, pharmacokinetic features and potential drug- drug interactions at the metabolism or transport level of monoterpenes may be relevant for obtaining effective concentrations against different nematodes. In this context, the prediction of absorption, distribution, metabolism and excretion (ADME) is essential to optimize the anthelmintic action of these compounds. The rapid absorption and elimination of monoterpenes after their oral administration may directly influence the drug concentration level attained at the target parasites and the resultant pharmacological effect. Therefore, investigations on the dose schedule, administration route and type of pharmaceutical formulation are necessary. The integration of *in vitro* assays, *in silico* analysis, and *in vivo* pharmaco-parasitological studies are relevant to corroborate the kinetic/metabolic interactions and the efficacy of bioactive natural products combined with synthetic anthelmintics.

Keywords: Goat, Natural Product, Nematode, Sheep, Small Ruminant, Synthetic Anthelmintics, Terpenes.

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INTRODUCTION

Gastrointestinal nematodes are especially relevant for small ruminant production. These parasites affect the animal's health and cause economic losses in meat, milk, and wool production [1]. Essential oils and their terpenoids have been shown to effectively control gastrointestinal nematodes [2 - 4]. The terpenoids, compounds from plants, are alternatives to control the gastrointestinal nematodes [5]. However, the mechanism of action of these composts is not quite clear yet.

Since 1950, studies have been performed to better understand anthelmintic compounds' mechanism to control human and animal parasites [6]. The anthelmintic action is associated with the interference of the product in the biochemical process of the parasites. This interference may be related to energy production, muscular coordination, microtubule dynamic, and procedures that can take the parasite's death [7]. Thus, the mechanism of action of the anthelmintic may be invalidated, with alterations that happen in nematode strains, as to the development of parasite defense and are known as resistance [8]. The great advantage of terpenoids is the possibility of acting on the parasite in a multidirectional way [6]. Although the use of control strategies must be well elaborated and planned, it has the most significant effect. For this, a broad knowledge of the mechanism of action is required.

Mechanism of Action of the Anthelmintic Compound

Neuromuscular System and Motility Control

Cys-loop receptors are ligand-gated ion channels activated by several neurotransmitters, like acetylcholine, serotonin, glycine, and GABA [9]. The nervous system of nematodes includes an exclusive and diverse family of cys- loop receptors linked in rapid synaptic transmission, fundamental for worm sensory and locomotor functions [10]. The Cys-loop receptors target widely used anthelmintics, such as levamisole, piperazine, and ivermectin [11 - 13]. TheLevamisol-sensitive nicotinic receptors (L-AChR) and GABA (A) (UNC-49) are two target muscular receptors of terpenoids that cause paralyzed effects. Thymol, carvacrol, and eugenol act as inhibitors of L-AChR and UNC-49 receptors from *Caenorhabditis elegans* muscle cells. This result is probably due to the double effects caused by terpenoids on muscle receptors that support antagonistic actions since L-ACHRs are involved in muscle contraction and UNC-49 receptors in muscle relaxation [11].

Terpenoids also act on other different transient receptors. There are 29 nAChR subunits present in *C. elegans*, demonstrating the importance of further studies to explore the selectivity of terpenoids in the nicotinic family [11]. Other terpenoids

like carvone, pulegone and eugenol, were also identified as inhibitors of the nAChRs.

Terpenoids with Action in GABA

γ-Aminobutyric acid (GABA) is a family of receptors widely distributed. Nematodes are responsible for regulating motility, feeding, and reproduction [14]. There are distinct forms of GABA receptors: GABAA and GABAB. GABAA is GABA-gated chloride channels located in post-synaptic membranes, while GABAB is G-protein coupled receptors located both in pre-and post-synaptic membranes [15, 16]. Some monoterpenes, such as thymol, thymoquinone, and borneol, are known as positive modulators for GABAA receptors [17]. Recently, with the use of *C. elegans,* it was identified that thymol and carvacrol might be causing paralyzing effects on the worm, linked to the critical receptors in its locomotion. Know well that the activation of neuronal GABA receptors generally results in hyperpolarization and muscle paralysis [11].

The blocking Ca^{2+} channels and positive allosteric activation of the GABAA receptor were attributed to menthol. Menthol, which is well-known for producing a cooling effect, is a TRPM8 agonist. The GABAB receptor activity was found to inhibit TRPV1 sensitization, and TRPV1 activation triggers GABA release [18]. 1.8-cineole, menthol (both (-)- and (+)-), carvone (both (-)- and (+)-), pulegone, linalyl acetate, linalool, carvacrol, estragole, bisabolol, carvone (both (-)- and (+)
-), terpinene-4-ol, are known to have analgesic properties targeting Na^{+} and TRP channels [19]. TRPV1-4 are temperature-sensitive channels activated by heatstimuli, whereas TRPM8 and TRPA1 are temperature-sensitive channels activated by cold stimuli [17]. The study suggested the role of glutamatergic neurotransmission and transient receptor potential cation channels (TRP channels) in these actions. Also, monoterpenes with chemical similarity, *e.g*., geraniol, limonene, α-phellandrene, and carvone, may similarly have anti-nociceptive action. These compounds may be ligands of the same receptors and have similar effects [20].

The Action of the Terpenoids on Tubulin

Microtubules are involved in the regulation of various cellular functions, such as cell division, cell motility, intracellular trafficking, and maintenance of cell shape [21]. Commercial anthelmintics can interfere with microtubules. Benzimidazoles block the dimerization of the a and b-tubulin, thus inhibiting microtubules formation, mitosis, and resulting in worm mortality [22]. Some terpenoids have microtubules as the target of action. Citral was lethal to *Arabidopsis* seedlings, interfered with cell division, and in microtubules disrupted, without acting on actin filamentous [23].

Structural Alterations

Nematodes cuticle is made up of concentric layers and plays a critical role in performing protective and selective absorption functions [24]. Molecules thatinduce significant morphological changes, such as nodulation along the body, degeneration of the body wall, and intense cytoplasmic vacuolization, are alternatives to new anthelmintics [25].

D-limonene, a lipophilic terpene with strong solvent capability, has already demonstrated efficacy against *H. contortus* infection in gerbils and sheep [26]. On *C. elegans*, *D*-limonene broke the waterproof protective layer of the worm's body surface and had strong penetrating properties so that worms die by suffocation [27].

Mentha pulegium essential oil, which has its main component a monoterpenepulegone, was also associated with the loss of the normal aspect of the parasiteand the occurrence of structural alterations manifested by aggregates bubbles around the body surface [28]. Such damage could be attributed to lipophilic components, which have an excellent affinity for the cell membrane and could induce structural and chemical alterations in the cell membrane [29].

The genus *Eucalyptus* is well known for its essential oils and used in the fragrance and pharmaceutical industries [30]. The *in vitro* effects of *Eucalyptus citriodora* essential oil and its main constituent, citronellal, presented effects, completely inhibiting the motility of *H. contortus* [31]. Through Transmission Electron Microscopy, it was observed that when *H. contortus* was exposed to citronellal, it demonstrated ultrastructural alterations, such as the mitochondrial profile [31]. The loss of homeostasis of the parasites exposed to the treatments can explain motility failure, such as the destruction of the muscular layer after exposure to citronellal [32].

The effect of carvacryl acetate and carvacrol on sheep gastrointestinal nematodes may also be related to changes in the cuticle. Analysis through the Scanning Electron Microscopy shows the wrinkling of the cuticle as the primary change after *in vitro* exposure to these compounds. The cuticular modifications may interfere with the permeability of the cuticle and motility, hindering the maintenance of homeostasis within these parasites [33].

Combination of Synthetic Anthelmintics and Terpenoids

Since the decade of 1960, when thiabendazole was reported, many synthetic anthelmintics have been developed to control nematodes in small ruminants [34]. These synthetic compounds have been a solution for many years, but resistant strains were selected due to overuse. The effect was a lowering efficacy of anthelmintics used are shown to be mainly in small ruminants production around the world [35 - 37].

The synthetics anthelmintic combination is an alternative to control resistant and susceptible parasites. The combination should be performed with different classes of anthelmintics to achieve good efficacy. Also, these combinations could show additive or synergic effects among the compounds [38, 39]. Although combining synthetic compounds increases the efficacy, the perspective is to select resistant strains to these mixtures over time [39]. Therefore, the research about natural products increased yearly, with many studies reporting terpenes as one alternative to control for small ruminant nematodes [2, 40]. The mixture of two or more terpenes demonstrated that more efficient combinations showed additive or synergic effects [3].

The strategy of combining terpenes and synthetic compound were used with commercial antibacterial and antifungal against resistant microorganisms. A combination of citral and norfloxacin and thymol and rifampicin was synergic against *Staphylococcus aureus* [41, 42]. The synergism was also shown in the combination of citronellol and amphotericin against a different strain of *Candida* spp. in the combination of terbinafine and the monoterpenes dihydrojasmone and terpinolene on dermatophytosis [43, 44]. Combining a low concentration of terpenes and synthetic anthelmintic is an alternative for increasing the efficacy of commercial synthetic compounds [38, 45].

The combination of albendazole or levamisole with r-carvone and s-carvone decreases the the hatchability of eggs and larval migration of *H. contortus*, respectively [45].

These authors showed damage to the egg wall resulting in an overflow of internal egg content when tested using albendazole and r-carvone. However, the synergism effect of the combination afore mentioned the antagonism was shown in some combinations [45]. The combination of synthetics, anthelmintics, and terpenes is a promising alternative to control of gastrointestinal nematode of small ruminants. However, pharmacological understanding and *in vivo* studies are necessary.

Influence of Pharmacological Properties of Monoterpenes on their Anthelmintic Effect

Helminth parasites affecting domestic animals are located in several tissues in the body, such as the gastrointestinal tract (GIT), liver, bile ducts and lung parenchyma. Chemical compounds, including bioactive phytochemicals, require effective and sustained concentrations at the sites of parasite location to achieve their anthelmintic effect. In this context, the physicochemical properties and pharmacokinetic disposition of these molecules have a direct influence on their anthelmintic activity [46]. Lipophilicity determines the ability of the compounds to pass through the cell membranes of parasites, a critical step for their accumulation at the target parasites. The higher the amount of a drug gaining access to a cell, the greater the effect, a classic axiom in pharmaco-toxicology. Drug diffusion across parasite tegument is crucial to reach the specific receptor and to produce its effect. Monoterpenes are non-polar and lipophilic compounds that easily penetrate membranes [47].

There is scarce information on the pharmacokinetics of these phytochemicals in ruminants. Most of the published information on this issue comes from studies on humans and rats. In general, after oral administration, monoterpenes are rapidly absorbed and highly metabolized. The fast absorption of monoterpenes might be favoured by the small size and the lipophilic characteristics of these molecules [48]. Metabolites of monoterpenes resulted from both liver phase I and phase II metabolism. Oxydized metabolites of thymol and carvacrol were found in the systemic circulation after the oral administration of these monoterpenes to rats. Phase-II metabolites, *i.e*., glucuronides or sulfates, were also detected in rats, rabbits, and humans [49, 50]. Due to their high clearance and short elimination half-lives, the accumulation of monoterpenes in body tissues seems to be unlikely [51]. Overall, the rapid absorption and elimination of monoterpenes after their oral administration may directly influence the drug concentration level attained at the target parasites and the resultant pharmacological effect. Therefore, investigations on the dose schedule, administration route and type of pharmaceutical formulation are necessary for the improvement of the anthelminticefficacy of these phytochemical compounds.

The complex anatomy-physiology of ruminant's gastrointestinal tract resides in the evolutionary development of a series of three chambers, the rumen, reticulum, and omasum, anterior to the true stomach, the abomasum. Frequently, this accounts for the often-dramatic differences observed between ruminants and monogastric in the oral absorption of drugs [52]. Although the liver plays a pivotal role in the metabolism of foreign compounds, biotransformation may also occur in the gastrointestinal tract, particularly in the rumen, due to the fermentative environment. In comparison with the liver, where oxidative metabolism predominates, the ruminal microflora is very active in reductive reactions of xenobiotics [53]. Ruminal metabolism may play a role in reducing thesystemic availability of orally administered compounds [54], thus being particularly important in the therapeutic outcome. In this context, bioactive natural products administered by the oral route should be stable in the ruminal environment to allow the active molecules to be in contact with the target gastrointestinal nematodes.

The chemical stability of carvacrol and thymol was evaluated in sheep ruminal content *in vitro*. Both monoterpenes were stable in the ruminal environment; the concentrations of unchanged (unmetabolized) carvacrol and thymol recovered after the incubation of both compounds were between 84-91% and 90-95%, respectively [55]. The lack of metabolic degradation of these phytochemicals was similar to that observed for synthetic anthelmintics such as monepantel [56, 57]. As carvacrol and thymol were found metabolically stable in the rumen, the oral administration of both compounds may assure their antiparasitic efficacy against GI nematodes. Among the different factors affecting the systemic availability of orally administered compounds in ruminants, the degree of adsorption to the particulate material of gastrointestinal contents plays a relevant role in their kinetic disposition. Thymol and carvacrol showed lower concentrations associatedwith the particulate material compared to those dissolved in the fluid phase of the ruminal content. The percentage of association to the particulate phase was higherfor thymol (43-49%) than that observed for carvacrol (31-34%). The fact that most of the monoterpenes are kept in the fluid phase of ruminal content may imply a shorter residence time of these compounds in the rumen and a faster flow rate to the abomasum and small intestine [55].

The concurrent use of natural compounds and synthetic drugs for the control of nematode parasites may give rise to pharmacokinetic interactions that should be carefully evaluated. Exposure to plant-derived products may cause the inductionor the inhibition of drug-metabolizing enzymes and/or transport proteins involved in the elimination of anthelmintic molecules. Certain anthelmintic drugs, such as albendazole and fenbendazole, are metabolized by different drug metabolizing enzymes from both hepatic and extrahepatic tissues. Metabolic conversions usually alter the polarity of the anthelmintic parent molecule and, consequently, the way in which the drug is distributed and excreted from the body. *In vivo* interference with the activity of certain drug metabolizing enzymes may give rise to pronounced modifications to both the pharmacokinetic behavior and the therapeutic outcome of active anthelmintic molecules. It has been shown that the flavin-monooxygenase (FMO) pathway is involved in roughly 65% of albendazole S-oxidation in sheep liver, whereas the rest of the production of the albendazole S-oxidized metabolite (*i.e.*, albendazole sulfoxide) depends on the cytochrome P450 (CYP) dependent metabolism [58]. In the presence of thymol, the metabolism of albendazole (ABZ) in sheep liver was decreased, particularly marked for its FMO-dependent production (54%). In contrast, the CYP-dependentproduction of the metabolite was less affected (25%) [59]. Sheep liver FMO and CYP1A1 enzyme activities were also significantly inhibited by thymol *in vitro* [59]. Overall, these *in vitro* investigations suggested that, in addition to its own anthelmintic effect, monoterpenes may potentiate ABZ anthelmintic activity by preventing its metabolic conversion into a less active metabolite. These

observations are in agreement with other studies on the *in vitro* inhibition of the hepatic CYP-dependent metabolism [60]. For instance, carveol decreased the catalytic activities of CYP2B and CYP2C in rats [61]. Inhibition of CYP1A1- andCYP1B1-dependent enzyme activities by eugenol was also observed in rats, whereas geraniol inhibited the catalytic activity of CYP2B6 in human liver microsomes [62, 63]. In order to confirm the clinical relevance of these interactions, further *in vivo* studies in ruminant species are necessary.

Efflux membrane transporters are energy-dependent protein complexes responsible for removing several compounds, including endogenous molecules and xenobiotics, from cells through active transport. They contribute to the preservation of cell homeostasis by removing potentially toxic compounds from the intracellular space; however, they may also limit the penetration of certain compounds. Therefore, the activity of these transport proteins may affect the bioavailability and elimination of numerous drugs and other xenobiotics in animals and humans. *P*-glycoprotein (*P*-gp) is one of the best-known efflux membrane transporters [64]. Modulation of *P*-gp accounts for the enhancement of the systemic exposure of antiparasitic drugs in ruminants. Besides, transport- related drug-drug interactions in parasite location tissues may contribute to enhance drug accumulation and efficacy against resistant worms [65].

Several monoterpenes were found to inhibit *P*-gp or to down-regulate its gene/protein expression. For instance, menthol led to a higher accumulation of rhodamine 123, a *P*-glycoprotein-specific substrate, in Caco-2 cells in a dose- dependent manner [66]. Citronellal caused down-regulation of *P*-gp mediated digoxin transport. However, these authors did not find a relationship between the monoterpenes' molecular structure and their inhibitory potential [67]. Among terpenoids, diterpenes have shown greater *P*-gp modulation properties than monoterpenes [68]. Recently, the presence of carvone significantly increased Rho123 accumulation in cattle intestinal explants. The presence of carvone also increased the intestinal concentration of the synthetic anthelmintic abamectin, a macrocyclic lactone [69]. Molecular docking studies may help to understand the interaction between monoterpenes and *P*-gp. Whereas abamectin showed specific binding to this transport protein *in silico*, this fact was not observed for carvone [69]. Therefore, the potential interaction of carvone with *P*-gp seemed to be unlikely. Thus, the influence of carvone on drug absorption and accumulation may be explained by other mechanisms. Different bioactive phytochemicals may increase intestinal absorption by enhancing enterocyte membrane permeability or by opening paracellular tight junctions [70].

The prediction of absorption, distribution, metabolism and excretion (ADME) helps to reduce the number of animal experiments, contributing to the so-called

3Rs principle (replacement, reduction and refinement). These methods use statistical and learning approaches, molecular descriptors and experimental data [71]. Table 1 shows ADME prediction for different monoterpenes. The enzymes CYP2C9 and CYP2C19 are well-known in the metabolism of carvone to carveol. However, it is not known if these enzymes are either induced or inhibited [72]. In ADME prediction, the carveol and carvone have shown inhibition activity on CYP2C9 and CYP 2C19 and were substrates for CYP 3A4. *In silico* analysis corroborated that carveol and carvone have a moderate capacity for binding to plasma proteins, dismissed a possible plasma transport mechanism [71]. The prediction also shows that geraniol and limonene will have a wide distribution across the blood-brain barrier (BBB). The molecules that pass-through BBB produce a quick, coordinated and effectively response [73]. Carvone's skin permeability is more effective than other compounds. This is an essential parameter for the transdermal delivery of drugs [74]. The ADME predictions showed that citronellal and citral would behave as *P*-gp inhibitors. Caco-2 and MDCK prediction are useful tools for measuring the absorption of the molecules [74]. Caco-2 permeability is more effective for nerol when compared to other compounds. Eugenol has the worst intestinal absorption (HIA) ~ 96.8% but has shown higher effective permeability across MDCK cells.

Table 1. Predicted ADME properties of compounds terpenoids.

ID	Carveol	Carvone	Citral	Citronellal	Eugenol	Geraniol	Limonene	Menthol	Nerol	*p*-Cimene
BBB	5.079	1.060	2.008	1.732	2.255	6.741	8.278	6.255	6.741	4.969
Caco-2	37.936	47.742	13.967	13.725	46.886	8.756	23.631	39.490	87.568	23.433
CYP 2C19 inhibition	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
CYP 2C9 inhibition	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
CYP 2D6 inhibition	No	No	No	No	No	No	No	No	No	No
CYP 2D6 substrate	No	No	No	No	Weakly	No	No	No	No	No
CYP 3A4 inhibition	No	No	No	No	No	No	No	Inhibitor	No	Inhibitor
CYP 3A4 substrate	Substrate	Substrate	Substrate	Weakly	No	Substrate	Substrate	Non	Substrate	Weakly
HIA	100.000	100.000	100.000	100.000	96.774	100.000	100.000	100.000	100.000	100.000
MDCK	97.813	97.545	252.255	265.594	342.148	271.032	238.434	96.054	271.032	23.750

(Table 1) cont.....

ID	Carveol	Carvone	Citral	Citronellal	Eugenol	Geraniol	Limonene	Menthol	Nerol	*p*-Cimene
PGP inhibition	No	No	Inhibitor	Inhibitor	No	No	Inhibitor	No	No	No
PPB	57.945	58.045	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000
Skin Permeability	-1.401	-1.403	-0.961	-1.019	-1.310	-1.059	-0.834	-1.607	-1.059	-0.805

BBB: blood–brain barrier (C.brain/C.blood); Caco-2: Caco2-cell model; CYP: cytochrome P450; HIA: human intestinal absorption model (HIA, %); MDCK: Madin–Darby canine kidney (nm/s); PGP inhibition: Inhibitor *P*-glycoprotein; PPB: plasma protein binding (%); Skin permeability: skin permeability (cm/h).

The physicochemical properties, pharmacokinetic features and potential drug- drug interactions at the metabolism or transport level of monoterpenes may be relevant for obtaining effective concentrations against different nematodes. In this context, the prediction of ADME is essential to optimize the anthelmintic use of these phytochemicals in ruminants.

In Vivo Anthelmintic Effect of Monoterpenes

Abomasal and intestinal nematodes are among the most pathogenic gastrointestinal parasites in sheep, cattle, and goats. An efficient deworming program is essential for obtaining rational control and sustained productivity. However, after years of intensive use, resistance to synthetic drugs has been spread worldwide [75]. There is an urgent need to find novel pharmacological tools in order to ensure efficient control of gastrointestinal nematodes. A wide variety of naturally sourced terpenes were shown to possess anthelmintic activity *in vitro* [45, 76, 77], but it is necessary to demonstrate whether these effects canbe observed *in vivo*. Although *in vitro* assays supply useful information, it is necessary to know the *in vivo* fate of terpenes after their administration [59]. This issue is crucial, as active compounds need to attain effective concentrations for some time at the sites of parasite location [78].

The anthelmintic activity of thymol was observed *in vitro*, particularly against *H. contortus* eggs, larvae and adult parasites [2, 3, 40, 45]. To evaluate whether effective concentrations of thymol can be obtained after the oral administration to sheep, the kinetic disposition of this monoterpene was evaluated in lambs. Thymol was chemically stable in metabolically active ruminal content of lambs and can be detected in the bloodstream after the oral administration of two high doses (150 mg/kg). Absorption of thymol after its oral administration was markedly fast in lambs, with a peak plasma concentration achieved between 1- and 2-hours post-administration. The highest thymol concentration detected in

plasma was 1.9 µg/mL [59]. However, *in vitro* tests on *Haemonchus* spp. eggs and larvae have corroborated that thymol concentrations necessary to obtain an efficacy above 90% were between 4 and 6 mg/mL [2, 3, 40]. Similarly, the concentrations necessary to affect the motility of adults of *Haemonchus* spp. were above 400 µg/mL [40]. It is evident that kinetics disposition features of thymol in sheep do not ensure a high exposure of parasites to the monoterpene as the measured concentrations *in vivo* were several times below the minimal effective concentrations. In agreement with these observations, administering one dose of 300 mg/kg of thymol was ineffective in reducing the egg count in faeces of infected sheep [2]. A lack of reduction of faecal egg counts was also observed after the administration of two oral doses of 150 mg/kg of this monoterpene to infected sheep [59]. On the contrary, André *et al*. [40] obtained an efficacy of 59% after the administration of thymol at 250 mg/kg to naturally infected sheep. Altogether these *in vivo* tests of efficacy suggest a low *in vivo* exposure ofparasites after the oral administration of thymol, leading to zero or low efficacy. Further research on its potential anthelmintic use may include the study of pharmaceutical strategies for providing sustained and effective concentrations. For instance, the encapsulation technique appears as a strategy to improve solubility, stability and the pharmacological response of thymol and other monoterpenes.

A semisynthetic derivative of carvacrol, carvacryl acetate, was synthesized in an attempt to reduce collateral effects. In fact, carvacryl acetate was shown to be safe; in an acute toxicity trial in mice, the LD50 of carvacryl acetate was 1544 (mg/kg), whereas for carvacrol was 919 mg/kg [33]. Further, carvacryl acetate andcarvacrol showed similar *in vitro* activity in a larval development test; the former compound showed a higher effect on the motility of adult specimens of *H. contortus*. After 24 h of exposure at 200 mg/mL, carvacryl acetate inhibited wormmotility by 100%, while the inhibition of carvacrol was 41.8%. In addition, both carvacrol and carvacryl acetate displayed morphological alterations in the cuticle and the vulvar flap of adult specimens of treated *H. contortus*, suggesting that these compounds have the same mechanism of action [79]. The *in vivo* trial addressed to evaluate the efficacy of carvacryl acetate showed a reduction faecal egg counts of 66% after its oral administration to infected sheep at 250 mg/kg. A similar improved *in vitro* and *in vivo* performance was shown after the acetylation of thymol [40]. Clearly, the aforementioned investigations show that chemical- pharmaceutical modifications are relevant to increase the exposure and effectiveness of bioactive phytochemicals.

Carvone is another terpene whose anthelmintic activity was tested *in vitro* and *in vivo.* The *in vitro* activity of R-carvone has been shown, particularly against *H. contortus*. The 99% lethal concentration of carvone that could inhibit hatching of

H. contortus was 366 µg/mL, and this effect was increased after the combination with different phytochemicals [3]. Therefore, carvone showed a higher potency compared to other monoterpenes such as carvacrol and thymol. Taking advantage of these properties, the evaluation of the long-term administration of encapsulated carvone plus anethole was recently evaluated in sheep [80]. Two oral doses (20 mg/kg and 50 mg/kg) of the bioactive phytochemical combination were administered to sheep for 45 days. A marked reduction of faecal egg counts was the main effect observed after the administration of the highest dose rate; total adult worm counts remained similar compared to the control group. Although further studies are necessary in order to find the ideal dose of carvone and the appropriate pharmaceutical formulation, the reduction faecal egg counts when animals are highly infected with gastrointestinal nematodes, can lead to a reduction in the infection of pasture. This fact may help to improve worm control programs.

Considering the *in vitro* and *in vivo* low pharmacological potency shown by terpenes as anthelmintic compounds, the combination with synthetic drugs may bean interesting approach to increase the clinical efficacy of phytochemicals. The rationale behind the use of combinations is the lower probability of parasites to beresistant to multiple active compounds compared with that observed after the administration of a formulation with a single active molecule [38, 82]. Despite thediverse chemical groups available to control parasitic diseases in ruminants, macrocyclic lactones (MLs) and benzimidazoles (BZD) have been the most widely used drugs. Considering the high level of resistance to both families [82] there is an urgent need to search for novel strategies to extend the life span of antiparasitic agents. The administration of different anthelmintic compounds in combination may lead to unpredictable pharmacokinetic (PK) and/or pharmacodynamic (PD) drug-drug interactions (DDI). PK interactions occurringat the absorption and metabolism/transport/excretion processes may affect the anthelmintic response [38]. PD interactions may take place when one drug may alter the intensity of the pharmacological effects of another drug given concurrently, resulting in additive, synergistic or antagonistic pharmacological effects [81, 83].

There is scarce information about the combinations involving terpenes and synthetic anthelmintics. Albendazole showed a pharmacodynamic synergism in combination with carvone after *in vitro* evaluation on eggs hatch assay [45]. As this synthetic anthelmintic is extensively metabolized in the liver by mixed function oxidases, an *in vitro* metabolic interaction was corroborated between albendazole and thymol [55]. In fact, thymol inhibited the hepatic sulphoxidation and sulphonation of the synthetic compound [55, 59]. Based on these *in vitro* findings, it was initially suggested that, in addition to its own anthelmintic effect,

thymol might improve albendazole anthelmintic activity by preventing its metabolic conversion into a less active metabolite [55]. In a further *in vivo* investigation, the pharmacokinetic and the efficacy of the combination of albendazole and thymol were evaluated in lambs naturally infected with resistant gastrointestinal nematodes [59].

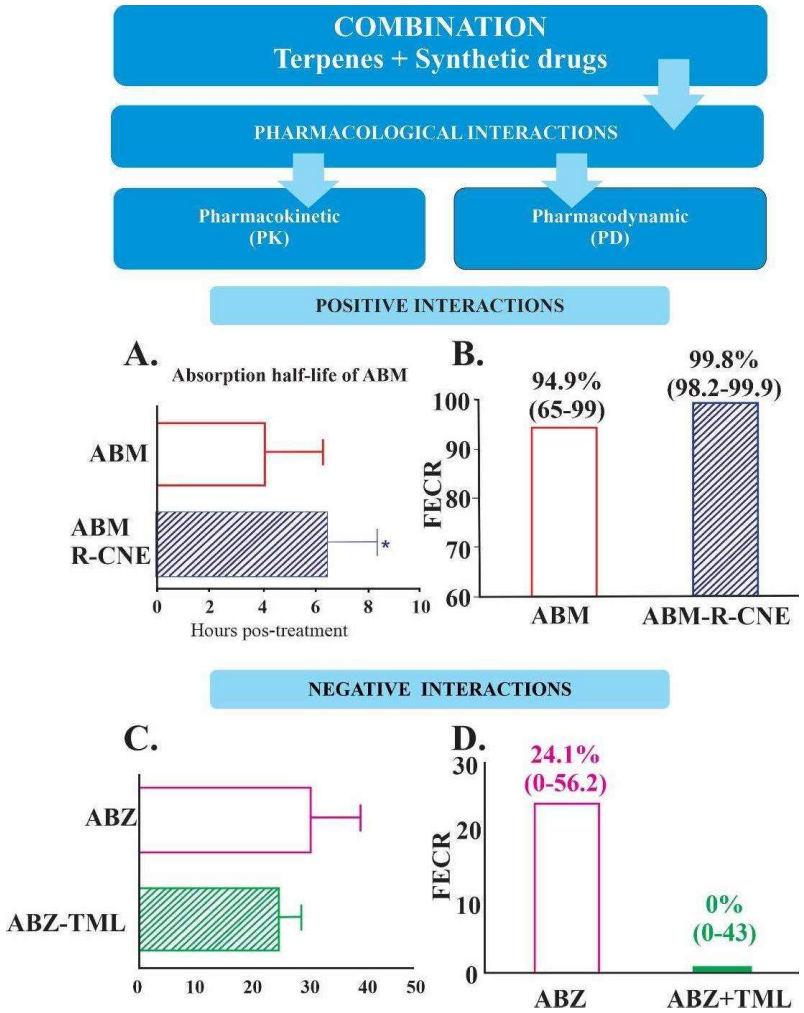

Fig. (1). Drug-drug interactions at pharmacokinetic (PK) and pharmacodynamics (PD) level after the combined administrations of terpenes and synthetic compounds (A) Positive PK interaction. The co- admiration of abamectin (AMB) and carvone (CNE) prolonged the absorption hael0life of ABM by 57% in lambs (Miró *et al*., 2020c). (b) positive PD interaction. The *in vivo* efficacy of AMB against resistant gastrointestinal nematodes increased from 94.9 to 99.8% in the presence of CNE (Miró *et al*., 2020c). (C) negative PK interaction. Albendazole suphoxide (ABZSO) systemic exposure measured as the area under concentration *vs.* time curve were lower after the combined treatment with albendazole and thymol (ABZ+TML) (Miró *et al*., 2020b). (D) Negative PD interaction. The presence of TML decreases the *in vivo* efficacy of ABZ from 24.1% to 0% (Miró *et al*., 2020b).

However, although the hepatic metabolism of albendazole was inhibited by thymol, the combined *in vivo* administration of the monoterpene and albendazole did not improve the kinetic plasma disposition of albendazole metabolites. Additional *in vitro* studies showed that thymol also inhibited the ruminal sulphoreduction of albendazole sulphoxide into albendazole, a metabolic reaction that renders a more active anthelmintic product. This metabolic step is considered of vital importance for the antiparasitic efficacy of benzimidazole thioethers. Therefore, the combined treatment of parasitized sheep with thymol and albendazole led to a negative pharmacokinetic interaction. In addition, the anthelmintic treatment of thymol with the synthetic drug caused a decline in the faecal egg count reduction test from 24% when albendazole was administrated alone to 0% when it was administrated in combination with the monoterpene [59].

Phytochemicals may also modulate the absorption and excretion processes of synthetic anthelmintics. The combination of carvone and the macrocyclic lactone abamectin was evaluated to test the potential modulation of the *P*-glycoprotein- dependent excretion of the synthetic drug. *In vitro* assays with intestinal explants showed that carvone improves the tissue accumulation of abamectin. Moreover, a prolonged absorption plasma half-life of the macrocyclic lactone was observed in naturally infected sheep receiving a combination of abamectin and carvone. An increased antiparasitic efficacy, from 94.9 (65-99%) to 99.8% (98-99.9%), was observed after the combined treatment [69]. However, a lack of interaction of carvone with the transport protein *P*-glycoprotein was revealed *in silico* [69]. Therefore, the beneficial effect of carvone on the pharmacokinetics of abamectin was attributed to the effect of the natural product on the enterocyte membrane permeability [70]. The potential drug-drug interactions at the pharmacokinetic andpharmacodynamic level after the combined administration of terpenes and synthetic anthelmintic compounds are shown in Fig. (1).

CONCLUDING REMARKS

Altogether, these observations clearly remark that the integration of *in vitro* assays, *in silico* analysis, and *in vivo* pharmaco-parasitological studies are relevantto corroborate the kinetic/metabolic interactions and the efficacy of bioactive natural products combined with synthetic anthelmintic. The development of pharmacology-based information is critical for the design of successful strategies for parasite control using pharmaceutical formulations of bioactive phytochemicals that ensure a high efficacy with low toxicity.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

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CAPÍTULO 2

Evaluation of a combination of *Citrus aurantium* var. Dulcis essential oil and albendazole for the treatment of sheep gastrointestinal nematodes

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Evaluation of a combination of *Citrus aurantium* var. Dulcis essential oil and albendazole for the treatment of sheep gastrointestinal nematodes

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ABSTRACT

Citrus fruits are consumed all over the world and their by-products are used for animal feed and essential oils production. This study aimed to evaluate the in vitro and in vivo activity of *Citrus aurantium* var. Dulcis essentialoil (CaEO) combined with ABZ against benzimidazole resistant *Haemonchus contortus*. *In vitro* egg hatching assays (EHA) were performed using CaEO and ABZ to estimate the effective concentration to achieve 50% egg death (EC_{50}) values and calculate the test essential oil and drug combinations using a simplex-centroid mixture design. These concentrations were used for a second round of EHAs. Sixteen sheep were randomly allocated into two groups and treated with ABZ and the combination of CaEO and ABZ, and faecal egg count reduction tests were performed. In the first round of EHA, CaEO and ABZ showed EC_{50} values of 0.57 and 0.0048 mg mL^{-1}, respectively. The *H. contortus* strain used in the study was shown to be highly benzimidazole resistant, with only 1.5% of parasites having susceptible ß-tubulin SNP genotypes. The ABZ reduced the shedding of nematode eggsby 78%, however, its combination with CaEO reduced faecal egg counts by only 9%. The present study is important to highlight the interferences of natural products in anthelmintic metabolism and consequently in drug efficacy.

1. Introduction

The control of gastrointestinal nematodes is mainly based on the frequent use of drugs, such as those belonging to the benzimidazole family. The overuse of these drugs has selected resistant populations and reduced its efficacy (Garbin et al., 2021). A population of benzimidazole-resistant parasites can be diagnosed by identifying one of three single nucleotide polymorphisms (SNP) in the isotype 1 gene of ß-tubulin (Barrère et al., 2013; Santos et al., 2014).

Albendazole (ABZ) is one of the most widely-used compounds in the benzimidazole group which has been used in human and veterinary medicine since 1960 (Lanusse et al., 2018). ABZ is metabolised to albendazole sulphoxide (ABZSO) and then albendazole sulphone (ABZSO2). The ABZ metabolites are more hydrosoluble than the parent drug which influences tissue distribution, elimination, and transcuticular accumulation in nematodes (Lanusse et al., 1993; Miró et al., 2020). Ruminal microorganisms have high reduction potential, hence are responsible directly for the transformation of ABZSO back toABZ. As ABZ has a higher affinity for the β-tubulin compared to its metabolites, the sulphoreduction activity by the ruminal microfloraplays a pivotal role in the availability and efficacy of orally or paren- terally administered benzimidazole drugs (Lanusse et al., 1992; Capeceet al., 2001; Cristofol et al., 2001).

Citrus fruits are highly consumed worldwide (Rafiq et al., 2018), with production estimated at 1.4 million tons for 2021/22 (USDA, 2020). This high consumption generates by-products that are used for animal nutrition and essential oil (EO) production (Guzmán et al., 2021; Luciano et al., 2017). The dehydrated citrus pulp and orange EO have been used in ruminant animal nutrition. These products have also been proposed as an alternatives to anthelmintic drugs for parasite control,

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reducing egg hatching in vitro and faecal egg count in vivo (Nordi et al., 2014; da Silva et al., 2021a). They have also been used to reduce methane emissions produced by the rumination process (Kamalak et al.,2011). EO changes the ruminal microbiota community, decreasing protozoa and bacterial activity (Cobellis et al., 2016; Miró et al., 2020; Zhou et al., 2019); and may, therefore, delay the ruminal biotransfor- mation of ABZSO to ABZ.

The combination of essential oil *Citrus aurantium* var. Dulcis (CaEO)and ABZ may give rise to modifications of the pharmacokinetics and therapeutic efficacy of benzimidazoles. The present study aimed to evaluate the effects of CaEO on the anthelmintic efficacy of ABZ against benzimidazole resistant *Haemonchus contortus* using in vitro and in vivo approaches.

2. Materials and methods

This project was approved by the Ethics Committee on Animal Experimentation of the Federal University of Maranhão (UFMA), Brazil, with protocol number 23115.005443/2017-51.

2.1. In vitro assay

2.1.1. Haemonchus contortus strain

Two crossbreed Santa Inês lambs were orally infected with a dose containing 2000 third-stage larvae (L_3) of *H. contortus*, maintained at the Laboratory of Parasites Control from UFMA. Previous in vitro egg hatch assay (EHA) tests had confirmed resistance to benzimidazole drugs. Artificial infection was verified by faecal egg counts (FECs) and coprocultures using previously described methods (Roberts and O' Sullivan, 1950; Ueno and Gonçalves, 1988).

2.1.2. Egg hatchability assay (EHA)

Fresh faeces were collected from the *H. contortus* donor lambs and processed in 1 mm, 105 μm, 55 μm and 25 μm graduated sieves. The eggs were suspended in saturated saline solution and washed with distilled water. The EHA was performed in 96-well sterile plates according with the method described by Coles et al. (1992). CaEO (Ferquima; Vargem Grande, São Paulo, Brazil) and ABZ (Sigma-Aldrich; São Paulo, São Paulo, Brazil) were used. The tests used a stock solution of ABZ at a concentration of 10 mg mL^{-1} in 100% dimethyl sulfoxide (DMSO). The CaEO was diluted in 3% tween-80 using distilled water. Concentrations of DMSO (1%) and Tween-80 (0.0015%) were included as a negative control.

In the first round of EHA, aliquots (100 μL) with approximately 100fresh eggs in distilled water were incubated with 100 μL of compoundswith final concentrations ranging from 5.00 to 0.15 mg mL^{-1} for CaEOand 0.02500 – 0.00078 mg mL^{-1} for ABZ. All tests were performed in quadruplicate. The plates containing different concentrations of the ABZ, CaEO, and negative controls were incubated for 48 h at 27 °C (Jackson and Hoste, 2010). Afterward, eggs and first-stage larvae (L1) were counted under a stereomicroscope at 40X magnification (Carl Zeiss, Oberkochen, Germany). The data were initially transformed into a log (X), normalised, and then nonlinear regression was calculated to esti- mate the effective concentrations of ABZ and CaEO for 50% egg hatching (EC50) using GraphPad Prism 8.0.2 software (GraphPad Inc.,San Diego, USA). The significance of each comparison was confirmed when the calculated confidence intervals did not overlap (Roditakiset al., 2005).

2.1.3. Combination design

A simplex-centroid design was used to evaluate the EHA efficacy of the tested compounds in combination (Ouedrhiri et al., 2022). The maximum and minimum effective concentrations of the ABZ and CaEOin the EHA were used to calculate the combinations that should be tested (Fig. 1). Statistica 7.0 software (Statsoft, USA) was used to calculate nine combinations of three centre point replicates. The results of the tests were used to calculate the efficiency and plot in the level curve graph.

2.2. In vivo assay

2.2.1. Genotype characterisation

Faeces were collected from thirteen ewes and eleven lambs to characterise the *H. contortus* population present on the study farm. Faeces were pooled into two groups (ewes and lambs) for L_3 culture andrecovery using the method described by Ueno and Gonçalves (1988).

The L_3 were fixed in 100% ethanol and stored at 4◦C until further genomic DNA extraction.

DNA extraction followed the methodology described by Costa-Junioret al. (2021), using pools of approximately 2000 L_3. The L_3 were centrifuged for two minutes at 7200 X g and the supernatant was discarded. The pellet was re-suspended in 40 μL lysis buffer and 10 mg mL^{-1} Proteinase K (New England BioLabs). The lysates were usedas templates for subsequent PCRs. Illumina Mi-seq deep amplicon sequencing of the isotype-1 β -tubulin locus was used for verifying the

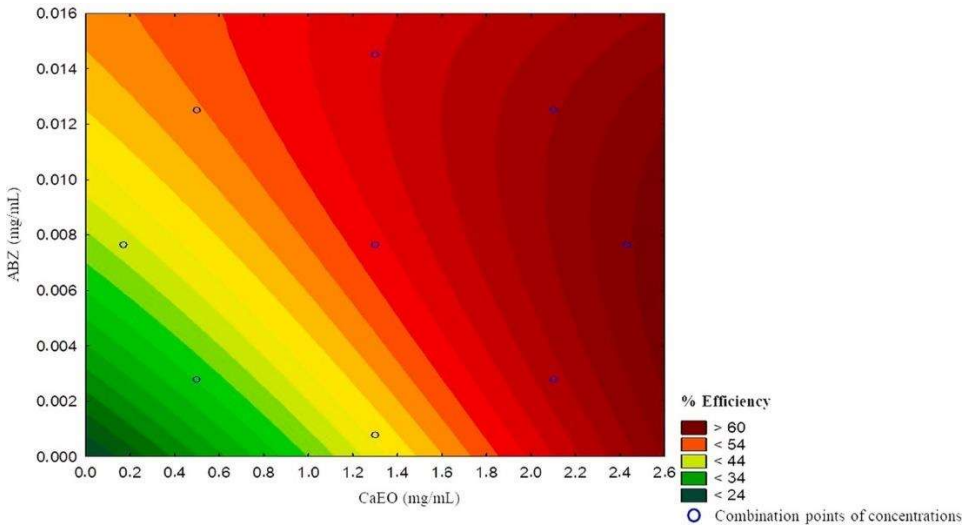

Fig. 1. Level curves generated using a simplex-centroid mixture design for the efficiency of albendazole (ABZ) and *Citrus aurantium* var. dulcis essential oil (CaEO) concentrations against *Haemonchus contortus* eggs. The blue circles indicate the nine concentrations selected for the study to calculate the efficiency of the combination.

distribution of the single nucleotide polymorphisms (SNP) in *H. contortus* populations according to previously described methods (Aliet al., 2019).

2.2.2. Faecal egg count reduction test

Sixteen lambs (average weight 34.3 kg), over three months of age, naturally infected with gastrointestinal nematodes were involved in this trial. The animals were kept under a semi-intensive breeding system with irrigated pasture. After a ten hours grazing period, the animals were housed in pens with access to fresh water. Animals had previous (Day −4) FECs above 200 eggs per gram (epg) using a McMaster technique with a detection threshold of 50 epg (Roberts and O' Sullivan, 1950). The groups were balanced according with age and FEC from days −4 and 0. The animals were classified into two experimental groups (n = 8 each). Group A received ABZ (3.5 mg kg^{-1}) in a single dose in commercial oral suspension (Endazol cobalto 10%; Hipra Saúde Animal). Group B received ABZ, at the same therapeutic dose in combination with CaEO (100 mg kg^{-1}). Faecal samples were collected directly from each experimental animal on days 0 and 10 after treatment, to estimate the FEC reduction.

The formula FECRT (%) 100=x (1 - T2/T1) was used to calculate theFEC reduction (McKenna, 1990), where T1 is the arithmetic mean of FEC at day 0 and T2 is the arithmetic mean FEC at days 10. The 95% con- fidence intervals were calculated as described by Coles et al. (1992). Faecal samples from each experimental group were cultured to obtain L_3 (Roberts and O' Sullivan, 1950). The nematode genus identification was determined according to published keys (van Wyk and Mayhew; 2013).

3. Results

3.1. Egg hatch assay combination model

The CaEO showed a dose-response effect with an EC_{50} of 0.57 mg mL^{-1} (95% CI 0.48 – 0.67), and ABZ had EC_{50} of 0.0048 mg mL^{-1} (95% CI 0.0045 – 0.0052). The EHA for the combinations showed an increase in efficiency when the least effective concentrations were combined (Table 1).

3.2. In vivo assay

Haemonchus contortus-associated Benzimidazole resistance SNPs F167Y and F200Y were found at a frequency of 46.1% and 52.3%,respectively. It was not possible using pipeline analysis verify the per- centage of homozygotes and heterozygotes at each mutation point.However, from the data below, possibly a part of these samples were heterozygotes. Only 1.6% of the *H. contortus* reads were genotyped as susceptible to benzimidazole. Animals from both groups showed FECs higher than 5000 epg on D0 (Table 2). The FECs decreased by 78% afterABZ treatment. However, animals treated with the combination of ABZand CaOE showed a mean FECR of 9% (Table 2).

Table 1
Egg hatching inhibition of *Haemonchus contortus* using different combinations of albendazole (ABZ) and *C. aurantium* var. Dulcis essential oil (CaEO).

CaEO (mg mL^{-1})	ABZ (mg mL^{-1})	Efficiency (%)
0.50	0.0028	26.17
2.10	0.0028	58.20
0.50	0.0125	49.25
2.10	0.0125	70.38
1.30	0.0076	65.79[a]
1.30	0.0076	50.82[a]
1.30	0.0076	49.44[a]
0.16	0.0076	52.18
2.43	0.0076	64.04
1.30	0.0007	55.90
1.30	0.0145	53.78

[a] Replicates of the combination at central point.

Table 2
Mean (± standard deviation) (n = 8) of faecal egg counts (FEC) of sheep treated at day 0 (D0) with albendazole (ABZ) and the combination of *Citrus aurantium* var. Dulcis essential oil (CaEO) and ABZ, and the reduction of faecal egg counts (FECR) 10 days (D10) after treatment.

Treatment	FEC		FERC (LCL - UCL)
	D0	D10	(%)
ABZ	5056.2 ± 9103.8	1081.2 ± 1333.9	78 (74 - 81)
ABZ+CaEO	5575.0 ± 4589.0	5031.2 ± 8003.6	9 (1 - 17)

ABZ: albendazole; CaEO: Citrus aurantium var. Dulcis essential oil; D0: day of the treatment; D10: ten days after the treatment; LCL: lower confidence limit; UCL: upper confidence limit.

There were few differences in the nematode genera present betweenpre and post-treatment populations. These differences were clearest in the combination treatment group, where the percentage of *Haemonchus*spp. increased from 72% to 88%, while *Trichostrongylus* spp. and *Oesophagostomum* spp. decreased from 11% and 7%, respectively, to unidentified in the post-treatment samples. Animals treated with ABZ alone had 99% Haemonchus spp. and 1% Oesophagostomum spp. at thepre-treatment, and 100% Haemonchus spp. in post-treatment.

4. Discussion

Citrus fruits are recognised for their nutritional value, and their bioactive compounds such as terpenes, flavonoids, and vitamins, particularly vitamin C (Bora et al., 2020). Citrus EOs have important applications in the pharmaceutical, sanitary, cosmetic, food, and agri- cultural industries (Bora et al., 2020; Dosoky and Setzer, 2018). EOs have a huge pharmacological potential that can contribute to the control of parasites. In our study, the hatchability of *H. contortus* egg was inhibited by CaEO with an EC_{50} of 0.574 mg mL^{-1}. These outcomeswere similar to EO from others species of the *Citrus* genera such as *Citrus sinensis* EO (0.390 mg mL^{-1}) and *Citrus aurantifolia* EO (0.694 mg mL^{-1}), where limonene is the major component (Ferreira et al., 2018; Gaínza et al., 2015). However, when this terpene was tested alone against *Haemonchus contortus* in vitro, it showed a low efficacy in the EHA (207.56 mg mL^{-1}) (Katiki et al., 2017). The in vitro egg hatch could correspond to faecal egg count reduction and has the ability to predict the anthelmintic efficacy in vivo (Babják et al., 2021). If so, a potential efficient strategy to increase the efficacy of conventional anthelmintics and reduce the selection of resistant gastrointestinal nematodes would be to combine synthetic and natural compounds with diverse and synergistic action mechanisms, for example CaEO and ABZ(Lanusse et al., 2018).

The EHA is widely used to diagnose benzimidazole resistant nema- todes. Benzimidazole resistance genotypes can be detected by mutationsof three SNPs in isotype-1 β -tubulin (Costa-Junior et al., 2021; George et al., 2021). In the present study, the strain of *H. contortus* had 98.5% ofSNPs of resistant nematodes. However, this does not necessarily imply the total inefficiency of the compound against mixed genus field pop- ulations (Samson-Himmelstjerna, 2006), as is shown in the nematode infections of the experimental animals and the ABZ efficacy of 78% of the current study.

No adverse effects of CaEO were seen in the present study. Studies have evaluated the toxicity of natural compounds in animal models and their results have been extrapolated to ruminants (André et al., 2020; Macedo et al., 2010). Side effects of citrus EO at 600 mg kg^{-1} with a duration of 90 min were observed in sheep (da Silva et al., 2021b, 2021a). This rapid effect has been attributed to fast absorption of terpenes through the rumen, which can achieve high concentrations in the central nervous system of lambs (da Silva et al., 2021b). In this sense, orange EOs administered at 200 mg kg^{-1} have caused ataxia, abnormal star gazing head posture and head shacking, that remained for 43 min after administration (da Silva et al., 2021b). Citrus EO showed an in vivo

efficacy ranging from 25.7% to 68.2% in the FECRT in sheep using the dose of 200 mg kg^{-1} (da Silva et al., 2021b, 2021a). To allow a safety margin, we used the dose of 100 mg kg^{-1} of CaEO in the present study. To the best of our knowledge, this is the first study that combines CaEO with a benzimidazole anthelmintic. The ABZ treatment reduced 78% of FEC, while the combination decreased only 9% of FEC in treatedanimals. These results could be explained by two independent or com- bined hypotheses. Firstly, ABZ is formulated as a suspension and showsonly limited gastrointestinal absorption due to relatively poor solubility in water. The dissolution of ABZ in the gastrointestinal fluids is the rate-limiting step in the systemic availability of the active drug or metabo- lites (Lanusse and Prichard, 1993). Only the dissolved drug is available to act on the gastrointestinal nematodes. Therefore, CaEO might be interfering with the ABZ dissolution, decreasing the parasites' drug exposure. Secondly, the interference of CaEO on the ruminal microfloramay affect the reduction of ABZSO to ABZ (Lanusse et al., 1993; Miró et al., 2020). Therefore, a negative pharmacological interaction may be occurring between ABZ and CaEO when administered in combination. The influence of the essential oils on ruminal flora has been well studied, with changes in the microbial community, ruminal pH, feed degrad- ability, and volatile fatty acids production (Cobellis et al., 2016; Zhouet al., 2020, 2019). Essential oils have promising potential to increase ruminant pro- ductivity and aid in nematode control (Štrbac et al., 2022). However, optimisation of their use as antiparasitic compounds, either alone or combined with the synthetic drugs, is still challenging. Safety and toxicity issues must be evaluated in the target species before these compounds can be used as therapeutic tools. Better understanding is needed of potential pharmacokinetic and pharmacodynamic in- teractions when using combinations of EOs and anthelmintic drugs. In conclusion, the present study highlights the reduction in benzimidazole anthelmintic efficacy caused by co-administration of essential oils. Further studies are necessary to improve phytochemical administrationto ruminants within rational nematode control programmes.

CRediT authorship contribution statement

Dauana Mesquita-Sousa: Data curation, Formal analysis, Investi- gation, Writing – original draft. **Nagilla R.C.L. Campos**: Investigation, Methodology, Formal analysis. **Juliana R.F. Pereira**: Investigation, Methodology. **Matheus N. Gomes**: Investigation, Methodology. **Car-olina R. Silva**: Data curation, Formal analysis, Supervision, Writing – review & editing. **Jose A.A. Cutrim-Júnior**: Investigation, Methodol- ogy. **Danilo R.B. Brito**: Methodology, Formal analysis, Supervision,Writing – review & editing. **Romildo M. Sampaio**: Conceptualization, Investigation, Methodology. **Neil D. Sargison**: Investigation, Method- ology, Writing – review & editing. **Adrian L. Lifschitz**: Conceptualiza- tion, Methodology, Formal analysis, Writing – review & editing. **Livio M. Costa- Junior**: Conceptualization, Data curation, Funding acquisi- tion, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors have no conflicts of interest to disclose.

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6. Considerações Finais

De modo geral, o desenvolvimento de produtos bioativos naturais combinados com anti-helmínticos sintéticos depende de vários processos, dentre eles os ensaios *in vitro*, análises *in silico* e estudos farmacoparasitológicos. Estes estudos agregam informações baseadas em farmacologia e são necessárias para que possamos elaborar associações estratégicas entre bioativos e anti-helmínticos. Contudo, a aplicação *in vivo* nos traz diversos fatores que podem contrapor com os resultados *in vitro,* como, as possíveis interações farmacocinéticas, farmacodinâmicas, a segurança e a toxicidade na espécie-alvo. Portanto, são necessários mais estudos para contribuir com informações sobre a administração de fitoquímicos a ruminantes, auxiliando no desenvolvimento de novas estratégias de controle de parasitas e na redução do uso excessivo de anti-helmínticos sintéticos.