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ASSOCIAÇÃO DE ANTI-HELMÍNTICOS SINTÉTICOS E MONOTERPENOS NO CONTROLE DE *Haemonchus contortus*

Carolina Rocha e Silva

São Luís, MA
Setembro/2020

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ASSOCIAÇÃO DE ANTI-HELMÍNTICOS SINTÉTICOS E MONOTERPENOS NO CONTROLE DE *Haemonchus contortus*

Trabalho de tese apresentado ao Programa de Pós-Graduação da Bionorte, Universidade Federal do Maranhão, como requisito para a obtenção do Título de Doutora em Biotecnologia.

Orientador: Prof. Dr. Livio Martins Costa Júnior
Coorientador: Prof. Dr. Adrian Lifschitz

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RESUMO

Nematoides gastrintestinais de pequenos ruminantes resistentes aos produtos anti-helmínticos sintéticos vêm sendo selecionados há vários anos. A utilização de monoterpenos, produtos presentes em óleos essenciais de plantas, pode contornar essa situação, ajudando no controle de *Haemonchus contortus*, através de um efeito sinérgico com anti-helmínticos sintéticos. No entanto, a fim de obter uma eficácia *in vivo* contra nematoides de ruminantes, os monoterpenos devem atingir os locais alvo do hospedeiro onde os nematoides estão presentes sem serem degradados após sua administração oral. O processo de fermentação ruminal afeta os componentes dos alimentos ingeridos pelo hospedeiro. Portanto, informações sobre o destino metabólico de monoterpenos no rúmen são necessárias antes que esses compostos possam ser usados *in vivo*. Ademais, a combinação de monoterpenos com produtos anti-helmínticos sintéticos pode ser uma estratégia interessante para alcançar a interação com o mesmo alvo em diferentes sítios de ligação, e potenciais efeitos aditivos ou sinérgicos entre produtos naturais e sintéticos devem ser avaliados. A propósito, a associação com produtos naturais e sintéticos pode agir alterando a ultraestrutura do nematoide. O presente trabalho objetivou avaliar a estabilidade ruminal, eficácia e o efeito na ultraestrutura e nos caracteres biofísicos da associação de compostos sintéticos e monoterpenos sobre *H. contortus*. Concluímos que os monoterpenos carvacrol e timol são estáveis em líquido ruminal. A associação de produtos sintéticos com monoterpenos melhora significativamente a eficiência de Albendazol e Levamisol sobre *H. contortus in vitro*, além de terem interações químicas e de causarem danos na ultraestrutura de ovos de *H. contortus*.

PALAVRAS-CHAVE: nematoides, pequenos ruminantes, resistência, monoterpenos, AFM.

ABSTRACT

Gastrointestinal nematodes from small ruminants resistant to synthetic anthelmintic products have been selected for several years. The use of monoterpenes, products present in essential oils of plants, can overcome this situation, helping to control *Haemonchus contortus*, through a synergistic effect with synthetic anthelmintics. However, to obtain an in vivo efficacy against ruminant nematodes, monoterpenes must reach target host sites where the nematodes are present without being degraded after their oral administration. The rumen fermentation process affects the components of food eaten by the host. Therefore, information about the metabolic fate of monoterpenes in the rumen is necessary before these compounds can be used in vivo. In addition, the combination of monoterpenes with synthetic anthelmintic products can be an interesting strategy to achieve interaction with the same target at different binding sites, and potential additive or synergistic effects between natural and synthetic products should be evaluated. By the way, the association with natural and synthetic products can act by altering the nematode's ultrastructure. The present work aimed to evaluate the ruminal stability, efficacy, and the effect on the ultrastructure and the biophysical characters of the association of synthetic compounds and monoterpenes on *H. contortus*. We conclude that the monoterpenes carvacrol and thymol are stable in rumen liquid. The association of synthetic products with monoterpenes significantly improves the efficiency of Albendazole and Levamisol on *H. contortus* in vitro, in addition to having chemical interactions and causing damage to the ultrastructure of *H. contortus* eggs.

KEYWORDS: nematodes, small ruminants, resistance, monoterpenes, AFM.

SUMÁRIO

1. INTRODUÇÃO

O parasitismo por nematoides gastrintestinais gera danos à saúde dos pequenos ruminantes e, consequentemente, prejuízos a produção de carne e de leite. O nematoide que mais se destaca é *Haemonchus contortus*, por ser um agente hematófago, causa intensa espoliação sanguínea, levando os ruminantes a anemia, perda de peso, diminuição na produção de leite, gerando grandes prejuízos na atividade, tendo a necessidade de mecanismos para inibir a ação desse endoparasito (Doyle *et al.*, 2019).

O controle de parasitos em pequenos ruminantes, durante muitos anos, foi realizado através da administração de produtos sintéticos, além de técnicas adequadas de manejo (Santos, dos *et al.*, 2019). Mas o uso inadequado dos anti-helmínticos sintéticos fez com que populações resistentes de nematoides fossem selecionadas (Mohammedsalih *et al.*, 2019; Niciura *et al.*, 2019, 2020; Santos, dos *et al.*, 2019). Para contornar essa situação, novos compostos que aumentem o efeito antiparasitário das moléculas já existentes vêm sendo estudadas no controle do parasitismo. Entre as alternativas estudadas, a utilização de produtos oriundos das plantas vem sendo bastante promissor (Araujo *et al.*, 2019; Dixit *et al.*, 2019; Silva *et al.*, 2019; Soares *et al.*, 2019).

Produtos e extratos das plantas vêm sendo utilizados no tratamento de diversas patologias devido aos seus efeitos farmacológicos. Elas produzem compostos bioativos sintetizados durante seu metabolismo secundário, entre eles os óleos essenciais (Ribeiro, Velozo e Guimarães, 2013). Os óleos essenciais podem ser encontrados em folhas, flores, caule, raízes, frutos, sementes e no rizoma de plantas do tipo angiospermas dicotiledôneas, mas também podem ser encontrados em angiospermas monocotiledôneas e raramente em gimnospermas, em gramíneas e zingiberáceas. São substâncias utilizadas pelas plantas para proteção contra microrganismos patogênicos, como bactérias e fungos, na atração de polinizadores e na proteção contra perda de água e controle da temperatura (Simões, 2001).

Os óleos essenciais são de baixo peso molecular e, geralmente, odoríficos, que podem ser obtidos por várias formas, como a destilação por arraste a vapor d'água das diversas partes das plantas (Almeida, 2015). Estes são substâncias complexas, podendo ser constituídos de hidrocarbonetos terpênicos, álcoois simples e terpênicos, aldeídos, cetonas, fenóis, ésteres, óxidos, peróxidos, furanos, ácidos orgânicos, lactonas cumarinas, até compostos com enxofre. Os terpenoides se destacam na composição dos óleos essenciais, e são formados pela condensação de um número variável de unidades pentacarbonadas, sendo mais frequente os sesquiterpenos e monoterpenos, sendo esse último o grupo mais encontrado (Almeida, 2015;

Simões, 2001). Os monoterpenos se destacam por suas atividades farmacológicas, incluindo ação antifúngica, antibacteriana, antioxidante, anticancerígena, antiespasmódica, hipotensiva, vaso-relaxante e antiparasitária (Romero *et al.*, 2013; Santos *et al.*, 2011).

A combinação de moléculas anti-helmínticas pode ser benéfica para a redução da resistência parasitária devido ao fato de cada composto pode ter mecanismo de ação diferente e atuar em locais distintos do parasito (Lanusse *et al.*, 2018; Lanusse, Alvarez e Lifschitz, 2014). O sinergismo entre compostos sintéticos e óleos essenciais ou seus compostos isolados já foi demonstrado em trabalhos *in vitro* (Dhinakaran, Mathew e Munusamy, 2019; Ji *et al.*, 2019; Silva, 2013). A utilização de monoterpenos pode ser uma alternativa no controle dos nematoides, entretanto é desconhecido a absorção e metabolização desses compostos e a interferência na farmacocinética e farmacodinâmica e consequentemente no efeito sinérgico de compostos sintéticos (Ribeiro, Velozo e Guimarães, 2013). Com isso, torna-se necessário o estudo farmacocinético dos monoterpenos associados aos anti-helmínticos sintéticos.

O objetivo desse trabalho foi fazer uma prospecção sobre a utilização de óleos essenciais e terpenos, avaliar o metabolismo ruminal e verificar a eficácia entre monoterpenos e anti-helmínticos sintéticos, além de analisar as características ultraestruturais e biofísicas de *H. contortus* tratados com essa associação. Para atingir esses objetivos dividimos a presente tese em três capítulos, sendo eles:

- Capítulo 1 – Avanços no desenvolvimento de tecnologias utilizando óleos essenciais para controle de parasitos de pequenos ruminantes. Advances in the development of technologies using essential oils for control of parasites of small ruminants. Artigo publicado na revista GEINTEC, v. 9, n. 2, 4966-4976, abr/maio/jun – 2019.

- Capítulo 2 – Chemical stability in rumen of terpenoids with anthelmintic activity against *Haemonchus contortus*. Esses dados fazem parte do artigo "Combination of bioactive phytochemicals and synthetic anthelmintics: *In vivo* and *in vitro* assessment of the albendazole-thymol association", publicado na revista Veterinary Parasitology, v. 281, 109121.

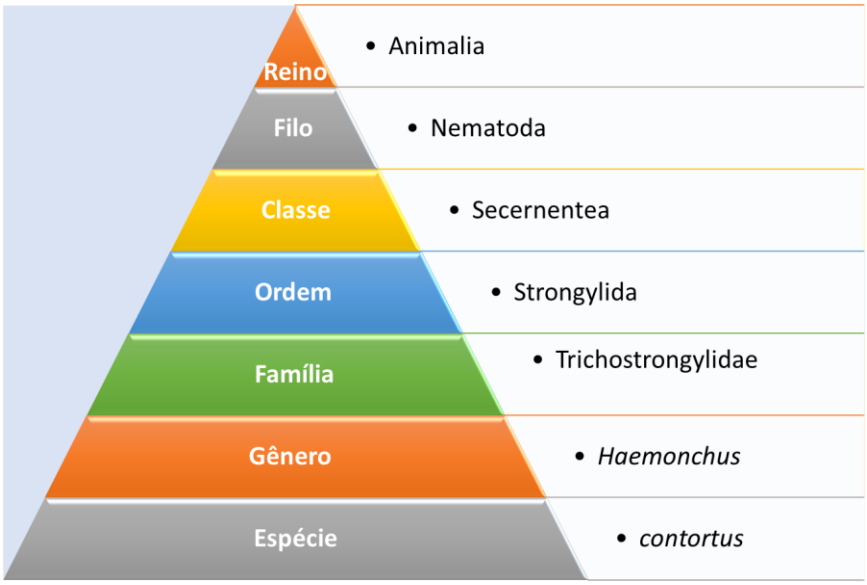
- Capítulo 3 – Combination of synthetic anthelmintics and monoterpenes: Assessment of efficacy and ultrastructural and biophysical properties of *Haemonchus contortus* using atomic force microscopy. Artigo submetido ao periódico Veterinary Parasitology.

2. REFERENCIAL TEÓRICO

2.1. *Haemonchus contortus* (Rudolph, 1803)

A espécie *H. contortus* é um nematoide abomasal hematófago pertencente à família Trichostrogylidae (Figura 1), responsável por grandes perdas econômicas em pequenos ruminantes. Tem distribuição mundial, sendo mais importante em regiões tropicais e subtropicais. Possuem de 2 a 3 cm e pode-se observar os ovários brancos enrolando-se em espiral no intestino repleto de sangue (Soulsby, 1965; Gordon, 2019).

Figura 1. Classificação taxonômica do nematoide *Haemonchus contortus*



O ciclo de vida é direto e envolve uma fase livre e outra fase parasitária. O ovo é liberado do hospedeiro através das fezes; em condições de temperatura e umidade favoráveis, este irá eclodir e a larva de primeiro estágio (L1) se desenvolverá para larva de segundo estágio (L2) e posteriormente larva de terceiro estágio (L3), dentro do bolo fecal. A L3 é a fase infectante, que irá sair do bolo fecal e ficar na pastagem, onde será consumida pelo hospedeiro. A L3 irá perder a bainha de proteção no rúmen e quando chegar ao abomaso, se desenvolverá para L4 e L5 e penetrar na mucosa abomasal para se alimentar de sangue nos vasos sanguíneos; então irá se desenvolver até adultos, que irão copular e posteriormente fazer a ovoposição, iniciando novamente o ciclo (Figura 2) (Selaive-Villarroel e Guimarães, 2019).

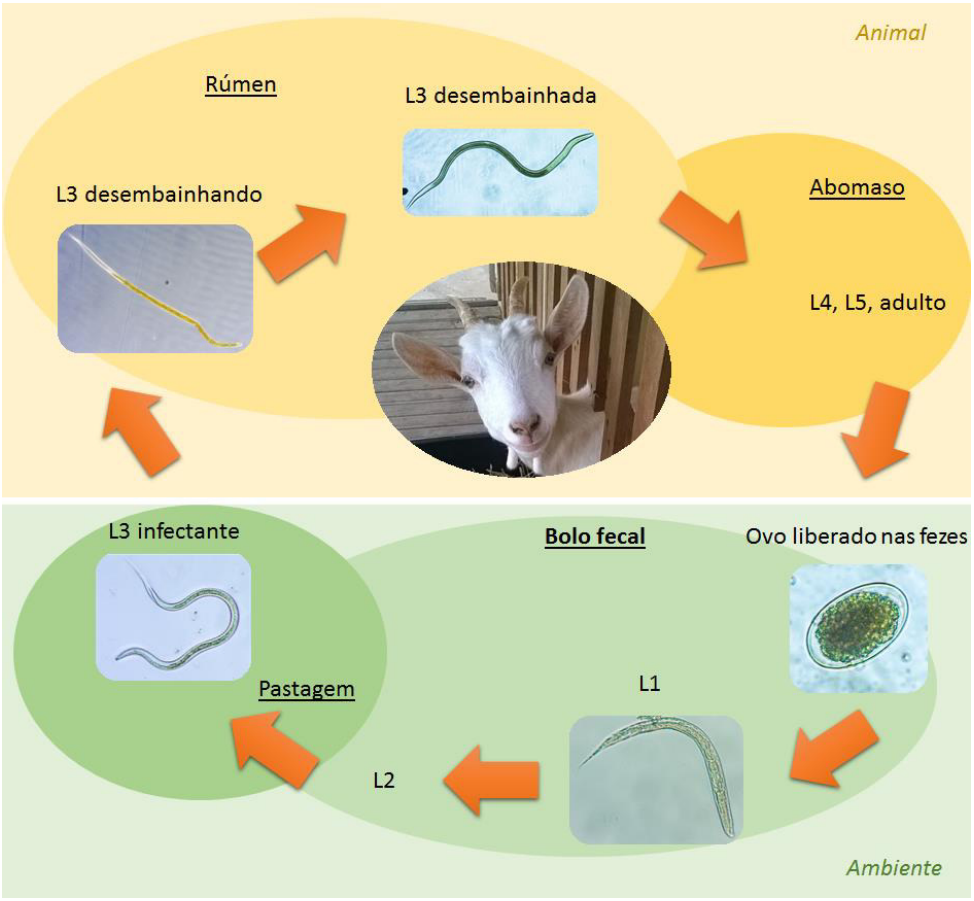


Figura 2. Ciclo de vida do nematódeo gastrointestinal *Haemonchus contortus* representativo da família Trichostrongylidae. L: estágios larvais.

A importância da infecção por *H. contortus* se deve a hábito de alimentação do parasito. Cada espécime pode ingerir cerca de 0,05 ml de sangue por dia, sendo que uma infeção por 5.000 vermes pode causar uma perda de 250 ml por dia, levando o animal a uma anemia hemorrágica aguda. Dentre a sintomatologia, pode-se observar edema submandibular e ascite devido a uma hipoproteinemia, letargia, fezes de coloração escura e queda de lã, e, como principal sintoma, anemia profunda (Urquhart, 2001). O diagnóstico laboratorial envolve a observação de ovos de triconstrongilídeos nas fezes, através de exames coproparasitológicos quantitativos como OPG (ovos por grama de fezes) através da técnica de McMaster e derivações ou centrifugo-flutuação, além dos sinais clínicos (Ueno e Gonçalves, 1998).

O tratamento de *H. contortus* envolve compostos antiparasitários, a base de benzimidazóis, lactonas macrocíclicas, imidazotiazóis, salicilanilidas, substitutos nitrofenólicos, organofosforados, derivados de amino-acetonitrila e spirindol (Selaive-Villarroel e Guimarães, 2019).

2.2. Anti-helmínticos comerciais

O controle de nematoides gastrintestinais vem sendo realizado, há muitos anos, através de anti-helmínticos sintéticos. São utilizados vários grupos químicos para controle dos nematoides gastrintestinais, sendo os que mais se destacam os benzimidazóis, imidazotiazóis e lactonas macrocíclicas, possuindo vários princípios ativos (Alvarez, Mottier e Lanusse, 2007; Amarante, 2015) (Tabela 1).

Tabela 1. Grupos químicos e princípios ativos utilizados no tratamento de nematoides gastrintestinais.

Grupo químico	Princípio ativo
Imidazotiazóis	Levamisol
Benzimidazóis	Albendazol, Fenbendazol, Oxifendazol, Tiabendazol, Mebendazol, Flubendazol, Oxibendazol
Lactonas Macrocíclicas	Doramectina, Ivermectina, Moxidectina, Abamectina, Eprinomectina, Selamectina
Salicilanilidas	Closantel, Rafoxanida
Substitutos Nitrofenólicos	Disofenol, Nitroscanato, Nitroxinil
Organosfosforados	Triclorfone
Derivados De Amino-Acetonitrila (AADs)	Monepantel
Spirindole	Derquantel

Adaptado de Lopes e Costa (2017)

Imidazotiazóis

O grupo químico imidazotiazóis atuam como agentes bloqueadores neuromusculares despolarizantes. Ligam-se aos receptores nicotínicos de acetilcolina, estimulando sua ação, o que resulta em excesso de despolarização de membranas com sucessivas contrações e morte de parasito por paralisia espásticas. Tem como principal composto o levamisol (Atchison *et al*., 1992; Coles, East e Jenkins, 1975).

Benzimidazóis

As drogas pertencentes ao grupo dos benzimidazóis agem sobre os parasitos impedindo a síntese de tubulinas, se ligando a β-tubulina no parasito. As tubulinas são proteínas responsáveis pela formação dos microtúbulos, auxiliares na movimentação dos cromossomos durante a divisão celular, fazendo parte dos processos de obtenção de energia. Isso resulta em falha no desenvolvimento do parasito, como ocorre nos ovos (Dustin, 1978; Lacey *et al*., 1978).

Dentre os princípios ativos, podemos destacar o albendazol. Este é classificado como um carbamato benzimidazólico, que tem ação anti-helmíntica devido a inibição da captação ou da utilização da glicose pelo parasito. Além disso, ele reduz a produção e o nível do ATP, diminuindo a respiração, resultando na imobilização e morte lenta do parasito. O metabolismo anaeróbico, fundamental para muitos helmintos, também é inibido pela ação do Albendazol (Lucia, 2016; Wolverton, 2015).

Lactonas Macrocíclicas

No grupo das lactonas macrocíclicas encontramos as avermectinas e as milbemicinas. O mecanismo de ação desse grupo está relacionado a abertura dos canais de cloro pela ligação com receptores de glutamato, com morte do parasito por inanição (Ardelli *et al*., 2009).

Nesse grupo podemos destacar a Ivermectina, um derivado anti-helmíntico semissintético oriundo dos produtos da fermentação da bacteria *Streptomyces avermitilis*. Sua ação se deve a imobilização dos parasitos através de paralisia tônica da musculatura, resultante da ação nos canais de cloro acoplado ao glutamato; liga-se também aos canais de cloro acoplado ao GABA (ácido gama-aminobutírico) (Bermudez *et al*., 1991).

Salicilanilidas

O grupo das salicilanidas atuam sobre o nematóide geralmente reduzindo as reações mitocondriais envolvidas no transporte de elétrons e, com isso, a síntese de ATP. Ocorre ainda rápida paralisia espástica pelo aumento de íons de cálcio nas células musculares dos parasitos. Destaca-se o closantel, um endoparasticida de amplo espectro, apresentando também ação contra ectoparasitos (Bacon *et al*., 1998).

Substitutos Nitrofenólicos

Os substitutos nitrofenólicos é um grupo com ação contra nematoides, cestoides e trematoides. Como mecanismo de ação inibem a fosforilação oxidativa das mitocôndrias e impedem a síntese de ATP. Dentre os substitutos nitrofenólicos temos o nitroxinil que atua

desacoplando a fosforilação oxidativa nas veias hepáticas. Reduz a espermatogênese em vermes sobreviventes, resultando em menos ovos férteis (Corbett e Goose, 1971).

Organofosforados

Os organofosforados são agentes anticolinesterásicos com baixo índice terapêutico. São lipossolúveis e a absorção desses compostos pode ocorrer por toda a superfície corporal, especialmente em trato gastrintestinal, pele, pulmões e olhos, com rápida distribuição e excreção. O mecanismo de ação está relacionado com a inibição irreversível da acetilcolinesterase, causando a morte do parasito por paralisia espástica (Leung e Meyer, 2019).

Derivados De Amino-Acetonitrila (AADs) e Spirindole

Dentre os produtos sintéticos comercializados, os mais recentes são os derivados de amino-acetonitrila (AADs) e o Spirindole. Ambos têm mecanismo de ação envolvendo receptores nicotínicos de acetilcolina. O monepantel é um composto químico AADs, sendo que esse antiparasitário tem um novo modo de ação envolvendo um clado único e específico de nematóides de subunidades de receptores de acetilcolina. Os AADs causam hipercontração dos músculos da parede corporal, levando à paralisia, contrações espasmódicas da porção anterior da faringe e, finalmente, a morte. Já o grupo Spirindole tem como principal composto o derquantel, que é associado à abamectina e não é comercializado no Brasil (Kaminsky *et al.*, 2008).

2.2.1. Resistência parasitária

Durante muito tempo o controle dos parasitos foi realizado quase que exclusivamente por meio de compostos químicos sintéticos. Entretanto, o uso indevido desses produtos vem antecipando a seleção de populações resistentes a diversas bases químicas. Diversos estudos descrevem a resistência de *H. contortus* aos compostos sintéticos disponíveis no mercado (Albuquerque *et al.*, 2017; Berton *et al.*, 2017; Knubben-schweizer e Pfister, 2017; Onzima *et al.*, 2017).

Com o intuito de controlar os parasitos resistentes, há a necessidade de desenvolver novos produtos que também sejam menos propícios a seleção de parasitos resistentes. Com isso, os produtos naturais têm sido destaque em muitas pesquisas (Castañeda-Ramírez *et al.*, 2017; Katiki *et al.*, 2017).

2.3. Monoterpenos

As plantas vêm sendo utilizadas no tratamento de diversas patologias devido aos seus efeitos farmacológicos. Elas produzem compostos bioativos sintetizados durante seu metabolismo secundário, entre eles os óleos essenciais, substâncias complexas de compostos lipofílicos, de baixo peso molecular e, geralmente, odoríficos (Ribeiro, Velozo e Guimarães, 2013). Eles podem ser obtidos por diferentes meios, como a destilação por arraste a vapor d'água de diversas partes das plantas. Possuem atividades farmacológicas, incluindo ação antifúngica, antibacteriana, antioxidante, anticancerígena, antiespasmódica, hipotensiva, vaso-relaxante e antiparasitária (Almeida, 2015; Romero *et al.*, 2013; Katiki et al., 2011 Santos *et al.*, 2011). Entre os constituintes dos óleos essenciais encontram-se principalmente os monoterpenos, que são hidrocarbonetos de cadeia curta (Almeida, 2015). Estudos vem demonstrando a ação de monoterpenos sobre *H. contortus* (André *et al.*, 2017; Ferreira *et al.*, 2016; Katiki *et al.*, 2017).

Devido à resistência parasitária, novas alternativas vêm surgindo nesse contexto, como a associação entre moléculas naturais e sintéticas, com o intuito de potencializar o efeito de produtos comercialmente já utilizados. A combinação de várias moléculas anti-helmínticas pode ser benéfica porque cada produto pode ter mecanismos de ação diferentes, agindo em locais distintos do parasito, auxiliando a driblar a resistência parasitária (Lanusse *et al.*, 2018; Lanusse, Alvarez e Lifschitz, 2014).

O sinergismo entre compostos sintéticos e óleos essenciais vem apresentando bons resultados. A combinação entre antimicrobianos e óleo essencial aumentou a eficiência sobre *Penicillium corylophilum* (Ji *et al.*, 2019). A associação de óleo essencial de *Croton ceanothifolius* com norfloxacino demonstrou efeito sinérgico sobre bactéria multirresistente (Araújo *et al.*, 2020). A associação de terpenos com larvicidas teve efeito sinérgico sobre o inseto *Aedes aegypti* (Dhinakaran, Mathew e Munusamy, 2019).

A utilização de moléculas naturais, como os monoterpenos, pode ser uma alternativa no controle e na interação sinérgica com compostos antiparasitários sintéticos, através dos estudos farmacocinéticos dessas moléculas associadas aos anti-helmínticos sintéticos, como uma alternativa mais eficiente frente cepas de nematoides gastrintestinais resistentes (Ribeiro, Velozo e Guimarães, 2013).

2.4. Interferência do metabolismo ruminal no desenvolvimento de produtos anti-helmínticos

Para obter uma eficácia *in vivo* ideal contra nematoides de ruminantes, os produtos com potencial nematicidas devem atingir os locais alvo do hospedeiro onde os parasitos estão presentes sem serem degradados após a administração oral. O processo de fermentação ruminal afeta os componentes alimentares ingeridos pelo hospedeiro. Esse processo de fermentação também pode modificar os medicamentos usados para tratar diferentes doenças (Malecky, Albarello e Broudiscou, 2012; Wu e Papas, 1997).

Os ruminantes são caracterizados por apresentarem estômago compartimentalizado e por serem fermentadores pré-gástricos. O estômago dos ruminantes domésticos é formado por quatro cavidades, rúmen, retículo, omaso e abomaso. A primeira câmera, o rúmen, é caracterizado por ser uma câmara de fermentação, contendo um líquido com pH não acidificado, constituído por micróbios heterótrofos como bactérias, protistas, leveduras e fungos (Melo *et al.*, 2013).

A microbiota ruminal é responsável pela degradação de carboidratos complexos, especialmente a celulose, que constitui grande parte da dieta regular de ruminantes, além de ser importante para a reabsorção dos ácidos graxo voláteis produzidos por fermentação (ácido acético, propiônico e butírico), e para a reabsorção de água. A microbiota também sintetiza as vitaminas do complexo B e aminoácidos essenciais (Lan e Yang, 2019).

Portanto, é necessário conhecer o destino metabólico de possíveis fármacos no rúmen, em particular suas taxas de degradação individuais, antes que esses compostos possam ser utilizados *in vivo*. Avaliar a estabilidade química ruminal *in vitro* de produtos com potencial anti-helmíntico é essencial no desenvolvimento farmacológico de produtos com via de administração oral para ruminantes. Isso permite avaliar se ocorre metabolização do produto durante um período, além de ser possível também observar em qual fase o produto se encontra, na fase sólida ou líquida do conteúdo ruminal. A presença da substância estudada na fase líquida demonstra que o produto tem uma taxa de passagem mais rápida, possibilitando um menor tempo no rúmen, além de alcançar o sítio de atuação de modo mais rápido, o abomaso, onde se encontram os nematoides na fase adulta (Hennessy, Ali e Tremain, 1994; Virkel *et al.*, 2002).

2.5. Microscopia de força atômica (AFM) e a ultraestrutura de *Haemonchus contortus*

Microscopia de luz, varredura e eletrônica de transmissão têm sido utilizadas com sucesso para avaliar danos ou parâmetros estruturais de *H. contortus*. No entanto, algumas limitações dessas técnicas estão impedindo avanços na compreensão do efeito de diferentes compostos no nematoide gastrintestinal *H. contortus*. O limite do comprimento de onda da radiação eletromagnética visível nos microscópios ópticos motivou o desenvolvimento de

microscopias avançadas não baseadas em luz. Através da microscopia de força atômica (AFM) podemos atingir estruturas antes não observadas. O AFM é uma técnica de microscopia de alta resolução que fornece informações sobre a topografia e a composição da superfície de uma grande variedade de materiais, desde células individuais vivas até tecido fixo (Oliveira *et al.*, 2017; Rebelo *et al.*, 2013).

Essa técnica permite atingir estruturas antes desconhecidas, em escala nanoscópica. Como o nome da técnica sugere, o AFM pode criar imagens de forças locais entre a superfície da amostra e a ponta, incluindo forças de van der Walls, repulsão de Born, forças eletrostáticas, forças magnéticas, fricção, adesão, bem como mapear propriedades estruturais de amostras, tais como rugosidade, viscosidade e elasticidade, correlacionando essas propriedades com a estrutura do material (Costa-Junior *et al.*, 2020).

Recentemente Costa-Junior *et al.* (2020) avaliaram a caracterização topográfica e biomecânica de ovos, larvas e a cutícula de formas adultas de *H. contortus* no AFM. Foram observadas redução qualitativa na rigidez quando os ovos se desenvolvem da mórula para o estágio de larva. A análise AFM do estágio L1 mostrou uma série de anéis separados periodicamente, com restos de larvas eclodindo na cutícula do nematoide. As imagens de *H. contortus* em adultos, L3 com bainha, estágios L2 e L1 permitiram comparar as alterações das estruturas anulares durante a evolução do parasita. O processo de desembainhamento artificial tornou evidente o primórdio genital de *H. contortus* no estágio L3. Os resultados revelaram um aumento da adesão na superfície do nematoide no estágio L3 devido à remoção da bainha. Esse estudo possibilitou uma visão inicial sobre as propriedades biomecânicas e ultraestruturais diferenciais desse nematoide, o que pode explicar as etapas biológicas e bioquímicas do ciclo de vida desses parasitas.

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CAPÍTULO 1

Avanços no desenvolvimento de tecnologias utilizando óleos essenciais para controle de parasitos de pequenos ruminantes

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AVANÇOS NO DESENVOLVIMENTO DE TECNOLOGIAS UTILIZANDO ÓLEOS ESSENCIAIS PARA CONTROLE DE PARASITOS DE PEQUENOS RUMINANTES

ADVANCES IN THE DEVELOPMENT OF TECHNOLOGIES USING ESSENTIAL OILS FOR CONTROL OF PARASITES OF SMALL RUMINANTS

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Resumo

O Brasil é um grande produtor de caprinos e ovinos, porém tem de enfrentar um dos principais problemas da criação, as parasitoses. Populações de parasitos resistentes vêm sendo selecionadas, motivando a busca de novos antiparasitários. Este trabalho objetivou prospectar trabalhos científicos e patentes relacionados ao uso de óleos essenciais no desenvolvimento desses novos produtos. Realizou-se uma pesquisa documental exploratória de abordagem quantitativa. Para prospecção tecnológica, pesquisaram-se as patentes depositadas no INPI (Instituto Nacional de Proteção Industrial) e presentes no Derwent Innovations Index. A prospecção científica foi realizada utilizando a coleção do Web of Science. Foi observado que o Brasil não possui patentes relacionadas a óleos essenciais, apesar disso, é o país que se destaca em publicações científicas no tema. Por se tratarem de produtos de elevado potencial inovador, percebe-se a urgente necessidade de se incentivar os pesquisadores brasileiros a protegerem os novos produtos obtidos a partir de óleos essenciais.

Palavras-chave: antiparasitários; caprinos; ovinos; produtos naturais.

Abstract

Brazil is a major producer of goats and sheep, but it has to face one of the main problems of breeding, parasites. Populations of resistant parasites have been selected, motivating the search by alternatives antiparasitic. This work aimed to prospect scientific works and patents related to the use of essential oils in the development of these new products. For technological prospection, the patents deposited with the INPI (National Institute of Industrial Protection) and present in the Derwent Innovations Index were searched. Scientific prospecting was done using the Web of Science collection. It was observed that Brazil does not have patents related to essential oils, nevertheless, it is the country that stands out in scientific publications on the subject. For being are products with a high potential for innovation, there is an urgent need to encourage Brazilian researchers to protect new products obtained from essential oils.

Keywords: antiparasitic; goats; sheep; natural products.

1. Introdução

O Brasil é um grande criador de pequenos ruminantes contando com um rebanho caprino e ovino de oito milhões e 17 milhões, respectivamente (IBGE, 2014), destacando-se as parasitoses como motivo de grandes prejuízos na criação. Os parasitos afetam à saúde e a produção de ovinos e caprinos em todo o mundo. Esses animais são acometidos tanto por endoparasitos quanto por ectoparasitos, causando irritação, espoliação sanguínea, diminuição da produção de carne e leite (ANGULO-CUBILLÁN *et al.*, 2007).

Durante muito tempo o controle dos parasitos foi realizado quase que exclusivamente por meio de compostos químicos sintéticos. Diversos estudos descrevem a resistência do principal parasito de pequenos ruminantes, o nematoide *Haemonchus contortus* aos compostos sintéticos disponíveis no mercado (ALBUQUERQUE *et al.*, 2017; BERTON *et al.*, 2017; KNUBBEN-SCHWEIZER e PFISTER, 2017; ONZIMA *et al.*, 2017), entretanto, o uso indevido desses produtos vem selecionando populações resistentes a diversas bases químicas.

Com o intuito de controlar os parasitos resistentes, há a necessidade de desenvolver novos produtos que também sejam menos propícios a seleção de parasitos resistentes. Com isso, os produtos naturais têm sido destaque em muitas pesquisas usando óleos essenciais (CASTAÑEDA-RAMÍREZ *et al.*, 2017; KATIKI *et al.*, 2017).

Os óleos essenciais são substâncias complexas de compostos lipofílicos, de baixo peso molecular e, geralmente, odoríficos. Eles podem ser obtidos por diferentes meios, como a destilação por arraste a vapor d'água de diversas partes das plantas. Possuem atividades farmacológicas, incluindo ação antifúngica, antibacteriana, antioxidante, anticancerígena, antiespasmódica, hipotensiva, vaso-relaxante e antiparasitária (ALMEIDA, 2015; ROMERO *et al.*, 2013; SANTOS *et al.*, 2011)

Estudos vêm demonstrando a eficiência dos óleos essenciais de *Thymus vulgaris*, *Melaleuca alternifolia*, *Ruta chalepensis* sobre nematoides de pequenos ruminantes, *Myrtus communis* sobre cestoides e *Cinnamomum camphora* e *Lavandula angustifolia* sobre dípteros (FERREIRA *et al.*, 2016; GRANDO *et al.*, 2016; MAHMOUDVAND *et al.*, 2015; ORTU *et al.*, 2017; SHALABY *et al.*, 2016).

Esse trabalho tem o objetivo de analisar as patentes e os trabalhos científicos publicados sobre óleos essenciais relacionados a parasitoses de ovinos e caprinos, afim de demonstrar o potencial inovador destes produtos e incentivar pesquisadores da área a protegerem seus produtos à base de óleos essenciais.

2. Metodologia

Realizou-se uma pesquisa documental exploratória de abordagem quantitativa. Para prospecção tecnológica, pesquisaram-se as patentes depositadas no INPI (Instituto Nacional de Proteção Industrial) e presentes no *Derwent Innovations Index*. A prospecção científica foi realizada utilizando a coleção do *Web of Science*.

Foram utilizadas diferentes palavras-chave relacionadas a óleos essenciais e parasitos de ovinos e caprinos (Tabela 1). Foram utilizados termos em português para a base de patentes do INPI, e termos em inglês para a base *Derwent Innovations Index* e para a principal coleção do *Web of Science*.

Tabela 1 – Palavras-chave empregadas na pesquisa sobre óleos essenciais, parasitos e ovinos e caprinos na base de dados do INPI.

Principal Coleção do *Web of Science* e *Derwent Innovations Index*	**INPI**
essential AND oil AND (sheep or goat)	óleo AND essencial
essential AND oil AND (sheep or goat) AND parasit*	óleo AND essencial AND parasit*
essential AND oil AND (sheep or goat) endoparasit*	óleo AND essencial AND (ovino OR caprino)
essential AND oil AND (sheep or goat) AND antihelmint*	
essential AND oil AND (sheep or goat) AND protozoan*	
essential AND oil AND (sheep or goat) AND helminth*	
essential AND oil AND (sheep or goat) AND ectoparasit*	
essential AND oil AND (sheep or goat) AND acaricid*	
essential AND oil AND (sheep or goat) AND acaricid* AND parasit*	
essential AND oil AND (sheep or goat) AND tick*	
essential AND oil AND (sheep or goat) AND flea*	
essential AND oil AND (sheep or goat) AND myiasi*	
essential AND oil AND (sheep or goat) AND louse*	
essential AND oil AND (sheep or goat) AND mite*	
essential AND oil AND (sheep or goat) AND mite* AND parasit*	

Fonte: INPI, 2017.

O Microsoft Excel 2016 MSO foi empregado para confecção do mapa e análise dos dados por meio de estatística descritiva.

3. Resultados e Discussão

3.1. Pesquisa INPI

A busca na base de dados do INPI detectou 242 processos de patenteamento usando como palavras-chave, óleo essencial ou óleo essencial combinado com parasitos, com 235 e 7 processos de patentes, respectivamente (Tabela 2). Em contrapartida, quando o óleo essencial combinado com ovinos ou caprinos foi objeto de busca no INPI, nenhuma patente foi encontrada. O uso prático de óleos essenciais na produção animal ainda é bem limitado, especialmente devido ao alto custo no desenvolvimento de pesquisas nesta área e o baixo rendimento dos óleos essenciais, que normalmente é abaixo de 2%, necessitando com isso de grandes áreas de plantio da espécie desejada (SCHMIDT, 2010).

Tabela 2 - Pesquisa por palavras-chave no Instituto Nacional de Proteção Industrial – INPI

Palavras-chave	Depósito de pedido	Patente	Total
Óleo AND essencial	74	161	235
Óleo AND essencial AND parasit*	3	4	7
Óleo AND essencial AND (ovino OR caprino)	0	0	0
Total	**77**	**165**	**242**

* Operador de truncagem
Fonte: INPI, 2017

Para superar alguns dos entraves acima citados, foi fundada a Associação Brasileira dos Produtores de Óleos Essenciais (ABRAPOE) que objetiva aproximar mais os produtores e as instituições de pesquisas agregando qualidade aos óleos por meio de pesquisas e estudos de padronização. Além disso, atua na disponibilização de dados atuais de mercado e ser representante na área frente aos órgãos governamentais, ajudando a angariar mais recursos para aproveitar melhor o grande potencial da diversidade da flora brasileira.

3.2. Pesquisa no *Derwent Innovations Index* e na Principal Coleção do *Web of Science*

Na pesquisa por patentes na base *Derwent Innovation Index*, observa-se que há muitas patentes relacionadas a óleo essencial, parasito e ovinos e/ou caprinos. Das 146 patentes encontradas envolvendo óleo essencial e ovinos e/ou caprinos, nota-se que apenas quatro são relacionadas a parasitos (Tabela 3).

Os dados encontrados sobre publicações científicas na Principal Coleção do *Web of Science*, observou-se que das 290 publicações envolvendo óleo essencial e ovino e/ou caprino, 37 estão relacionadas a parasitos (Tabela 3).

Tabela 3 - Pesquisa por palavras-chave na Principal Coleção do *Web of Science* e no *Derwent Innovation Index*

Palavras-chave	Principal Coleção do *Web of Science*	*Derwent Innovations Index*
Essential AND oil AND (sheep or goat)	290	146
Essential AND oil AND (sheep or goat) AND parasit*	37	4
Essential AND oil AND (sheep or goat) endoparasit*	0	1
Essential AND oil AND (sheep or goat) AND antihelmint*	2	1
Essential AND oil AND (sheep or goat) AND protozoan*	2	1
Essential AND oil AND (sheep or goat) AND helminth*	9	2
Essential AND oil AND (sheep or goat) AND ectoparasit*	7	0
Essential AND oil AND (sheep or goat) AND acaricid*	11	4
Essential AND oil AND (sheep or goat) AND acaricid* AND parasit*	5	1
Essential AND oil AND (sheep or goat) AND tick*	10	4
Essential AND oil AND (sheep or goat) AND flea*	0	2
Essential AND oil AND (sheep or goat) AND myiasi*	3	0
Essential AND oil AND (sheep or goat) AND louse*	3	0
Essential AND oil AND (sheep or goat) AND mite*	8	3
Essential AND oil AND (sheep or goat) AND mite* AND parasit*	1	0
Total	**388**	**169**

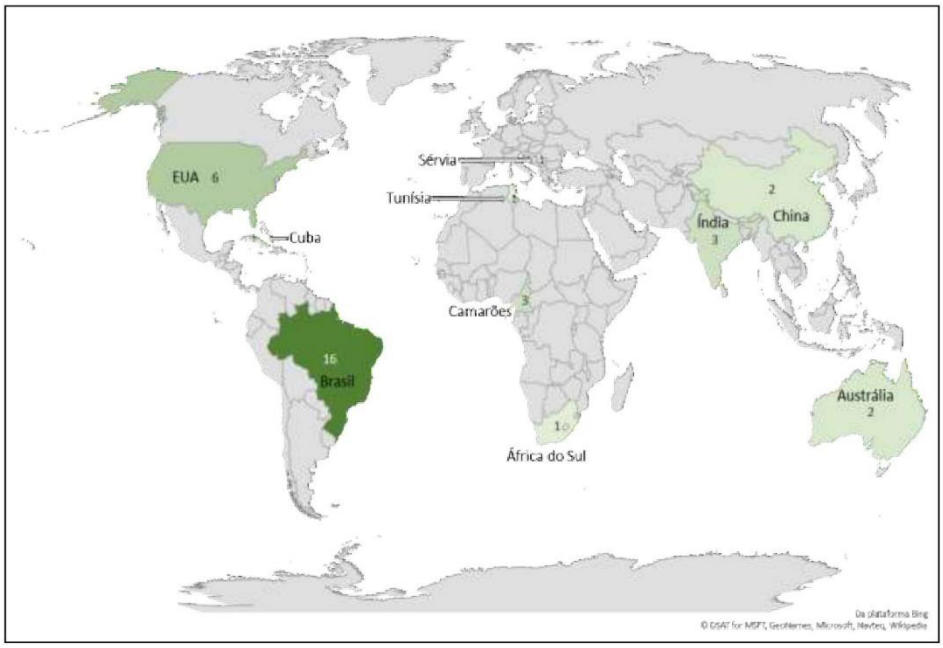
* Operador de truncagem
Fonte: *Web of Science* e *Derwent Innovation Index*, 2017

De uma forma geral, o maior número de patentes relacionado a óleo essencial no mundo é devido a sua ampla utilização para diversas finalidades, como atividade larvicida, anti-inflamatória, antioxidante, antimicrobiana, analgésica, fungicida e parasiticida (RAJKUMAR et al., 2010; WANNES et al., 2010, MENDES et al. 2010, CARMO et al., 2008, SILVA, 2008; RIBEIRO, 2013; DRUINS, 2017).

Assim como no Brasil, o depósito de patentes de produtos à base de óleos essenciais para pequenos ruminantes em todo mundo também é escasso, apesar de já estarem disponíveis no mercado produtos para ruminantes com ação anti-septica e analgésica, à base do óleo essencial óleo essencial *Lavandula angustifólia* e *Gaultheria procumbens,* respectivamente (BASER e FRANZ, 2010). Esses achados demonstram que, em detrimento da aplicabilidade, ainda há grande carência nos pedidos de depósitos de patentes nacionais e internacionais.

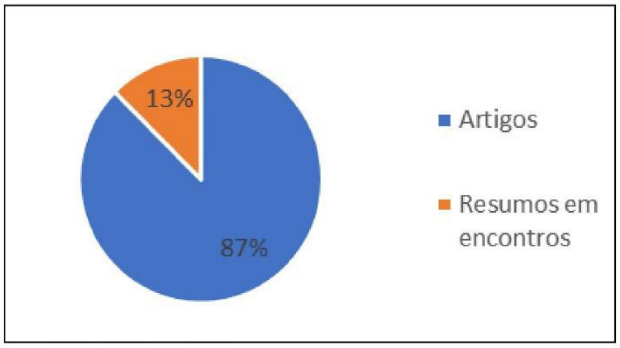
Das 35 publicações que continham as palavras óleo essencial, ovino e/ou caprino e parasit, 24 registros foram encontrados no continente americano, 5 no africano, 1 na Europa, 3 na Ásia e 2 na Oceania (Figura 1). A maioria das publicações foi encontrada no Brasil (16 registros). Dentre esses registros, 87% são artigos e 13% são resumos de encontros nacionais (Figura 2). As publicações citam óleos essenciais de plantas dos gêneros *Calotropis*, *Citrus Cymbopogon*, *Eucalyptus, Hesperozygis*, *Melaleuca*, *Mentha, Ocimum* e *Thymus*, além de citar terpenoides, componentes marjoritários dos óleos, e extratos de plantas. A grande participação brasileira nesses achados pode ser justificada pela ampla e diversidade da flora que esse país apresenta. São mais de 46 mil espécies, além do interesse dos pesquisadores brasileiros em desenvolver antiparasitários com produtos oriundos das plantas (JARDIM BOTÂNICO, 2017).

Figura 1 - Distribuição geográfica de patentes encontradas na Principal Coleção do *Web of Science*, com a pesquisa das palavras-chave (essential AND oil AND (sheep or goat) AND parasit*)



* Operador de truncagem
Fonte: *Web of Science,* 2017.

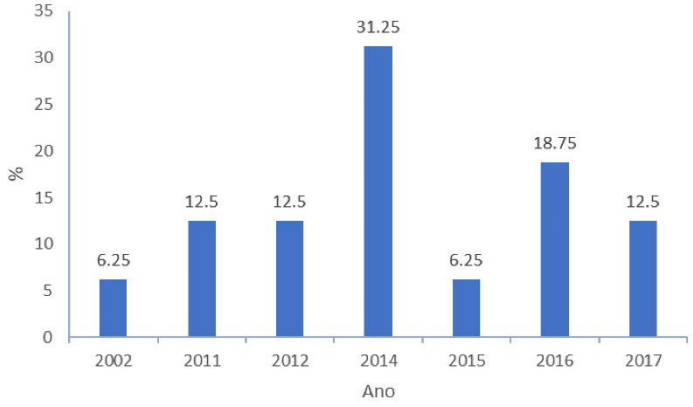
Figura 2 - Tipos de registros brasileiros de publicações na Principal Coleção do *Web of Science*, com palavras-chave (óleo and essencial and (ovino or caprino) and parasit*)



Fonte: *Web of Science*, 2017

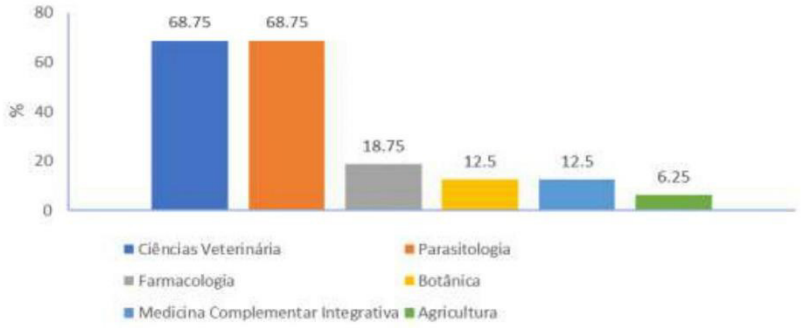
As publicações brasileiras utilizando as palavras-chave óleo essencial, ovino, caprino e parasito foram realizadas em sua maioria no ano de 2014 (31,25%), seguido do ano de 2016 (18,75%) e mais recentemente o ano de 2017 (12,5%) (Figura 3). Quanto à área de pesquisa, os trabalhos brasileiros foram mais publicados nas áreas das Ciências Veterinárias (68,8%) e na Parasitologia (68,8%) (Figura 4), demonstrando a importância científica da parasitologia e da saúde animal dentro deste contexto.

Figura 3 - Registros brasileiros de publicações, segundo o ano, na Principal Coleção do *Web of Science*, com palavras-chave (óleo and essencial and (ovino or caprino) and parasit*)



Fonte: *Web of Science*, 2017

Figura 4 – Registros/ brasileiros de publicações, segundo a área de concentração, na Principal Coleção do *Web of Science*, com palavras-chave (óleo and essencial and (ovino or caprino) and parasit*)



Fonte: *Web of Science*, 2017

4. Considerações finais

A falta de processos de patenteamento com produtos a base de óleo essencial para pequenos ruminantes demonstra a importância de se investir em mais pesquisas e em processos que incentivem o aumento de depósitos de patentes para a proteção do produto. A necessidade de se trabalhar nestes processos se deve principalmente no fato de melhor aproveitar o potencial diversificada em biomas naturais que o Brasil possui, além da importância dos pequenos ruminantes para produção de carne e leite no sul e nordeste. O maior uso de produtos contendo óleos essenciais para pequenos ruminantes é bastante promissor uma vez que, além de possibilitar alternativas naturais em detrimentos dos já utilizados, é uma forma de proteger o meio ambiente e ter subprodutos com agregado valor no mercado.

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CAPÍTULO 2

Chemical stability in rumen of terpenoids with anthelmintic activity against *Haemonchus contortus*

Os dados abaixo referentes a estabilidade de timol fizeram parte do artigo "Combination of bioactive phytochemicals and synthetic anthelmintics: *In vivo* and *in vitro* assessment of the albendazole-thymol association", publicado na revista Veterinary Parasitology – Qualis A1. Os dados de estabilidade do carvacrol ainda são inéditos.

ABSTRACT

The intensive use of synthetic drugs has led to the development of antiparasitics resistance. There is an urgent need to find novel approaches in order to ensure efficient control programs. Carvacrol (CVC) and Thymol (THY) are phenolic monoterpenes found in several essential oils of plants. This study evaluated the ruminal stability of CVC and THY against *Haemonchus contortus*. Eggs hatch and larval migration inhibition assays were performed to assess CVC and THY activity against *H. contortus*. The ruminal stability of both compounds and the relative distribution between the fluid and solid phase of ruminal content was evaluated. CVC and THY concentrations were analyzed by HPLC. CVC and THY showed a higher potency against nematode eggs than those observed against the larvae. The metabolism of both CVC and THY in the ruminal content was rather low. The unchanged CVC and THY concentrations were 84 and 95 % of the total natural products measured in control samples. CVC and THY have shown a low degree of association (31-49 %) with the particulate phase of ruminal content. Further *in vivo* trials in ruminants are needed to evaluate the potential pharmaco-chemical interactions between these monoterpenes and the traditional anthelmintic compounds.

Keywords: Carvacrol; Thymol; Ruminal metabolism; *In vitro* efficacy; Small ruminants

RESUMO

O uso inadequado de anti-helmínticos sintéticos vem selecionando parasitos resistentes, sendo necessário o desenvolvimento de programas eficientes de controle de nematoides gastrintestinais. Carvacrol (CVC) e timol (THY), monoterpenos fenólicos encontrados em óleos essenciais de plantas, foram estudados nesse trabalho. Avaliou-se a estabilidade ruminal de concentrações anti-helmínticas eficazes desses terpenóides contra *Haemonchus contortus*. Foram realizados teste de eclodibilidade de ovos e teste de inibição da migração larval de CVC e THY sobre esse nematoide gastrintestinal. A estabilidade ruminal de ambos os compostos e a distribuição relativa entre a fase sólida e o conteúdo ruminal foram analisados. As concentrações dos monoterpenos foram medidos por HPLC. CVC e THY foram mais eficientes contra ovos do que larvas. A metabolização de CVC e THY no líquido ruminal foi muito baixa, sendo que 84 e 95%, respectivamente, se mantiveram inalterados em relação ao encontrado no controle. CVC e THY mostraram baixo grau de associação (31 e 49%, respectivamente) com a fase particulada do conteúdo ruminal. São necessários ensaios *in vivo* adicionais em ruminantes para avaliar as potenciais interações farmacoquímicas entre estes monoterpenos e os compostos anti-helmínticos tradicionais.

Palavras-chave: carvacrol; timol; metabolismo ruminal; eficácia *in vitro*; pequenos ruminantes

INTRODUTION

Parasitic diseases are a relevant problem affecting domestic animals and humans. *Haemonchus contortus* is particularly pathogenic to small ruminants in tropical and temperate farming areas due to its intense hematophagous habits, (Emery et al., 2016). Nematode control in livestock is primarily based on the use of antiparasitic drugs. The overuse of synthetic drugs for many years has led to the development of antiparasitics resistance in different countries (Kaplan & Vidyashankar, 2012). Therefore, there is an urgent need to find novel approaches in order to ensure efficient control programmes.

The essential oils of a wide variety of plants or the administration of their secondary metabolites could be an interesting alternative for parasite control (Hoste & Torres-Acosta, 2011; Oliveira et al., 2017). Among the wide variety of existing secondary metabolites, monoterpenes are short chain hydrocarbons compounds with many corroborated pharmacological effects (Rajput et al., 2018). Carvacrol (CVC) and Thymol (THY) are phenolic monoterpenes found in several essential oils of plants belonging to the genera *Origanum* and *Thymus*, respectively (Elandalousi et al., 2013; Guimarães et al., 2015). Both compounds have shown *in vitro* activity against different stages of *H. contortus* (Andre et al., 2016; Ferreira et al., 2016; Katiki et al., 2017). However, in order to obtain an optimal *in vivo* efficacy against ruminant nematodes, monoterpenes must reach the host target sites where the nematodes are present without being degraded following their oral administration. The process of ruminal fermentation affects food components ingested by the host. This fermentation process may also modify drugs used to treat different diseases (Wu & Papas, 1997). A wide degree of ruminal degradation of several terpenes was observed after their incubation in goat ruminal fluid (Malecky, et al., 2012). However, information on the metabolic fate of CVC and THY in the rumen, in particular their individual degradation rates, is needed before these compounds could be used *in vivo.* Therefore, the objective of this study was to evaluate the chemical stability of anthelmintically effective concentrations of CVC and THY in the sheep ruminal content.

Additionally, the relative portioning of both phenolic monoterpenes between the fluid and particulate phases of the ruminal content was evaluated.

MATERIALS AND METHODS

In vitro evaluation of anthelmintic activity of carvacrol and thymol

Egg hatch and larval migration inhibition assays were performed to assess CVC and THY activity against *H. contortus*. This work was done with the acceptance of the Ethics Committee on Animal Use – UFMA, Brazil, under number 23115.005443/2017-51 protocol. Eggs and larvae were obtained from fresh feces of sheep artificially infected with *H. contortus*.

The egg hatch test (EHT) of *H. contortus* was carried out according to Coles et al. (Coles et al., 1992). CRC and THY were diluted in 1% Tween 80 to obtain a concentration range between 2,500 and 75 µg/mL. Approximately 100 eggs/well were placed in 96 well plates adding the different dilutions (four replicates). The plate was incubated in an incubator (27 °C, relative humidity > 80%), during 48 hours and the eggs and larvae were quantified under an inverted microscope. The assay was repeated three times.

For larval migration inhibition assay, L3 larvae were unsheathed with 2% sodium hypochlorite and sodium chloride solution and then washed in distilled water. CRV and THY were diluted in 3% Tween 80 to obtain six concentrations between 10,000 and 312.5 µg/mL. In total, 500 larvae were added to the mixture and incubated for 2 hours (27 °C and RH > 80%). To assess migration, the larvae were placed in an apparatus of 20-µm granulometric mesh in a 96-well plate containing the six dilutions, with four replicates, for another 2 hours. Thereafter, the apparatus was washed with distilled water in 24-well plates, and the larvae that migrated and did not migrate were counted (Rabel, Mcgregor, & Douch, 1994). The migration assay was repeated three times.

Ruminal stability

Experimental animals and collection of ruminal content

Four (4) healthy untreated Corriedale sheep (30–40 kg) were used for obtaining ruminal contents. Animal procedures were carried out according to the Animal Welfare Policy (Academic Council Resolution 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar). Animals were housed in stalls, fed with high-quality lucerne hay and provided with water *ad libitum*. Ruminal fluid was collected from sheep by an oesophageal tube, filtered through hydrophilic gauze and kept at 37 ºC. The incubation process was performed immediately after obtaining the ruminal content. Incubations were done by duplicated.

Evaluation of ruminal stability of CVC and THY

Stock solutions of CVC and THY (Sigma-Aldrich Chemical Co.,St. Louis, MO, USA) were prepared in methanol at 32 mg/mL and conserved at -18 °C. Ruminal metabolism was studied using the technique described by Virkel et al. (2002). Either CVC or THY dissolved in 50 µL of methanol were added to 2 mL of ruminal content to reach a final concentration of 0.8 mg/mL. Unfortified ruminal content samples with the same volume of methanol were incubated as blanks. Control samples of boiled ruminal content were prepared and incubated under the same conditions to measure CVC and THY concentrations in the absence of active ruminal bacteria. Incubation tubes with ruminal content were gassed with pure N_2 and then they were shaken in a water bath at 38 ºC under anaerobic conditions for 30, 120 and 240 min.

The anthelmintic drug albendazole sulphoxide (ABZSO), which is metabolized to albendazole (ABZ) by the ruminal bacteria (Lanusse et al., 1992), was used to assess the metabolic viability of the ruminal content (positive control). Four replicates of ruminal content

151 spiked with 40 µM of ABZSO (dissolved in 50 µL of methanol) were incubated for 30 min.
152 Additionally, ABZSO was also incubated for 30 min with an aliquot of ruminal content kept
153 during 240 min at 38 ºC in anaerobiosis. After each incubation period, an aliquot (1 mL) was
154 frozen at -20 ºC until high-performance liquid chromatography (HPLC) analysis.
155
156 Relative distribution between the fluid and solid contents
157 To evaluate the relative partition of CVC and THY between the fluid and solid phases
158 of ruminal contents, both compounds were incubated in ruminal content for 30, 120 and 240
159 min as described above. From each incubation vial 0.5 mL was taken and centrifuged at 18,000
160 g for 8 min to separate the solid (particulate) and fluid phases of the ruminal content (Hennessy
161 et al., 1994). Both phases were frozen at -20 °C until HPLC analysis.
162
163 **Drug extraction and chromatographic analysis**
164 A liquid-liquid extraction of fortified and experimental samples was performed to
165 determine CVC and THY concentrations from the ruminal content. An aliquot of 0.5 mL of
166 each sample was mixed with 0.5 mL of cold acetonitrile (Baker Inc., Phillipsburg, NJ, USA)
167 and mixed for 10 min (Multi-Tube Vortexer; VWR Scientific Products, Wilmington, NC,
168 USA). The solvent-sample mixture was centrifuged at 6,000 g for 10 minutes at 5 °C. The
169 supernatant was transferred into a tube and ultrapure water was added to a final volume of 1
170 mL. CVC and THY were analyzed by HPLC following the technique described by Shekarchi
171 et al. (2010). An aliquot (50 µL) of each sample was injected directly into the chromatograph
172 (Shimadzu 10 A HPLC system, Shimadzu Corporation, Kyoto, Japan). The HPLC analysis was
173 performed using a reverse phase C18 column (Kromasil, Eka Chemicals, Bohus, Sweden, 5
174 µm, 4.6 mm x 250 mm). The mobile phase was acetonitrile (53 %) and ultrapure water (47 %),

175 pumped at a flow rate of 1.5 mL/min. Both analytes were measured by a UV detector (SPD-
176 10A; Shimadzu) reading at 274 nm.

177 ABZSO and its parent drug ABZ were analyzed by HPLC from positive control samples
178 following the methodology described by Lanusse et al. (1992) with modifications. Validation
179 of the analytical procedures for quantification of CVC and THY was performed. Known
180 amounts of each analyte were added to aliquots of boiled (inactivated) samples of ruminal
181 content. The fortified samples were extracted and analyzed by HPLC (3 replicates) to obtain
182 calibration curves and determine the percentages of recovery and the precision of the method.
183 Calibration curves were prepared using the least squares linear regression analysis (Instat 3.00,
184 GraphPad Software, Inc., San Diego, CA, USA) of HPLC peak area of analytes and nominal
185 concentrations of spiked samples. The range of the calibration curves was between 100 and 800
186 µg/mL. The departure from linearity of the calibration curves was determined using ANOVA
187 and runs test (Instat 3.0; GraphPad Software, Inc.).

188

189 **Data analysis**

190 In the egg hatch and larval migration inhibition assays, the mean of each treatment was
191 compared to its respective control. The data were initially transformed to Log(X), normalized
192 and then a nonlinear regression was carried out to obtain the IC_{50} (50% of maximal inhibitory
193 concentration) for CVC and THY using GraphPad Prism 7.0 software (GraphPad Inc., San
194 Diego, CA, USA).

195 The percentages of unchanged parent drug (CVC and THY) were determined by
196 comparison between the concentrations measured in the incubated samples and those in control
197 (inactivated) samples of ruminal content. Concentrations of CVC and THY determined in the
198 fluid and solid phases of ruminal contents were expressed as a percentage (mean ± SD) of the
199 total drug measured. The amount of ABZ formed in the positive control samples was expressed

as the percentage of the total amount of analytes (substrate plus metabolite) detected after the incubation period. Data were statistically compared by Student t-test (Instat 3.0; Graph Pad software Inc.). A value of $P < 0.05$ was considered significant.

RESULTS

CVC and THY demonstrated efficiency against *H. contortus*. IC_{50} in EHT was 185.9 µg/mL (±57.9) for CVC and 187.0 µg/mL (±7.9) for THY. Similarly, CVC and THY showed activity on larvae motility with an IC_{50} of 1,785.3 µg/mL (±372.7) and 1,846.6 µg/mL (±968.7), respectively (Table 1). The analytical method for measuring CVC and THY in samples of ruminal content by HPLC was validated. The correlation coefficients of the calibration curves were > 0.99 and there was no significant departure from linearity. The mean absolute recovery of CVC and THY from ruminal content was 82 % and 88 %, respectively. The precision of the method showed a CVs of 7.41 % (CVC) and 4.62 % (THY). The limits of detection and quantification for both molecules were 0.5 µg/mL and 2 µg/mL, respectively.

Immediately after the collection of the ruminal content, the percentage of ABZSO reduced into ABZ under anaerobic conditions was 58% in samples incubated during 30 min. In the samples kept at 38 ºC during 240 min before the metabolic assay, a 55% of the substrate was reduced into ABZ after 30 min of incubation This result demonstrated the metabolic viability during the whole period of incubation of the natural products. Further, the metabolism of both CVC and THY in the ruminal content was rather low. The unchanged CVC and THY concentrations were between 629 µg/mL and 758 µg/mL, representing 84 and 95 %, respectively, of the total natural products measured in control (inactivated) samples. Small peaks of novel products were observed in the chromatograms of CVC and THY incubated in the active ruminal content. These hypothetical metabolites represented 0.20 % of the total amount of products. Figure 1 shows the chromatograms of CVC and THY incubated in the

225 blank (not fortified), control (boiled/inactive) and active samples of ruminal contents. The CVC
226 and THY concentrations obtained after their incubations in control and active ruminal content
227 are shown in Figure 2.

228 A partition between the solid and fluid phases of the ruminal content was observed for
229 CVC and THY. Higher concentrations of CVC ($P < 0.05$) were measured in the fluid phase
230 compared to the solid phase of the ruminal content. The percentage recovered in the fluid phase
231 was between 66 and 69 % of the total CVC recovered. There was no effect of the incubation
232 time on the relative distribution of CVC in the ruminal content. The concentrations of THY in
233 the fluid phase of the ruminal content were higher compared to the solid phase after 30 min and
234 120 min of incubation, but they were similar after 240 min of incubation in the ruminal content.
235 The CVC and THY concentrations in the fluid and solid phases of ruminal contents at the
236 different incubation times are shown in Table 2.

237

238 **DISCUSSION**

239 In the current trial, the activity of CVC and THY was corroborated by assessing
240 nematode egg hatching and larval motility. A higher potency was observed on nematode eggs
241 compared with the effect on larvae, with an-IC_{50} that was 10-fold lower for both compounds
242 under study. The comparison of the potency of CVC and THY showed a similar IC_{50} for both
243 compounds (Table 1). The IC_{50} obtained in the current trial for the activity of CVC and THY
244 on eggs hatching was in the similar range than those previously reported (Andre et al., 2016;
245 André et al., 2017; Katiki et al., 2017). Ferreira et al. (2016) reported a higher THY IC_{50} for the
246 egg hatching (442 µg/mL) and lower (497 µg/mL) against larvae motility compared to those
247 obtained in the current study. This divergence may be related to the differences in the
248 methodology used for the *in vitro* assays or genetic differences among the strains.

Although *in vitro* assays supply useful information on the activity of monoterpenes, it is necessary to know the fate of these compounds when they are administered *in vivo*. This issue is relevant as active compounds need to attain effective concentrations at the sites of parasite location for a certain period of time (Lifschitz et al., 2000). Besides the metabolism in the liver, drugs are biotransformed in extra-hepatic tissues such as the gastrointestinal tract, particularly in the rumen (Renwick et al., 1986). Ruminal metabolism may reduce the bioavailability of orally administered compounds (Irazoqui et al., 2015; Vynckier & Debackere, 1993), 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 current trial evaluated the chemical stability of CVC and THY in the sheep ruminal environment. The metabolic activity of the ruminal content was clearly demonstrated by incubating ABZSO as a positive control. Thus, the reduction of ABZSO to ABZ reflects the metabolic activity of the ruminal microflora (Lanusse et al., 1992).

CVC and THY were stable in the ruminal content of sheep. The concentrations of CVC and THY recovered after the incubation of both compounds in the active ruminal content were between 84-91 % and 90-95 % of the control samples, respectively. The ruminal degradation of these natural phytochemicals was higher for CVC than for THY. Although the "new unknown peaks" observed in the chromatograms of samples incubated with metabolically active ruminal content represent a very low percentage (0.20 %) of the total parent compound, it is possible that some degradation products of the natural compounds were not detected under the chromatographic conditions used in the current assays. The percentages of the intact CVC and THY after their incubation in the ruminal content of sheep were similar to those obtained for monepantel (93 %) (Ballent et al., 2016) and lower than those measured for IVM (98 %)

273 (Lifschitz et al., 2005), two anthelmintic molecules metabolically stable in the rumen after their
274 oral administration.

275 Among the different factors that may affect the bioavailability of orally administered
276 compounds in ruminants, the degree of adsorption to the particulate material of gastrointestinal
277 contents plays a relevant role in their kinetic disposition. The degree of adsorption of different
278 synthetic anthelmintics to the particulate material of ruminal content was between 90 and 99 %
279 (Ali & Hennessy, 1995; Lifschitz et al., 2005; Ballent et al., 2016). The bioactive terpenoids
280 under study in the current experiment showed significantly lower concentrations associated to
281 the particulate material compared to those measured in the fluid phase of the ruminal content
282 (Table 2). The percentage of association to the particulate phase was higher for THY (43-49 %)
283 than those observed for CVC (31-34 %). Whereas for benzimidazole anthelmintics the
284 association to the particulate material of rumen benefits the absorption process (Lanusse et al.,
285 2018), the high degree of association in the case of the macrocyclic lactones may decrease the
286 systemic availability (Lifschitz et al., 2005). CVC and THY are mainly kept in the fluid phase
287 of ruminal content, that may imply a shorter residence time in the rumen and a faster flow rate
288 to the abomasum and small intestine.

289 The search of novel molecules with different mechanisms of action than the traditional
290 drugs is important for the future control of nematodes. Different plant secondary metabolites
291 have demonstrated anthelmintic effects. Whereas the pharmacological features of anthelmintic
292 drugs and their impact on sustained parasite control in ruminants have been extensively
293 reviewed (Lanusse et al., 2018), the information that describes the fate of natural bioactive
294 products in ruminants is scarce. This information is relevant as monoterpenes with *in vitro*
295 anthelmintic activity require effective *in vivo* drug concentrations in order to inhibit the
296 development of nematodes in the host. The current work is a first great step addressed to
297 understand the kinetic fate of CVC and THY in the sheep ruminal environment. Both

298 compounds were chemically stable in a large proportion in metabolically active ruminal
299 content. Moreover, CVC and THY have shown a lower degree of association with the
300 particulate phase or ruminal content compared with other synthetic anthelmintic drugs. Further
301 *in vivo* trials in ruminants are needed to evaluate the kinetic behavior of these bioactive
302 monoterpenes as well as to assess potential pharmaco-chemical interactions if they are
303 combined with traditional synthetic anthelmintic compounds.

304

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410 **Table 1.** Effects of monoterpenes on the egg hatch test (EHT) and the larval migration
411 inhibition test (LMIT) of *Haemonchus contortus*.

EHT			LMIT		
Concentration (µg/mL)	**Efficiency % ± SD**		**Concentration µg/mL**	**Efficiency % ± SD**	
	CVC	**THY**		**CVC**	**THY**
2500.0	-	99.8 ± 0.3	10000.0	89.9 ± 7.2	85.0 ± 13.2
1250.0	100.0 ± 0.0	99.5 ± 0.4	5000.0	80.1 ± 11.6	81.6 ± 13.3
625.0	99.8 ± 0.3	98.9 ± 1.1	2500.0	60.2 ± 2.2	70.9 ± 21.0
312.5	88.8 ± 6.1	76.1 ± 9.1	1250.0	41.6 ± 9.7	39.4 ± 16.7
156.3	35.9 ± 29.1	42.4 ± 6.0	625.0	20.9 ± 4.1	23.7 ± 12.4
78.1	7.4 ± 7.3	4.6 ± 3.4	312.5	7.4 ± 2.2	6.5 ± 2.6
39.1	2.9 ± 3.3	-	-	-	-
IC_{50} ± SD	185.9 ± 57.9	187.0 ± 7.9	**IC_{50} ± SD**	1785.3 ± 372.7	1846.6 ± 968.7

412 **IC_{50} –** 50% of maximal inhibitory concentration

413 **SD -** Standard deviation

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Table 2. Mean concentrations (± SD) of carvacrol (CVC) and thymol (THY) associated to the particulate (PP) (solid) and fluid phases (FP) of sheep (n=4) after 30, 120 and 240 minutes of incubation. The percentage of the compounds associated with each phase is also indicated.

Incubation Time (min)	**CVC Concentrations (µg/mL)**		**THY Concentrations (µg/mL)**	
	FP	**PP**	**FP**	**PP**
30	456 ± 37.3* (66 %)	237 ± 46.8 (34 %)	388 ± 23.8* (57 %)	298 ± 18.2 (43 %)
120	449 ± 38.2* (68 %)	216 ± 18.4 (32 %)	380 ± 22.5* (54 %)	326 ± 19.3 (46 %)
240	449 ± 9.37* (69 %)	203 ± 4.08 (31 %)	360 ± 10.3 (51 %)	344 ± 10.0 (49 %)

Data are the mean concentrations measured in samples of ruminal contents taken from 4 animals

*Mean concentrations associated with the fluid phase are statistically different (P<0.05) vs. those observed in the particulate phase.

444 **FIGURE LEGENDS**

445

446 **Figure 1** Typical chromatograms after carvacrol (1a) and thymol (1b) incubation in ruminal
447 content during 240 min): a) unfortified blank, b) carvacrol or thymol incubated in metabolically
448 active ruminal content and c) carvacrol or thymol incubated in inactive (boiled) ruminal content

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451 **Figure 2** Mean (± SD) carvacrol (CVC) (a) and thymol (b) concentrations observed after its
452 incubation (between 30 and 240 min) in metabolically active and inactive (boiled) sheep
453 ruminal content under anaerobic conditions. The percentages of unchanged parent drug
454 recovered from the incubation assays are shown in brackets

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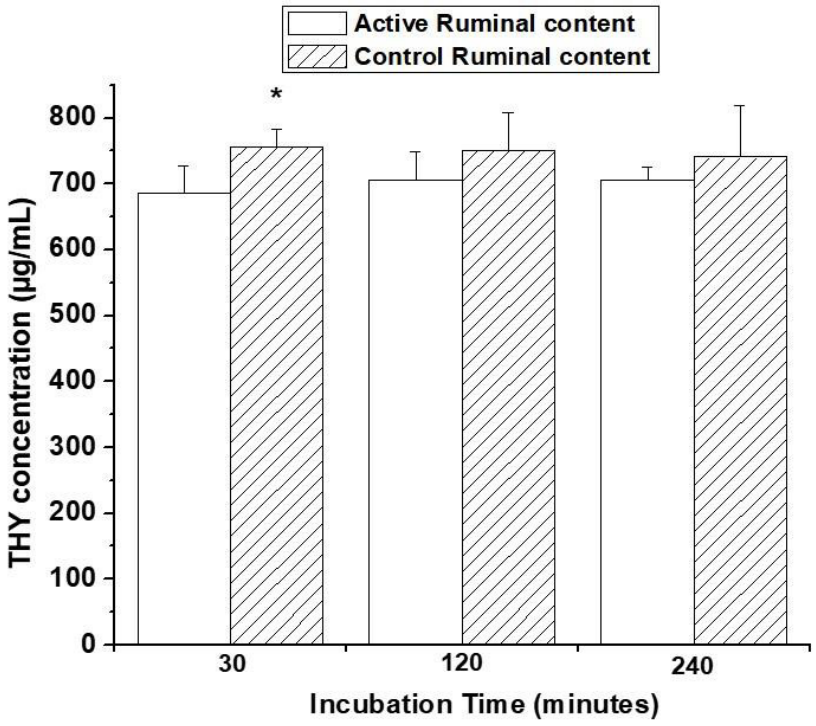
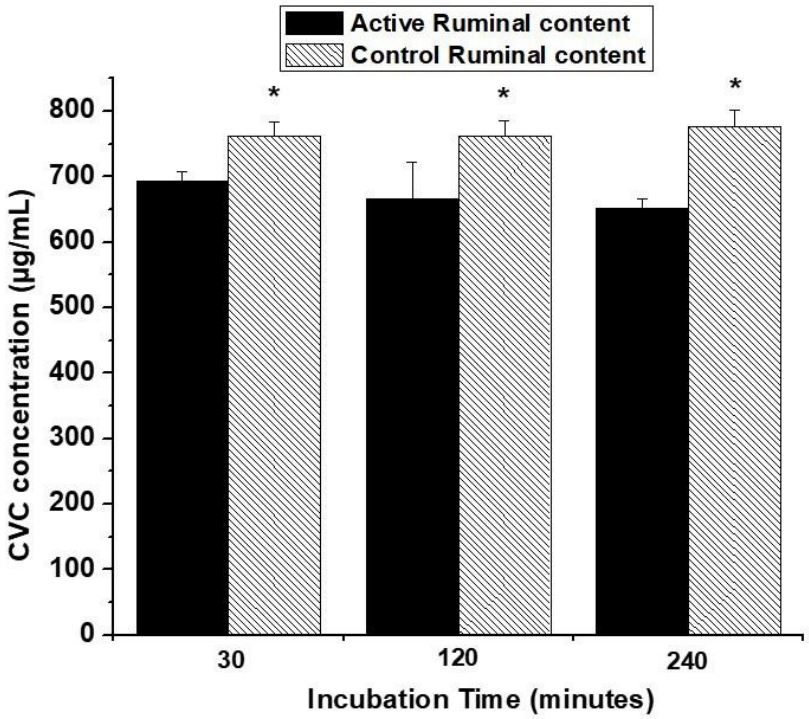
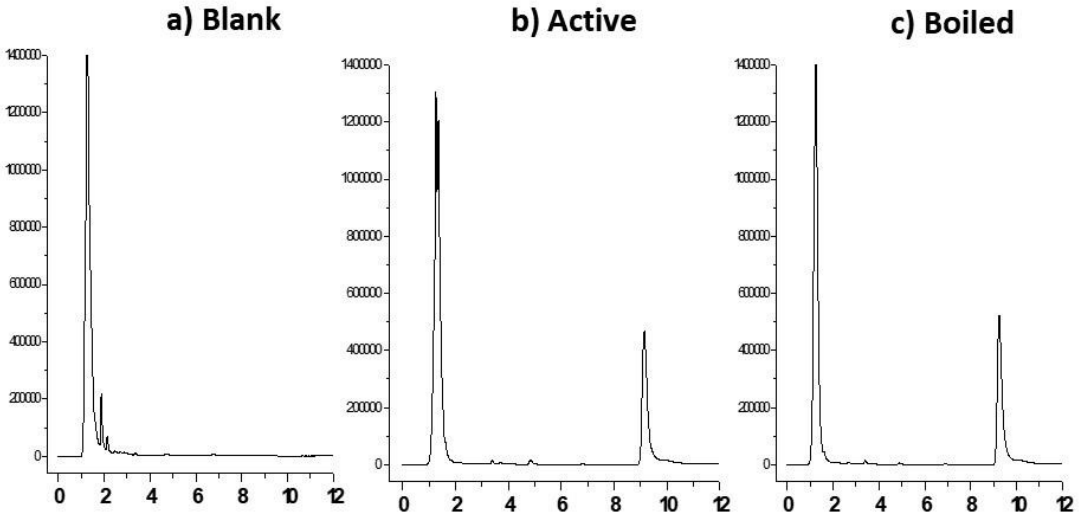
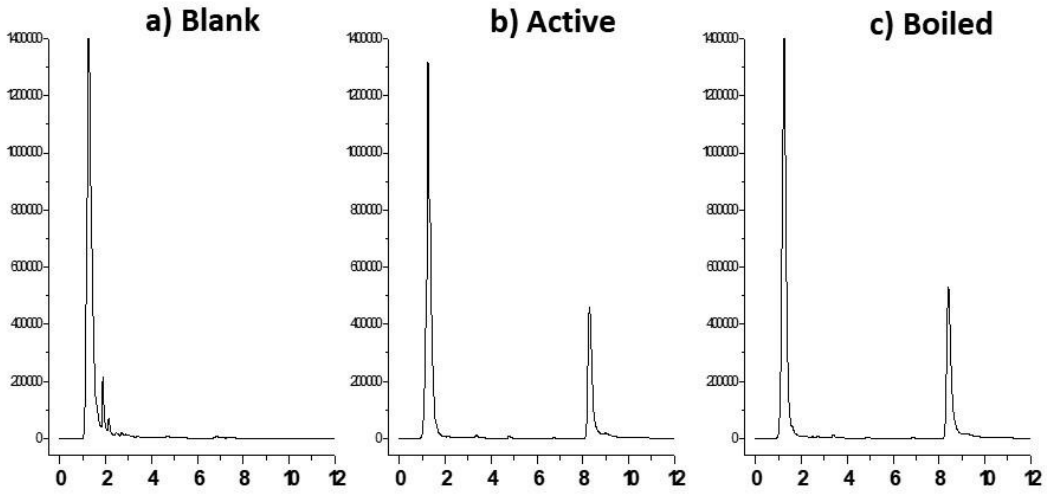

Active Ruminal content
Control Ruminal content
THY concentration (µg/mL)
0
100
200
300
400
500
600
700
800
*
30
120
240
Incubation Time (minutes)


Active Ruminal content
Control Ruminal content
CVC concentration (µg/mL)
0
100
200
300
400
500
600
700
800
*
*
*
30
120
240
Incubation Time (minutes)

RUMINAL CONTENT: Thymol incubation



RUMINAL CONTENT: Carvacrol incubation



CAPÍTULO 3

Combination of synthetic anthelmintics and monoterpenes: Assessment of efficacy and ultrastructural and biophysical properties of *Haemonchus contortus* using atomic force microscopy

Artigo submetido ao periódico Veterinary Parasitology - Quallis A1

Combination of synthetic anthelmintics and monoterpenes: Assessment of efficacy and ultrastructural and biophysical properties of *Haemonchus contortus* using atomic force microscopy

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Abstract

The resistance of *Haemonchus contortus* to synthetic anthelmintic is of increasing concern, and different strategies are being evaluated to improve parasite control. The present study investigated the in vitro effect of combinations of synthetic compounds and monoterpenes. The chemical association of the best combinations and their impact on the ultrastructural and biophysical properties on eggs of *H. contortus* were also evaluated. The monoterpenes carvacrol, thymol, r-carvone, s-carvone, citral and p-cymene, and anthelmintic albendazole and levamisole were assessed using the egg hatch test (EHT) and larval migration inhibition test (LMIT), respectively. The lowest effective concentration of monoterpene by the EHT (< 10% of efficacy) and LMIT (< 14% of efficacy) was applied in combination with different concentrations of synthetic compounds, and the IC50 and synergism rate (SR) were calculated. For analyzing the chemical association between the best combinations according to the in vitro tests (albendazole and levamisole with r-carvone and s-carvone), Fourier-transform infrared spectroscopy (FTIR)was utilized. The atomic force microscopy (AFM) was used to assess the ultrastructural and biophysical properties of *H. contortus* eggs treated with albendazole and r-carvone combination. The highest efficiency of monoterpenes in the EHT was obtained with carvacrol (IC50 = 185.9 µg/mL) and thymol (IC50 = 187.0 µg/mL) and in the LMIT with s-carvone and carvacrol (IC50 = 1526.0 and 1785.3 µg/mL, respectively). The combination with albendazole in the EHT showed synergism for r-carvone (SR= 3.8), and s-carvone (SR= 3.0). The results of the LMIT for levamisole in combination with monoterpenes showed the best association for r-carvone (SR = 1.7) and s-carvone (SR = 1.7). FTIR of albendazole, as well as levamisole associated with r-carvone and s-carvone indicated the establishment of chemical interactions between both synthetic and natural molecules, contributing to a possible synergistic effect of these associations. Eggs treated with albendazole and r-carvone showed an increase in the roughness and decreased in the height, suggesting damage to the egg surface and overflow

of internal content occasioning by the treatment. Overall, the combination of albendazole with r-carvone and s-carvone was efficient on *H. contortus*, having a chemical association between the compounds and significant changes in egg ultrastructure that justify the efficacy.

Keywords: monoterpenes; anthelminthic resistance; carvone; levamisole; albendazole; AFM.

1. Introduction

Haemonchus contortus is one of the most pathogenic gastrointestinal nematodes (GINs) of small ruminants, causing substantial economic losses to the livestock industry worldwide (Climeni et al., 2008; Raza et al., 2016). Antiparasitic drugs have been developed since 1960, including benzimidazoles, imidazothiazoles and macrocyclic lactones, which are the most important chemical families for the control of helminth infections (Alvarez et al., 2007; Amarante, 2015).

Although the use of synthetic anthelmintic has had satisfactory results, an indiscriminate application has led to the selection of resistant helminths. Indeed, anthelmintic resistance is a problem in animal production around the world (Demeler et al., 2012). To overcome this situation, alternative pharmacological and epidemiological strategies are currently being investigated. The combination of natural compounds with synthetic anthelmintics appears to be a highly feasible alternative (Lanusse et al., 2018).

Plants are used to treat several diseases due to their various pharmacological properties, producing bioactive compounds synthesized as essential oils (Ribeiro et al., 2013), the main compounds of which are monoterpenes. These compounds exhibit notable pharmacological properties such as antifungal, antibacterial, antioxidant, anticancer, antispasmodic, hypotensive and antiparasitic activities (Santos et al., 2011). *In vitro* and *in vivo* tests have corroborated the potent anthelmintic effect of monoterpenes of essential oils from different sources (Andre et al., 2016; Trailović et al., 2015; Echeverrigaray et al., 2010). However, there is an associated high cost of isolating individual monoterpenes, limiting their practical use in livestock production.

The combination of anthelmintic molecules has been proposed as a beneficial strategy because each product possesses a different mechanism of action, which may help in delaying parasitic resistance (Lanusse et al., 2018). Furthermore, combining monoterpenes with

synthetic anthelmintic products may be an interesting strategy to achieve interaction with the same target at different binding sites (Ferreira et al., 2016), and potential additive or synergistic effects between natural and synthetic products should be evaluated against resistant parasites. Synergism between terpenes with antibiotics has been demonstrated for resistant bacteria (Honório et al., 2015; Ribeiro et al., 2013) Monoterpenes may constitute an alternative for the control of GINs, and these compounds may act synergistically with synthetic antiparasitic drugs.

Light and electron microscopy has been successfully used to assess, in micrometer scales, damage or structural parameters of *H. contortus* in different life cycle stages in several in vitro studies (Campos et al., 2008; Engstrm et al., 2016). However, some limitations of these techniques are that the difficulty of the advances in understanding the effect of different compounds on *H. contortus*. Atomic force microscopy (AFM) is a nanometer-scale technique with high resolution that provides information about the topography and mechanical characteristics from a wide variety of materials. Studies using AFM with nematode still scarce and very new and challenging. Recently, Costa-Junior et al. (2020) described the ultrastructural and biophysical properties of several stages from *H. contortus* open the possibility to studies to understanding the effect of the synthetic or natural compounds on this parasite. The present study investigated the in vitro effect of combinations of synthetic compounds and monoterpenes. The chemical association of the best combinations and their impact on the ultrastructural and biophysical properties on eggs of *H. contortus* were also evaluated.

2. Materials and methods

2.1. Products

The synthetic compounds albendazole and levamisole and the natural monoterpenes carvacrol, thymol, r-carvone, s-carvone, citral and p-cymene (Figure 1) were purchased from

Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). *In vitro* tests were performed using stock solutions of albendazole and levamisole, each was diluted in dimethylsulfoxide (DMSO), with the concentration of 10000 µg/mL and stored at 6 ºC. Initially, the tests were performed with the isolated products to obtain the IC_{50}, and the lowest effective concentration of monoterpene was then used for evaluating combinations with the synthetic anthelmintic products.

2.2. *Haemonchus contortus* strain

This experiment was approved by the Ethics Committee on Animal Experimentation of Federal University of Maranhão, Brazil under protocol number 23115.005443/2017-51. Adult worms of *H. contortus* were collected of goat abomasum in slaughterhouses of Maranhão state, Brazil. The worms were macerated, mixed with goat feces uninfected and L3s were obtained according to Robert and O'sullivan (1950) and Ueno and Gonçalves (1998).

2000 L3 of *H. contortus* were used for the experimental lambs infection. Lambs received hay and water *ad libitum* and 1% live weight of commercial feed with 20% of crude protein. The infection of *H. contortus* was confirmed with fecal eggs count and fecal culture (Robert and O'sullivan, 1950; Ueno and Gonçalves, 1998).

2.3. Acquisition of eggs and third-stage larvae (L3)

H. contortus eggs were recovered from artificially infected sheep by the methodology described by Coles et al. (1992). Briefly, fresh feces samples from artificially infected lambs were macerated, washed with distilled water and passed through 1-mm, 105-µm, 55-µm and 25-µm graduated screens. The eggs were suspended in saturated saline and then washed with distilled water.

H. contortus L3 larvae were obtained from artificially infected sheep according to Roberts and O'Sullivan (1950) and Baermann technique (Ueno and Gonçalves, 1998). In brief,

133 feces samples from lambs artificially infected with *H. contortus* were macerated, mixed with
134 vermiculite, placed in glass beakers and incubated at 27°C for 15 days. The beakers containing
135 the feces were then filled with warm water, the contents were poured into Petri dish, and the L3
136 larvae could migrate. After two hours, the larvae were placed in Falcon tubes and stored at 6°C
137 until use in *in vitro* tests.
138
139 2.4. Egg hatch test (EHT)
140 Monoterpenes were diluted in 3% Tween-80. Six serials dilutions of the monoterpenes
141 and ten serials dilutions of albendazole were used to evaluate the efficacy of the compounds
142 separately (Table 1). Approximately 100 eggs/well were placed in 96-well plates, and the
143 different dilutions were added (four replicates). The plate was incubated at 27°C and Relative
144 Humidity (RH) > 80%; after 48 hours, the eggs and larvae were quantified under an inverted
145 microscope (Coles et al., 1992). The tests were repeated three times.
146 The EHT was also carried out with the combination of monoterpenes and albendazole.
147 The lowest effective concentration of each monoterpene in the EHT (< 10% of eggs hatch
148 inhibited) was used as a fixed value in combination with serial dilutions of the synthetic
149 anthelmintic product (ten concentrations) (Table S1).
150
151 2.5. Larval migration inhibition test (LMIT)
152 L3 larvae were unsheathed with 2% sodium hypochlorite and sodium chloride solution
153 and then washed in distilled water. Monoterpenes were diluted in 3% Tween-80, with six serial
154 dilutions (Table 1). The test with levamisole alone was realized with 10 serial dilutions (Table
155 1). In total, 500 larvae were added to the mixture and incubated for 3 hours at 27°C and RH>
156 80%. The larvae were placed in an apparatus of 20-µm granulometric mesh in a 96-well plate
157 containing the six dilutions, with four replicates, for another 2 hours for migration. Thereafter,

158 the apparatus was washed with distilled water in 24-well plates, and the larvae that migrated
159 and did not migrate were counted (Rabel et al., 1994). The tests were performed with three
160 replicates.

161 To perform the test with the combinations, the lowest effective concentration of
162 monoterpene in the LMIT (≤ 14 % of inhibition larvae migration) was used as a fixed value in
163 combination with serial dilutions of levamisole (Table 1).

164

165 2.6. Data analysis of EHT and LMIT

166 The mean of each treatment was compared to its respective control in egg hatch test
167 (monoterpenes and albendazole) and larval migration inhibition test (monoterpenes and
168 levamisole). The data were initially transformed to Log(X), normalized and then nonlinear
169 regression was calculated to get IC50 (50% inhibition concentration) using GraphPad Prism 7.0
170 software (GraphPad Inc., San Diego, CA, USA). The synergism rate (SR) was calculated using
171 the formula: IC_{50} synthetic product alone / (IC_{50} combination of synthetic product +
172 monoterpene).

173

174 2.7. Fourier-transform infrared spectroscopy (FTIR)

175 For analyzing the chemical association between synthetic anthelmintic and
176 monoterpenes Fourier-transform infrared spectroscopy (FTIR) was utilized. The combinations
177 evaluated in this study were albendazole and r-carvone, albendazole and s-carvone, levamisole
178 and r-carvone, and levamisole and s-carvone, which were the best combinations according to
179 the *in vitro* tests (Table 3). For this study, combinations were prepared in the same way of the
180 first dilution of albendazole and levamisole association on *H. contortus in vitro* tests, diluted
181 only in DMSO 3%. The analysis was recorded with a Prestige-21 IR spectrometer (Shimadzu,
182 Tokyo, Japan) in the spectral range of 4000 - 400 cm^{-1} at a resolution of 2 cm^{-1} (16 scans). In

183 general, solid samples were diluted in KBr (~ 1%) and formed as pellets under a pressure of 10
184 Ton; 5 µL of liquid sample was placed on the KBr pellets.
185
186 2.8. Atomic force microscopy (AFM) analysis
187 The AFM analysis was performed to assesses the topography and mechanical
188 characteristics of eggs treated with albendazole 5 µg/mL, r-carvone 156 µg/mL, and the
189 combination of albendazole and r-carvone which had the highest synergic effect in vitro (Table
190 3). Eggs treated with 3% DMSO, 3% Tween-80, and the combination 3% Tween-80 and 3%
191 DMSO, which were used to dilute albendazole, r-carvone, and the combination respectively
192 were used as control. The eggs were incubated at 27°C and Relative Humidity (RH) > 80%
193 until larvae eggs stages. Eggs treated with albendazole and the combination albendazole and r-
194 carvone were incubated for 48 hours to allow the possibility of hatching, which did not occur.
195 All eggs were collected and fixed in 5% formalin (Costa-Júnior et al., 2020).
196 AFM measurements were performed in 10 eggs/sample using a Multimode 8
197 microscope (Bruker, Santa Barbara, CA) in PeakForce Tapping Quantitative Nanomechanics
198 mode, using probes model SCANASYST-AIR (Bruker), with nominal spring constant of 0.4
199 N/m and nominal tip ratio of approximately 2 nm, however, the actual spring constant of each
200 probe used in this work was measured by the thermal noise method. Images were taken at
201 approximately the center region of the eggs, with a size of 5x5 µm. The resolution of images
202 was 256 lines per sample. The data of height was obtained through ASCII matrices, stiffness
203 was obtained through log modulus ASCII matrices and roughness was obtained analyzing areas
204 of 1x1 µm from each image, got 150 roughness data for each treatment (Costa-Júnior et al.,
205 2020). Height, roughness, and stiffness data from *H. contortus* eggs were analyzed using
206 D'Agostino & Pearson for normality, and means were compared using the Kolmogorov-
207 Smirnov test.

3. Results

The individual *in vitro* efficacy of the different monoterpenes was evaluated in this study. The monoterpenes showing the best efficiency in the EHT were carvacrol (IC_{50} 185.9 µg/mL), followed by thymol (IC_{50} 187.0 µg/mL), r-carvone (IC_{50} 301.6 µg/mL) and s-carvone (IC_{50} 361.9 µg/mL) (Table 2). The IC_{50} for albendazole according to the EHT was 0.82 µg/mL (Table 3).

In the LMIT, s-carvona and carvacrol exhibited the lowest IC_{50} (1526 and 1785 µg/mL, respectively), followed by r-carvone and thymol (1805 and 1847 µg/mL, respectively). In contrast, p-cymene and citral with an IC_{50} higher than 10000 µg/mL, demonstrated no effect on larval migration and not used in the association (Table 2). The mean IC_{50} for levamisole was 0.26 µg/mL (Table 3).

The activity of the monoterpenes combined with albendazole compounds was also evaluated by the EHT. Combination with albendazole showed synergism for r-carvone (SR= 3.8), s-carvone (SR= 3.0), citral (SR= 1.7) and carvacrol (SR= 1.6) (Table 3). In the case of levamisole, a low synergistic effect was found by the LMIT for combination with r-carvone (SR = 1.7) and s-carvone (SR = 1.7), and a possible antagonistic effect with carvacrol (SR= 0.8) and thymol (SR= 0.8) was observed (Table 3).

In order to investigate the possible interactions between the synthetic compounds and monoterpenes, FTIR analyses were carried out using the best combinations obtained by the *in vitro* tests. Pure albendazole, levamisole, r-carvone and s-carvone display characteristic bands related to the vibration of functional groups present in their respective molecular skeleton (Table S3). However, it was observed that bands related to pure albendazole (Figure 2A) or levamisole (Figure 2B) were shifted toward lower or higher wavenumber values, or even not appreciated once associated with r-carvone or s-carvone, indicating a strong interaction between the synthetic compound and the monoterpenes.

233 Optical microscopy images of treated *H. contortus* eggs reveal differences in the larvae
234 development among the treatments (Figure 3). Indeed, AFM images reveal differences in
235 biomechanical and ultrastructural properties compared to control samples (Figure 4 and 5).
236 Eggs treated with albendazole (Figure 5 H and I) show an increase in height differences (1301.0
237 ± 269.6) and average square surface roughness (105.0 ± 63.1) when compared to the control
238 sample (1069.0 ± 178.1 and 59.6 ± 46.1 to height and roughness, respectively) ($p < 0.0001$).
239 Eggs treated with the combination of albendazole and r-carvone (Figure 4 M and N) show a
240 decrease in height differences (1096.0 ± 225.3) on the surface, however, they show an increase
241 in the average roughness (83.1 ± 44.1) of the surface when compared to the control samples
242 (1494.0 ± 245.6 and 67.8 ± 36.4 to height and roughness, respectively), that may suggest an
243 overflow of internal content. There is no statistically significant difference for r-carvone treated
244 eggs and its control for roughness. All treatments reduced the values of surface stiffness when
245 compared to their respective controls (Figure 5 E, J and O).
246
247 **4. Discussion**
248 Several studies have demonstrated the *in vitro* efficacy against *H. contortus* of essential
249 oils and their major components such as carvacrol, thymol, r-carvone, s-carvone, citral and p-
250 cymene (Carvalho et al., 2012; Elandalousi et al., 2013; Macedo et al., 2015; Andre et al., 2016;
251 Ferreira et al., 2016). There are several studies reporting promising results for combinations of
252 natural and synthetic compound in the control of fungi, bacteria, and ticks (Ahmad et al., 2015;
253 Castro et al., 2015; Araújo et al., 2016; Moon and Rhee, 2016; Chang et al., 2017). However,
254 there is scarce information on the nematodicidal activity of monoterpenes in combination with
255 other natural or synthetic compounds. To the best of our knowledge, the present study is the
256 first to evaluate combinations of natural terpenes and synthetic anthelmintic compounds by
257 two different *in vitro* tests.

Our FTIR spectra analysis showed an indicative of perturbations originated from strong interactions between albendazole or levamisole in combination of r-carvone and s-carvone involving the carbonyl or amine groups (Table S3) (Gunasekaran and Uthra, 2008; Cavalcanti et al., 2012; Chakraborty et al., 2015; Carvalho Neto, 2017). In addition to these interactions at the molecular level, it is important to highlight that in the case of the combination of monoterpenes with albendazole (Figure 2), possible hydrogen bonds can be easily evidenced by the strong perturbation of the –OH groups in 3490cm^{-1} region. This additional interaction may be the justification for the high synergism observed in albendazole-based combinations compared to those based on levamisole, as observed by *in vitro* tests.

Albendazole combined with r-carvone (SR= 3.8), s-carvone (SR= 3.0), citral (SR= 1.7) and carvacrol (SR= 1.6) exerted synergistic effects on the hatchability of *H. contortus* eggs (Table 3), with albendazole and r-carvone showing the greatest effect. Levamisole combined with r-carvone (SR= 1.7) and s-carvone (SR= 1.7) exerted synergistic effects on the larval migration of *H. contortus* (Table 3). The synergistic effect of combinations of various compounds may be explained by different mechanisms of action, an increase in antiparasitic activity with the same mechanism of action, or a change in the chemical structure of the combined compounds (Blanco et al., 2017).

Previous studies on monoterpene mechanisms of action have revealed several possible biochemical targets in the nervous system of vertebrates and invertebrates that indicate acetylcholinesterase inhibition (Miyazawa et al., 1997). However, evidence for a correlation between toxicity toward insects and acetylcholinesterase inhibition after monoterpene exposure is contradictory (Ryan and Byrne, 1988; Lee et al., 2001a, 2001b). Carvone, carvacrol, citral and thymol are reported acetylcholinesterase inhibitors (Ryan and Byrne, 1988; Jukic et al., 2007; López and Pascual-Villalobos, 2010; Kurt et al., 2017). Overall, acetylcholinesterase inhibition is the classical mechanism of action of organophosphates, potent anthelmintics with

283 high toxicity toward mammals (Miller et al., 1986; Ross et al., 2013), and the potential
284 advantages of monoterpenes with low mammal toxicity are under study (OECD, 2007; Suntres
285 et al., 2015).

286 The anthelmintic synergic effect of monoterpenes with albendazole may be explained
287 by their different mechanisms of action, as was showed by the activity of a combination of
288 organophosphates with macrocyclic lactones against insects (Khan et al., 2013; Chen et al.,
289 2015). Additional modes of action may be involved in the effects of combinations of
290 monoterpenes and anthelmintic drugs. Carvacrol inhibits gamma aminobutyric acid (GABA)
291 and induces acetylcholine-mediated contractions in *Ascaris suum* (Trailović et al., 2015).
292 Carvacrol and its isomer thymol also showed nematodicidal activity possibly mediated via the
293 nematode tyramine receptor (Lei et al., 2010). Thymol acts on glutamate-gated chloride
294 channels (GluCls) (Lynagh et al., 2014; De Lucia, 2016).

295 In the current work, it was observed a decrease in stiffness in all treated eggs. However,
296 r-carvone did not prevent the eggs hatching suggesting that stiffness is not a good marker to
297 egg hatch, although the stiffness of eggs shell is different in morula and larvae eggs (Costa-
298 Junior et al., 2020). The egg hatch assay is generally referred to as an 'ovicidal' assay, when
299 the eggs failing to hatch are considered dead (Lancey et al., 1987). The benzimidazoles, like
300 albendazole, have a common primary mechanism of action the inhibition of the formation of
301 microtubules, which are hollow structures formed by heterodimers of a-b-tubulin (Mandelkow
302 and Mandelkow, 1990). The microtubule inhibitors, manifesting their effect in rapidly dividing
303 cells, such as developing eggs, via the inhibition of formation of the mitotic spindle with
304 resultant failure of normal cellular division (Dustin, 1978; Lancey et al., 1987). In the present
305 study, we showed that the shell of treated eggs with albendazole increase the height and
306 roughness (Figure 5) characteristic of an interruption of the development of the eggs. While the
307 combination of albendazole with r-carvone changed the surface of the egg decreasing the height

and increasing the roughness which is characteristic of injury on the surface and suggests the overflow of the egg content (Kambli et al., 2015; Oh et al., 2017).

To our knowledge, the current study is the first to assess combinations of natural terpenes and synthetic anthelmintic compounds and analyzing its effect on the ultrastructural and biophysical properties of *H. contortus*. Our results suggest chemical interactions between synthetic and natural compounds with alteration of biophysical properties and overflow of internal egg content that explain the synergist effect against nematode. Further studies are needed to evaluate if these synergistic effects are in vivo achieved and therefore may be useful to improve the control of gastrointestinal nematodes.

Conflict of Interest

The authors declare that they have no conflict of interest.

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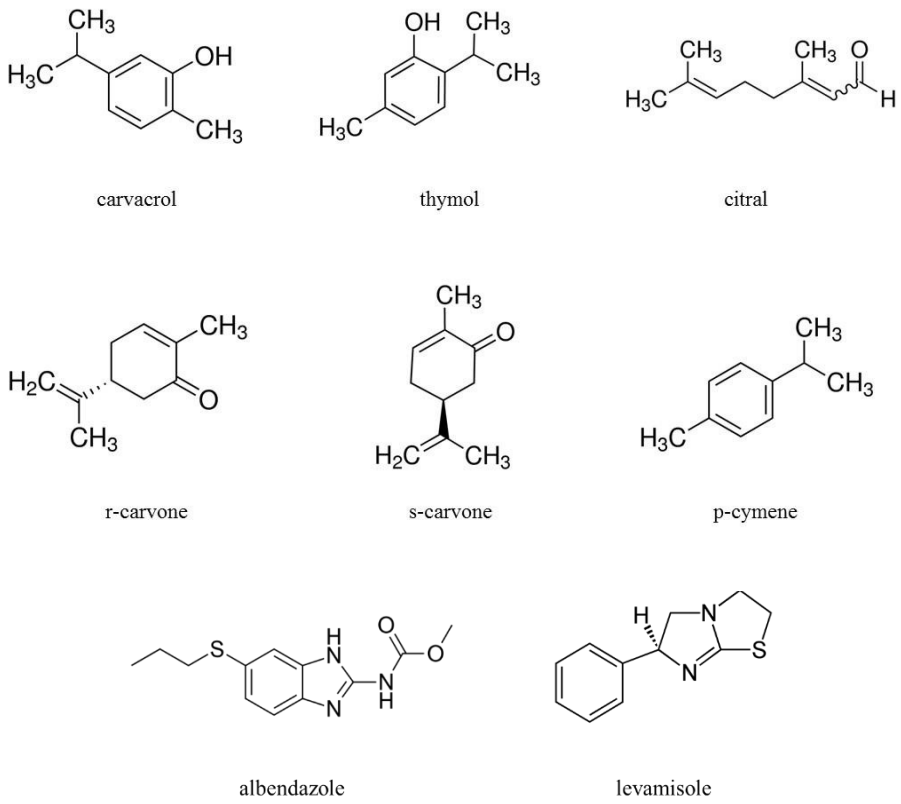

Figure 1. Chemical structure of monoterpenes and synthetics anthelmintic used in the present study.

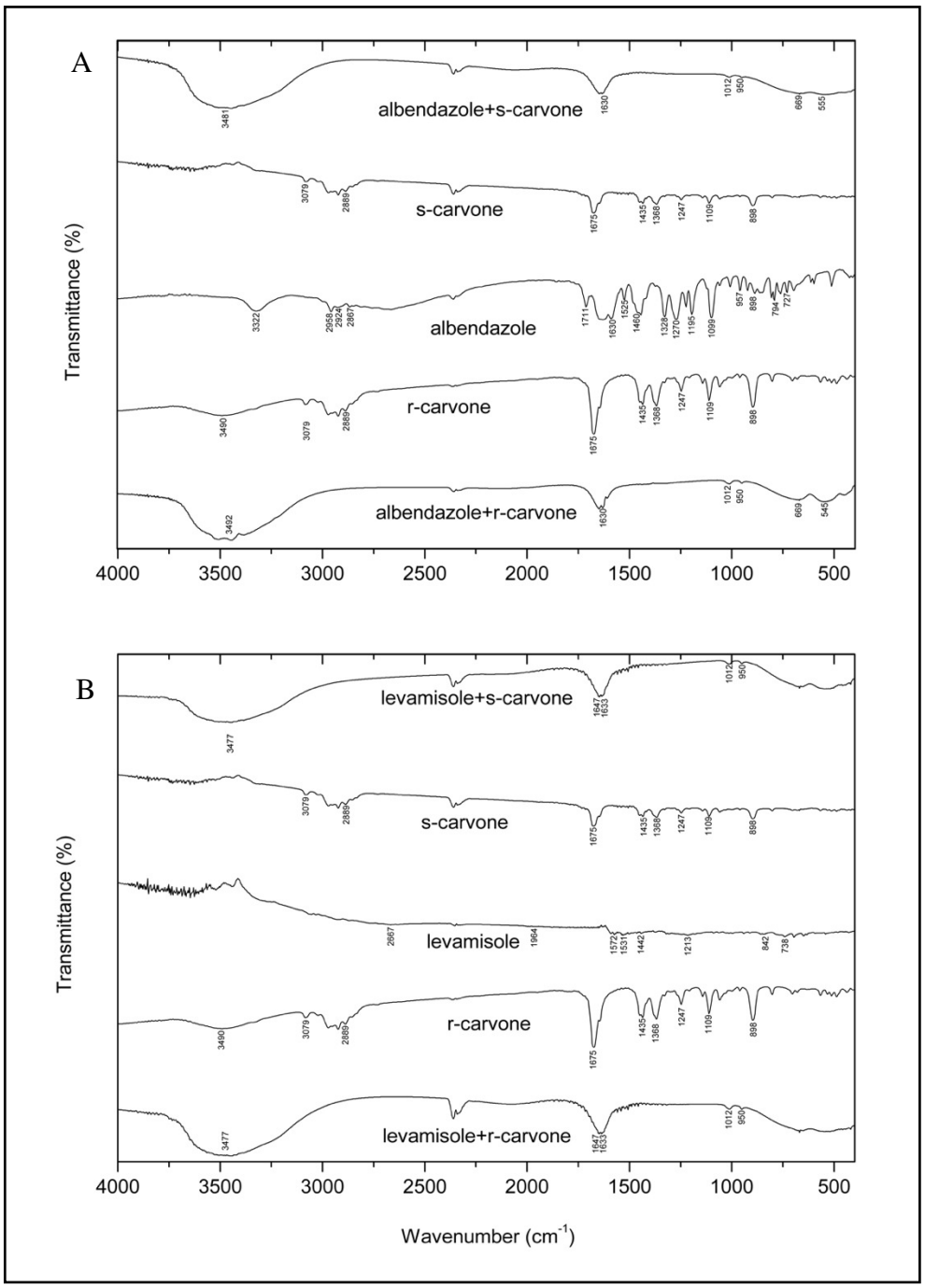

Figure 2. Fourier-transform infrared spectroscopy (FTIR) spectra of albendazole (A) and levamisole (B) and its combinations with s-carvone and r-carvone.

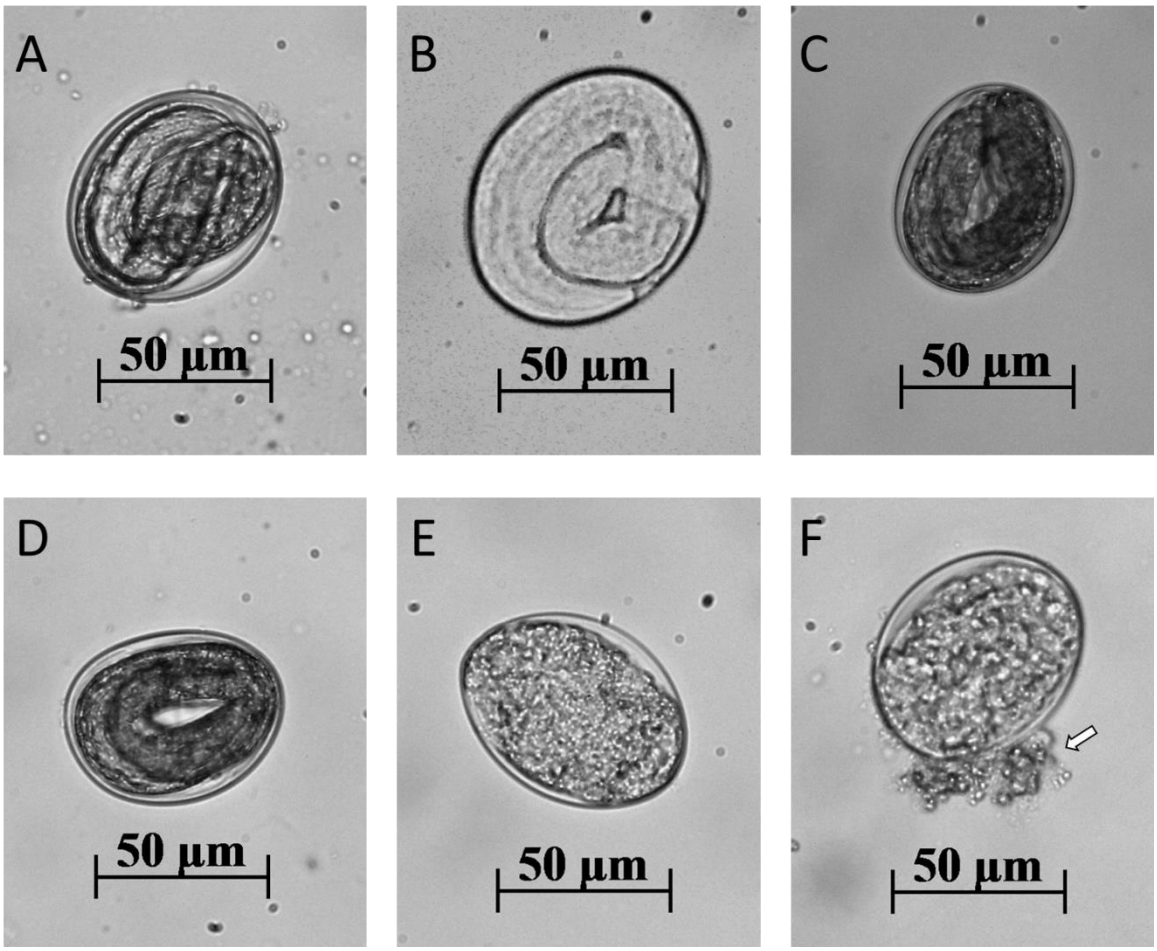

Figure 3. Optical microscopy of *Haemonchus contortus* eggs incubated with 0.03% tween (A), 2% DMSO (B), 0.03% tween and 2% DMSO (C), r-carvone at 156 µg/mL (D), albendazole at 5 µg/mL (E), and albendazole 5 µg/mL and r-carvone 156 µg/mL (F). The eggs incubated with tween, DMSO, tween and DMSO, and r-carvone were analyzed after 8 hours, and eggs incubated with albendazole, and the combination albendazole and r-carvone were analyzed after 48 hours. The white arrow shows the overflow of internal egg content.

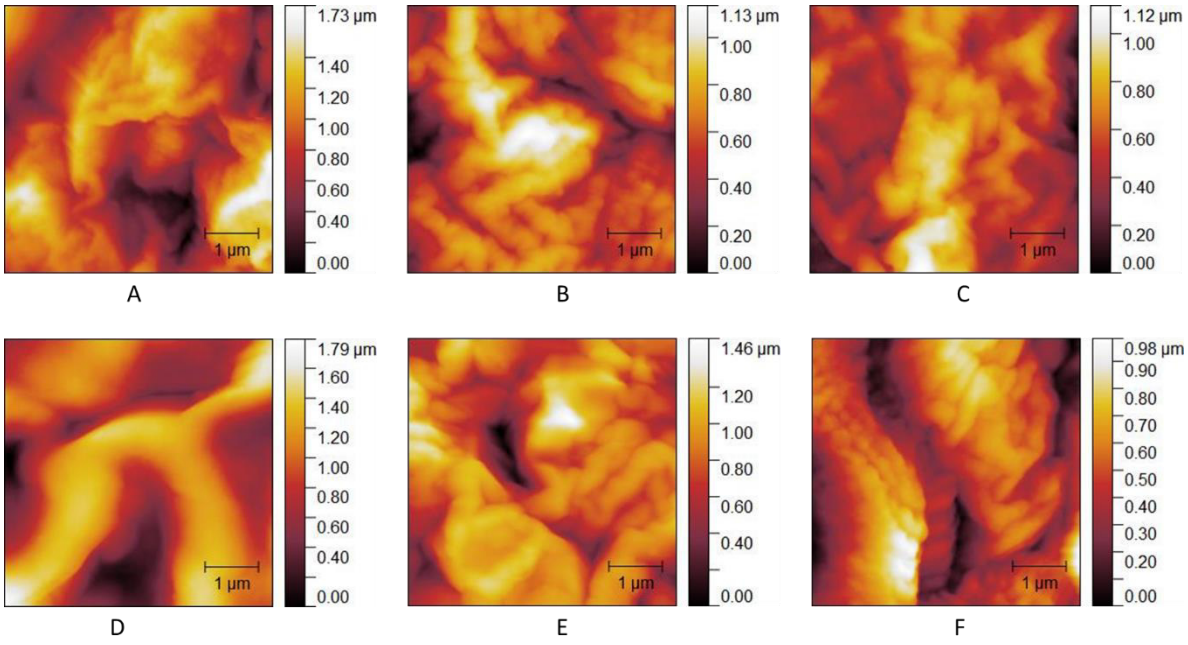

Figure 4. Topographic images obtained by atomic force microscopy (AFM) of *Haemonchus contortus* eggs treated with 0.03% tween (A), 2% DMSO (B), 0.03% tween and 2% DMSO (C), r-carvone (D), albendazole (E), and the combination of albendazole and r-carvone (F). The lower structures are the darker regions, and the higher are the lighter regions.

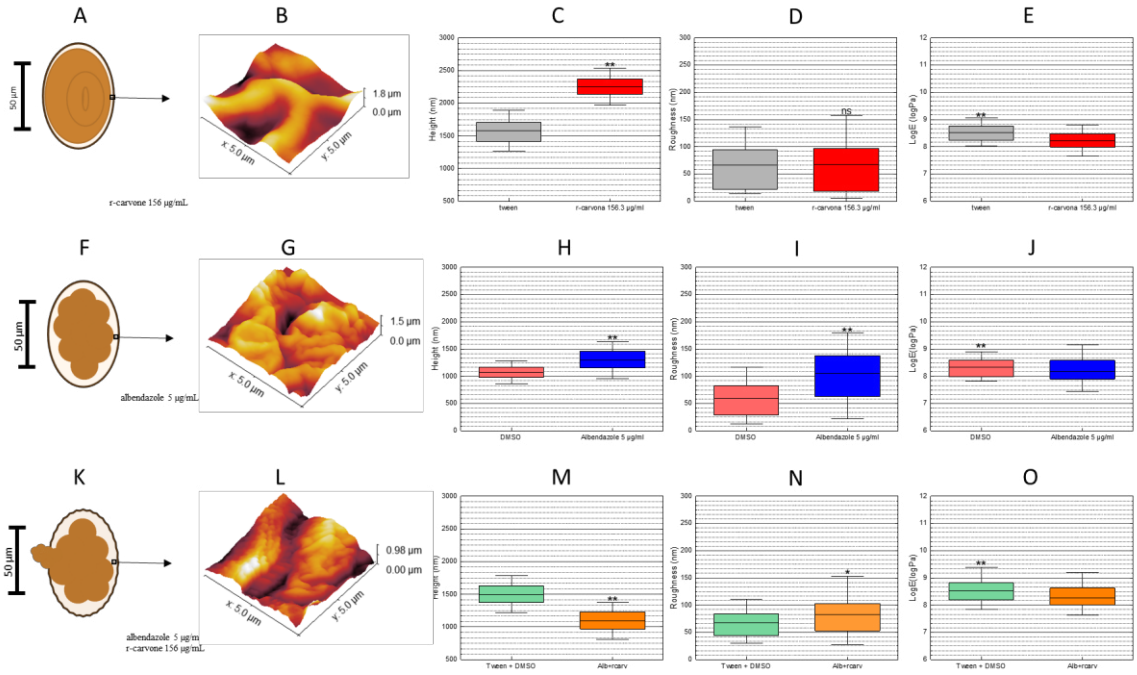

Figure 5. Schematic drawing of *Haemonchus contortus* egg treated with r-carvone (A), albendazole (F) and the combination albendazole and r-carvone (K), with a zoom to height map obtained in the atomic force microscopy (AFM) of the respective treatment (B, G, and L) and the box plot with the measures of height, roughness, and stiffness of eggs incubated in 0.03% tween or r-carvone (C, D, and E, respectively), in 2% DMSO or albendazole (H, I, and J, respectively) and the eggs incubated in 0.03% tween and 2% DMSO or the combination of albendazole and r-carvone (M, N and O, respectively). ns – not significant $p > 0.05$, * $p < 0.001$, and ** $p < 0.0001$.

Table 1. Concentration of synthetic and natural compounds used in egg hatching (EHT) and larval migration inhibition tests (LMIT).

Compound	Concentration (µg/mL)	
	EHT	**LMIT**
albendazole	10.0 – 0.002	-
levamizole	-	2.0 – 0.039
carvacrol	1250.0 – 39.1	10000.0 – 312.5
thymol	2500.0 – 78.125	10000.0 – 312.5
r-carvone	5000.0 – 156.2	10000.0 – 312.5
s-carvone	5000.0 – 156.2	10000.0 – 312.5
citral	5000.0 – 156.2	10000.0 – 312.5
p-cymene	5000.0 – 156.2	10000.0 – 312.5

Table 2. The mean and standard deviation of half-maximal inhibitory concentration (IC_{50}) of monoterpenes on *Haemonchus contortus* in egg hatching (EHT) and larval migration inhibition test (LMIT).

Compound	IC_{50} ± SD (µg/ml)	
	EHT	LMIT
carvacrol	185.9 ± 57.9	1785.3 ± 372.7
thymol	187.0 ± 7.9	1846.6 ± 968.7
r-carvone	301.6 ± 76.8	1805.3 ± 649.2
s-carvone	361.9 ± 23.4	1526.0 ± 696.5
citral	352.8 ± 48.4	> 10000
p-cymene	1705.3 ± 89.8	> 10000

SD - standard deviation.

Table 3. The mean and standard deviation (SD) of half-maximal inhibitory concentration (IC_{50}) and synergist rate (SR) of combinations of monoterpenes with albendazole or levamisole on *Haemonchus contortus* in egg hatching test and larval migration inhibition test.

			Monoterpene		**IC_{50} ± SD (µg/ml)**	**SR**
			Concentration (µg/ml)	**Efficiency (%)**		
Egg hatch test	Albendazol	none	-	-	0.82 ± 0.42	-
		carvacrol	78.0	9.51	0.52 ± 0.09	1.6
		thymol	78.0	7.46	1.17 ± 0.70	0.7
		r-carvone	156.0	8.26	0.21 ± 0.14	3.8
		s-carvone	156.0	6.74	0.27 ± 0.12	3.0
		citral	78.0	5.35	0.48 ± 0.10	1.7
		p-cymene	625.0	5.82	0.80 ± 0.16	1.0
Larval migration inhibition test	Levamisole*	none	-	-	0.26 ± 0.19	-
		carvacrol	312.5	5.53	0.32 ± 0.31	0.8
		thymol	312.5	13.84	0.33 ± 0.15	0.8
		r-carvone	312.5	3.66	0.15 ± 0.09	1.7
		s-carvone	312.5	9.93	0.15 ± 0.08	1.7

*citral and p-cymene were not combinate with levamisole because did not inhibited larval migration.

Supplementary material

Table S1. Action of monoterpenes on inhibition of egg hatching of *Haemonchus contortus*.

µg/ml	Efficiency (%) ± SD					
	carvacrol	**thymol**	**r-carvone**	**s-carvone**	**citral**	**p-cymene**
5000.0	-	**-**	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.8	92.8 ± 6.8
2500.0	**-**	99.8 ± 0.3	100.0 ± 0.0	100.0 ± 0.2	99.6 ± 0.3	67.9 ± 12.3
1250.0	100.0 ± 0.0	99.5 ± 0.4	100.0 ± 0.0	100.0 ± 0.0	96.8 ± 1.9	28.3 ± 22.7
625.0	99.8 ± 0.3	98.9 ± 1.1	98.4 ± 4.0	96.2 ± 3.5	75.9 ± 16.3	10.8 ± 5.9
312.5	88.8 ± 6.1	76.1 ± 9.1	54.5 ± 26.8	28.8 ± 10.4	44.1 ± 5.5	4.5 ± 2.6
156.3	35.9 ± 29.1	42.4 ± 6.0	9.3 ± 3.6	4.4 ± 3.8	11.8 ± 2.8	1.9 ± 2.4
78.1	7.4 ± 7.3	4.6 ± 3.4	**-**	**-**	**-**	**-**
39.1	2.9 ± 3.3	**-**	**-**	**-**	**-**	**-**

SD - standard deviation; - concentration not performed.

Table S2. Action of monoterpenes on larval migration inhibition of *Haemonchus contortus.*

µg/ml	Efficiency (%) ± SD					
	carvacrol	**thymol**	**r-carvone**	**s-carvone**	**citral**	**p-cymene**
10000.0	89.9 ± 7.2	85.0 ± 13.2	78.0 ± 8.3	87.1 ± 3.6	19.5 ± 18.2	14.7 ± 11.3
5000.0	80.1 ± 11.6	81.6 ± 13.3	74.7± 8.3	84.7 ± 15.0	-	-
2500.0	60.2 ± 2.2	70.9 ± 21.0	70.0 ± 16.7	72.6 ± 18.5	-	-
1250.0	41.6 ± 9.7	39.4 ± 16.7	43.2 ± 19.4	52.4 ± 11.3	-	-
625.0	20.9 ± 4.1	23.7 ± 12.4	20.1 ± 10.9	17.4 ± 5.6	-	-
312.5	7.4 ± 2.2	6.5 ± 2.6	5.6 ± 5.3	6.7 ± 5.1	-	-

SD - standard deviation; - concentration not performed.

Table S3. Assigned Fourier-transform infrared spectroscopy bands for albendazole, levamisole, r-carvona and s-carvone

Absorption peaks (cm^{-1})	**Assigned bands**
3481	OH
3477	OH
3322	N-H stretching of the so-called amide A groups
3079	CH of alkenes
2889	Aliphatic CH
2667	C=N stretching
1964	S $(=O)_2$ asymmetric stretching
1711	υ_{CO} (amide I) of the C–N–H bond of amide II vibration modes
1675	C=C band of cycloalkene
1572	C=C (aromatic) stretching
1531	C=C (aromatic) stretching
1525	υ_{C-N} of the C–N–H bond of amide II vibration modes
1442	C=C (aromatic) stretching
1247	C=O bond, characteristic of ketone
1213	C-N stretching
1109	C=O bond, characteristic of ketone
1012	C-O
898	Angular deformation of C=O outside the cycloalkene
842	C-Cl symmetric stretching
738	C-S

Highlights

- Monoterpenes and synthetic products have shown synergistic antiparasitic effect.
- Monoterpenes and synthetic anthelmintic interact chemically.
- Synthetic compound plus monoterpenes affect the ultrastructure of *H. contortus* eggs.
- This combined treatment may be an alternative against resistant nematodes.

CONSIDERAÇÕES FINAIS

Patentes de fármacos à base de óleos essenciais para pequenos ruminantes são escassas. Dentre os componentes dos óleos essenciais, os monoterpenos se destacam por possuírem potencial anti-helmínticos contra o nematoide que mais causa dados a pequenos ruminantes, o *H. contortus*. Dentre os monoterpenos, o carvacrol e timol são quimicamente estáveis em líquido ruminal, além de demonstrarem um menor grau de associação com a fase particulada do conteúdo ruminal em comparação com anti-helmínticos sintéticos.

A associação de monoterpenos com produtos sintéticos é uma alternativa para aumentar a eficiência em cepas de nematoides resistentes. Os monoterpenos r-carvona e s-carvona demonstraram potencializar *in vitro* o efeito de Albendazol e Levamisol contra o nematoide *H. contortus*. Também possuem interações químicas entre esses compostos naturais e os sintéticos. A associação entre r-carvona e Albendazol causa danos a ultraestrutura e sugerem extravasamento de conteúdo de ovos de *H. contortus*.

A associação entre monoterpenos e produtos sintéticos é uma alternativa promissora contra cepas resistentes de nematoides de pequenos ruminantes. Portanto, são necessários mais estudos farmaco-parasitológicos *in vivo* para avaliar melhor a utilidade dessa abordagem terapêutica.

PRODUÇÃO CIENTÍFICA REALIZADA DURANTE O DOUTORADO

Artigos completos publicados em periódicos

1. MIRÓ, V. *et al. In vitro* inhibition of the hepatic S-oxygenation of the anthelmintic albendazole by the natural monoterpene thymol in sheep. **Xenobiotica**, v. 50, n. 4, p. 408–414, 2020.

Este artigo avaliou o efeito *in vitro* do monoterpeno timol (TML) no metabolismo do albendazol através de estudos com microssomas hepáticos de ovinos.

2. MIRÓ, M. V. *et al.* Combination of bioactive phytochemicals and synthetic anthelmintics: *In vivo* and *in vitro* assessment of the albendazole-thymol association. **Veterinary Parasitology**, p. 109121, 2020.

Este artigo demonstrou a interação fármaco-química *in vivo* e *in vitro* e a eficácia *in vivo* da combinação de albendazol (ABZ) com timol (TML) em cordeiros naturalmente infectados com nematóides gastrointestinais resistentes.

3. COSTA-JUNIOR, L. M. *et al.* Assessment of biophysical properties of *Haemonchus contortus* from different life cycle stages with atomic force microscopy. **Ultramicroscopy**, v. 209, 2020.

Esse artigo caracterizou as várias fases de vida do *H. contortus* através da microscopia de força atômica (AFM).

4. MALIK, S. *et al.* Chemical profile and biological activities of essential oil from *Artemisia vulgaris* L. Cultivated in Brazil. **Pharmaceuticals**, v. 12, n. 2, 2019.

Esse trabalho avaliou o óleo essencial *A. vulgaris* L. cultivadas no Brasil, analisando a composição química e atividades biológicas, incluindo antibacteriana, antifúngica e anti-helmíntica.

5. SILVA, R. R. S. *et al. Parkia platycephala* lectin enhances the antibiotic activity against multi-resistant bacterial strains and inhibits the development of *Haemonchus contortus*. **Microbial Pathogenesis**, v. 135, 2019.

Este artigo avaliou a capacidade de ligação de glicose / manose de uma lectina oriunda de sementes de *P. platycephala* (PPL) para inibir o desenvolvimento de *H. contortus* e para

modular a atividade antibiótica contra cepas bacterianas multirresistentes, confirmando assim sua eficácia quando usado em combinação com gentamicina.

6. SILVA, C.R. *et al.* Advances in the development of technologies using essential oils for control of parasites of small ruminants. **Revista GEINTEC-Gestão, Inovação e Tecnologias**, v. 9, n. 3, p. 5067–5075, 2019.
Este trabalho objetivou prospectar trabalhos científicos e patentes relacionados ao uso de óleos essenciais no desenvolvimento desses novos produtos para uso em pequenos ruminantes.

7. ARAÚJO, S. A. *et al. In vitro* anthelmintic effects of *Spigelia anthelmia* protein fractions against *Haemonchus contortus*. **PLoS ONE**, v. 12, n. 12, 2017.
Este trabalho teve como objetivo avaliar o potencial anti-helmíntico e acaricida de um extrato hidroetanólico de folhas e galhos de *I. imperati*, mais conhecida como salsa da praia e a qual é popularmente utilizada com antiparasitário.

8. SILVA, N. C. S. *et al. In vitro* and *in vivo* activity of hydrolyzed *Saccharomyces cerevisiae* against goat nematodes. **Veterinary Parasitology**, v. 254, 2018.
Este artigo demonstrou através estudos *in vitro* e *in vivo* o efeito da parede celular de levedura na prevenção da infecção de caprinos por helmintos gastrintestinais.

9. SOARES, A. M. S. *et al. Myracrodruon urundeuva* seed exudates proteome and anthelmintic activity against *Haemonchus contortus*. **PLoS ONE**, v. 13, n. 7, 2018.
Este estudo identificou proteínas em exsudatos de sementes de *M. urundeuva* e avaliou a atividade anti-helmíntica contra *H. contortus*.

10. SOARES, A. M. S. *et al. Myracrodruon urundeuva* seed exudates proteome and anthelmintic activity against *Haemonchus contortus*. **PLoS ONE**, v. 13, n. 7, 2018.
Este trabalho investigou o efeito anti-helmíntico da lectina de *C. brasiliensis* (ConBr) contra *H. contortus* e avaliou a interação do ConBr com os glicanos deste parasita por docagem molecular.

11. WANDERLEY, L. F. *et al.* A cysteine protease from the latex of *Ficus benjamina* has in vitro anthelmintic activity against *Haemonchus contortus*. **Revista Brasileira de Parasitologia Veterinária**, v. 27, n. 4, p. 473–480, 2018.

Este artigo demonstrou o potencial anti-helmíntico da protease purificada do látex de *F. benjamina* contra *H. contortus*.

12. OLIVEIRA, A. F. *et al.* Anthelmintic activity of plant extracts from Brazilian savanna. **Veterinary Parasitology**, v. 236, p. 121–127, 2017.
Este estudo demonstrou a atividade *in vitro* contra *H. contortus* de plantas utilizadas popularmente e selecionadas naturalmente por caprinos no cerrado brasileiro.

13. ARAÚJO, S. A. *et al.* In vitro anthelmintic effects of *Spigelia anthelmia* protein fractions against Haemonchus contortus. **PLoS ONE**, v. 12, n. 12, 2017.
Este estudo avaliou a atividade anti-helmíntica de frações protéicas de *S. anthelmia* sobre *H. contortus*.

Capítulo de livro publicado

1. TEIXEIRA, M. et al. Doenças parasitária de caprinos. *In*: Arturo Bernado Selaive-Villarroel; Vinicius Pereira Guimarães. (Org.). Produção de caprinos no Brasil. 1ed.Brasilia: Embrapa, 2019, v. 1, p. 311-353.
Esse livro trata da produção de caprinos no Brasil e trata de diversos temas relacionado a produção de leite e de carne, sistemas de produção, genética, alimentação, reprodução, sanidade e comercialização. O capítulo de livro de nossa autoria trata sobre as doenças parasitárias de caprinos.