UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José do Rio Preto

CAROLINE SPRENGEL LIMA

Avaliação de extratos e substâncias de *Pterogyne nitens* Tul. (Fabaceae) contra nematoides de interesse veterinário

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Tese apresentada como parte dos requisitos para obtenção do título de Doutora em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Financiadora: CAPES

Orientador: Prof. Dr. Luis Octavio Regasini
Coorientadora: Dr.ª Ana Carolina de Souza Chagas

São José do Rio Preto
2020

L732a

Lima, Caroline Sprengel
Avaliação de extratos e substâncias de Pterogyne nitens Tul. (Fabaceae) contra nematoides de interesse veterinário / Caroline Sprengel Lima. -- São José do Rio Preto, 2020
151 f. : tabs.

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto
Orientador: Luis Octavio Regasini
Coorientadora: Ana Carolina de Souza Chagas

1. Leguminosa. 2. Fenóis. 3. Anti-helmínticos. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca do Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto. Dados fornecidos pelo autor(a).

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São José do Rio Preto
03 de abril de 2020

Dedico este trabalho à minha família,

em especial às minhas avós (*in memoriam)* **Aparecida Rodrigues de Lima** (vó Cida) e

Maria Alfa Lahr Sprengel (vó Alfa).

AGRADECIMENTOS

Aos meus pais, Eduardo e Silvana, obrigada pelo amor abundante, cuidado diário, pela dedicação e educação que sempre me proporcionaram. Minha eterna gratidão por respeitarem minhas escolhas, apoiar minhas decisões e por sempre acreditarem em mim. Através de seus ensinamentos, aprendi, cresci, me tornei mais forte e amadureci.

Minhas irmãs, Gabriele e Isabele, pelo companheirismo, amizade, afeto e incentivo todos esses anos. Com vocês a vida sempre foi mais alegre. À minha família, muito obrigada pelo apoio, afeto, respeito e por entenderem que nem sempre posso estar presente fisicamente. Ao meu companheiro de vida André Luiz por estes 4 anos de cuidado, amor, companheirismo, apoio, e muitas afirmações de que eu sou capaz.

Agradeço ao meu orientador Prof. Dr. Luis Octavio Regasini por dispor de seu tempo e laboratório, por acreditar e confiar no meu trabalho, pela oportunidade de estudo, suporte e incentivo durantes estes anos. Sem dúvida, foram anos de muito aprendizado como profissional, mas também como ser humano. Agradeço pelo comprometimento ao longo dessa jornada.

À Dra. Ana Carolina de Souza Chagas, minha coorientadora, que também foi fundamental para essa conquista, obrigada por estar presente em todos os anos do meu doutoramento. Meus sinceros agradecimentos pela confiança, paciência, atenção e cuidado. Agradeço pela oportunidade oferecida, por disponibilizar seu laboratório, junto a EMBRAPA Pecuária Sudeste, para que eu pudesse desenvolver este trabalho, e por me acolherem e me ensinarem com paciência a rotina de um laboratório de parasitologia

Agradeço ao Prof. Dr. Hervé Hoste pela colaboração nesta pesquisa. Obrigada por acreditar em meu trabalho e capacidade, por disponibilizar seu tempo e laboratório para eu pudesse aprender e conhecer novos costumes, bem como vivenciar a pesquisa em outro país. Com certeza, sua orientação e apoio foram essenciais para a conclusão deste trabalho.

Gostaria de estender minha gratidão aos pesquisadores, Dr. Alessandro Minho, Dr. Waldomiro Barioni Junior, Dra. Simone Cristina Meo Niciura, Dr. Eric Baranowski, Dr. Danilo Grünig Humberto da Silva, aos analistas de laboratório Me. Reinivaldo Ferraz

Junior e Setha Ketavong por seu tempo, pela colaboração, atenção e pela ajuda na realização de alguns dos ensaios/análises contidos neste trabalho.

Aos meus amigos que pude encontrar e conhecer aos longos dos meus 11 anos de IBILCE. Obrigada pela admiração, carinho e por todos os bons momentos de risada e descontração. Obrigada pela amizade, conversas, happy-hours e comilanças.

Aos amigos de pesquisa Matheus Henrique Pereira, meu filho científico, Me. Marta Oliveira, Dra. Ana Carolina Nazaré, Dra. Mariana Bastos dos Santos, Dra. Daiane Bertolin Anselmo, Me. Carlos Roberto Polaquini, Me. Beatriz Carvalho Marques, Me. Gabriela Miranda Ayusso, Me. Mayara Aparecida Rocha Garcia, Me. Griselda Meza, Ms. Yousmel Alemán Gainza, Ms. Amanda Figueiredo, Ms. Rafaela Fantatto, Hayala Caroline, Yngrid Veltroni obrigada por me ajudarem a enfrentar os desafios lado a lado, por compartilhar conhecimento, por me apoiarem.

Agradeço as minhas amigas da Republica Disfarça (São Carlos), por estes dois últimos anos de convívio diário, obrigada pela amizade, amor, carinho, conversas, festas e pelas risadas que não foram poucas.

Ao Dr. Fábio Rogério de Moraes, técnico responsável do Centro Multiusuário de Inovação Biomolecular do IBILCE/UNESP, Campus São José do Rio Preto, pelas análises de Ressonância Nuclear Magnética nos equipamentos Bruker Avance III 9,397 T (400 MHz) e 14,095 T (600 MHz). Meus agradecimentos aos funcionários do Instituto de Biociências, Letras e Ciências Exatas, da Empresa Brasileira de Pesquisa Agropecuária, a toda equipe do French National Institute for Agriculture, Food, and Environment (INRAE) pela paciência, suporte e ajuda durante meu doutorado e todos que de alguma forma me ajudaram nesta caminhada.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001. Agradeço à CAPES também pela bolsa de doutorado sanduíche concedida (88881.187940/2018-01) para realização de parte de meu doutorado no INRAE – Toulouse, na França.

À Comissão Examinadora, pela disponibilidade e aceite em tornar esse trabalho mais completo. E a todos, que direta ou indiretamente contribuíram para essa realização, meu mais sincero reconhecimento.

Acredite e não se explique
pois poucos vão entender:
só se compreende um sonho
se o sonhador for você.
há quem possa lhe animar
há quem possa duvidar,
há quem lhe faça seguir.
mas não descuide um segundo
pois muita gente no mundo
quer lhe fazer desistir

Acredite, pense e faça,
Use sua intuição,
transforme sonho em suor,
pensamento em ação.
Enfrente cada batalha
sabendo que a gente falha
e que isso é natural,
cair e se levantar,
aprender para ensinar
que o bem é maior que o mal.

Bráulio Bessa, 2019

RESUMO

Um dos maiores problemas sanitários encontrados pela pecuária são os nematoides gastrintestinais (NGIs), os quais causam perdas significativas aos pecuaristas de pequenos ruminantes. O controle de NGIs é realizado com base na administração repetida e imprópria dos anti-helmínticos (AHs) convencionais, havendo uma crescente presença de resíduos no meio ambiente ou em produtos de consumo. Acima de tudo, o desenvolvimento de resistência em populações de NGIs tornou-se um problema mundial. A fim de preservar a saúde dos hospedeiros, bem como retardar a resistência, torna-se necessário identificar métodos alternativos ao uso de AHs, tais como o uso de plantas, seus extratos e substâncias. *Pterogyne nitens* Tul. (Fabaceae), árvore nativa brasileira, apresenta indicações de uso popular como vermífugo, bem como de diversas substâncias bioativas, tais como: ácidos fenólicos, flavonoides, alcaloides guanidínicos, terpenos e esteróis. O presente estudo teve como objetivo investigar os efeitos de extratos e substâncias de *P. nitens* em diferentes estágios do ciclo de vida de três isolados susceptíveis originários do Brasil e da França de duas espécies de NGIs, *Haemonchus contortus* e *Trichostrongylus colubriformis*. Foram preparados três extratos etanólicos a partir das folhas (EEL), frutos (EEFR) e flores (EEFL); e oito substâncias fenólicas isoladas [duas flavonas (sorbifolina e pedalitina), dois flavonóis (quercetina e rutina), um flavan-3-ol (ourateacatequina) e três ácidos fenólicos (ácido caféico, ácido ferúlico e ácido gálico)]. E, adicionalmente, dois flavonoides (crisina e morina) foram obtidos comercialmente da Merck®, para auxiliar os estudos de relação entre estrutura química e atividade anti-helmíntica. Os extratos e substâncias foram avaliados contra eclosão de ovos, desenvolvimento larval (L_1 para L_3) e desembainhamento larval (L_3). O EEFL não foi testado nos ensaios, pois sua massa obtida foi suficiente apenas para o isolamento das substâncias. Além de ser uma parte da planta de difícil acesso, em que se dá apenas uma vez e em poucos meses do ano. Ambos extratos etanólicos demonstraram potente atividade anti-helmíntica *in vitro* contra as duas espécies de NGIs nos três ensaios analisados. No ensaio de eclosão de ovos, o extrato etanólico de folhas (EEL, de 2,9 mg/mL e 316 µg/mL) apresentou atividade anti-helmíntica duas vezes mais

potente do que o extrato etanólico de frutos (EEFR, 5,9 mg/mL e 512 µg/mL) contra *T. colubriformis* e *H. contortus*, respectivamente. No ensaio de desenvolvimento larval, EEL e EEFR apresentaram valores de EC_{50} de 8,9 e 20 µg/mL contra *T. colubriformis*, e EC_{50} de 47 e 35 µg/mL contra o isolado brasileiro de *H. contortus*, respectivamente. No caso do ensaio de desembainhamento larval, os valores de EC_{50} de ambos extratos variaram de 78,6–158 µg/mL para os três isolados de ambas espécies de NGIs. Esses resultados nos encorajaram a investigar as substâncias que poderiam ser responsáveis pela atividade anti-helmíntica dos extratos etanólicos. Ácidos fenólicos e flavonas foram os mais ativos contra a eclosão de ovos (0,56–4,93 µg/mL) e desenvolvimento larval (18 e 83 µg/mL) do isolado brasileiro de *H. contortus*, respectivamente. Quercetina foi a substância mais ativa contra eclosão de ovos (0,4 µg/mL) e o desenvolvimento larval (100 µg/mL) de *T. colubriformis*. Além disso, apresentou eficácia de 1,62 e 5,83 µg/mL contra o desembainhamento larval dos isolados francês e brasileiro de *H. contortus*, respectivamente. Vale ressaltar que este é o primeiro estudo avaliando a atividade anti-helmíntica de *P. nitens*, bem como algumas substâncias contra *H. contortus* e *T. colubriformis*. Contudo, análises *in vivo,* incluindo estudos de eficácia e segurança, são necessários para melhor entendimento da atividade anti-helmíntica dessas substâncias.

Palavras-chave: anti-helmíntico, *Pterogyne nitens*, compostos fenólicos, *Haemonchus contortus, Trichostrongylus colubriformis*.

ABSTRACT

One of the considerable health problems encountered by livestock is gastrointestinal nematodes (GINs), as they cause significant losses to small ruminant breeders. The control of GINs is based on the repeated and improper administration of synthetic anthelmintics (AHs), causing an increase of environmental residues and in food. Furthermore, the development of resistance in GIN populations has become a worldwide problem. In order to preserve the health of the hosts, as well as reduce resistance, it became necessary to identify AH alternative methods, such as the use of medicinal plants and their extracts and compounds. *Pterogyne nitens* Tul. (Fabaceae), brazilian native tree, presents popular use as a vermifuge and several bioactive compounds, such as phenolic acids, flavonoids, guanidine alkaloids, terpenes, and sterols. The aim of the current study was to investigate the effects of extracts and compounds from *P. nitens* against the different stages of the life cycle of three susceptible isolates from Brazil and France of two GIN species, *Haemonchus contortus*, and *Trichostrongylus colubriformis*. Three ethanolic extracts were prepared from leaves (EEL), fruits (EEFR), and flowers (EEFL). Eight phenolic compounds were isolated [two flavones (sorbifolin and pedalitin), two flavonols (quercetin and rutin), one flavan-3-ol (ouratecatechin) and three phenolic acids (caffeic acid, ferulic acid, and gallic acid)]. In addition, two flavonoids (chrysin and morin) were obtained commercially from Merck®, to derive the chemical structure and AH activity relationship studies. Extracts and compounds were evaluated against hatching eggs, larval development, and larval exsheathment (L_3). EEFL was not tested, because as the extracted mass obtained was sufficient only for the isolation of the compounds. As well as it is an element of the plant that is difficult to access once the flowering occurs in a few months of the year. Ethanolic extracts demonstrated a potent *in vitro* AH activity against both GINs in the three assays. In the egg hatch assay, the ethanolic extract of leaves (EEL, 2.9 mg/mL and 316 µg/mL) showed AH activity twice as potent as the ethanol extract of fruits (EEFR, 5.9 mg/mL and 512 µg/mL) against *T. colubriformis* and *H. contortus*, respectively. In the larval development assay, EEL and EEFR showed EC_{50}

values of 8.9 and 20 µg/mL against *T. colubriformis*, and EC_{50} of 47 and 35 µg/mL against the brazilian isolate of *H. contortus*, respectively. In the case of the larval exsheathment assay, both extracts EC_{50} values varied from 78.6–158 µg/mL for the three isolates of both NGI species. These results encouraged us to investigate the compounds that could be responsible for the AH activity of ethanol extracts. Phenolic acids and flavones were the most active against hatching eggs (0.56–4.93 µg/mL) and larval development (18 and 83 µg/mL) of the *H. contortus* brazilian isolate, respectively. Quercetin was the most active substance against hatching eggs (0.4 µg/mL) and larval development (100 µg/mL) of *T. colubriformis*. In addition, it showed the efficacy of 1.62 and 5.83 µg/mL against the larval exsheathment of *H. contortus* French and Brazilian isolates, respectively. It is worth mentioning that this is the first anthelmintic evaluation of *P. nitens*, as well as some substances against *H. contortus* e *T. colubriformis*. However, *in vivo* analyzes, including studies of efficacy and safety, are necessary to better understand the AH activity of these compounds.

Keywords: anthelmintic, *Pterogyne nitens*, phenolic compounds, *Haemonchus contortus*, *Trichostrongylus colubriformis.*

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LISTA DE ABREVIATURAS E SÍMBOLOS

°C	Grau Celsius
μg	micrograma
μm	micrometro
AH	anti-helmíntico ou anthelmintic
CI	confidence interval
cm	centímetro
EC_{50}	Effective Concentration having 50% of the maximal response
EHA	Egg Hatch Assay
EPG	egg per gram
g	grama
G	glucose
GINs	gastrointestinal nematodes
GluCls	Glutamate-gated Chloride Channels
H	hidrogênio ou hydrogen
HcEch91	*Haemonchus contortus* Echevarria 1991
HcINRAE	*Haemonchus contortus* Institut National de Recherche pour l'agriculture, l'alimentation et l'environnement
HCV	Hepatite C virus
IBGE	Instituto Brasileiro de Geografia e Estatística
Km^2	quilômetro quadrado
L	litro
L_1	estágio Larval 1
L_2	estágio Larval 2
L_3	estágio Larval 3
L_4	estágio Larval 4
LDA	Larval Development Assay
LEA	L_3 stage Exsheathment Assay
M	Molaridade
MCA	Multiple Correspondence Analysis
Mg	miligrama
mL	mililitro
mm	milímetro
mm^3	milímetro cúbico
MPO	Mieloperoxidase
nAChR	Nicotinic Acetylcholine Receptor

NMR	Nuclear Magnetic Resonance
nt	not tested
OPG	ovos por grama
PBS	Phosphate-buffered saline
PCO	Proantocianidinas Oligoméricas
PD/ PC	Prodelfinidina/ Procianidina
pH	Potencial hidrogeniônico
PIB	Produto Interno Bruto
PVPP	Polyvinyl polypyrrolidone
R	Radical
R$	Real (Moeda Brasileira)
rpm	Rotações por minuto
RU	Relative humidity
S.E.M.	Standard Error
TC	Taninos Condensados
TH	Taninos Hidrolisáveis
TIAL	Teste de Inibição da Alimentação Larval
TIDL	Teste de Inibição do Desenvolvimento Larval
$TIDL_3$	Teste de Inibição do Desembainhamento de Larvas Infectantes (L_3)
TIEO	Teste de Inibição da Eclosão de Ovos
TIMA	Teste de Inibição da Motilidade de Adultos
TIML	Teste de Inibição da Migração Larval
Tot	Total
US $	Dólar (Moeda Norte Americana)
UV	Ultravioleta
w/v	weight/volume

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Capítulo I

INTRODUÇÃO

A pecuária desempenha papel importante no agronegócio global e contribui, em média, com 40% do PIB de diversos países, nos quais pequenos ruminantes (ovinos e caprinos) representam números significativos dos rebanhos (OIE & FAO, 2015). No Brasil, atualmente, a pecuária é responsável por aproximadamente 7% e 31% do PIB nacional e do agronegócio, respectivamente (CEPEA; CNA; FEALQ, 2019).

No entanto, um dos maiores problemas sanitários encontrados pela pecuária das áreas temperadas e tropicais é o parasitismo causado por nematoides gastrintestinais (NGIs), em que suas infecções resultam na redução do crescimento, redução da produção de carne, lã e leite, bem como, morte dos hospedeiros, causando perdas significativas aos pecuaristas (URQUHART, 1996; HOSTE et al., 1999).

Sabe-se que o controle dos NGIs é convencionalmente realizado com base na administração sucessiva de anti-helmínticos (AHs) convencionais, os quais por muitos anos demonstraram eficácia. Contudo, devido ao uso constante e irracional desses fármacos, há um crescente número de limitações, particularmente relacionadas à presença de resíduos no meio ambiente ou em produtos de consumo. Acima de tudo, o desenvolvimento de resistência em populações de nematoides tornou-se um fenômeno mundial e de crescente preocupação, especialmente no tratamento de pequenos ruminantes (ABONGWA; MARTIN; ROBERTSON, 2017).

A fim de preservar a saúde dos pequenos ruminantes, bem como retardar o recrudescimento da resistência, tornou-se necessário identificar métodos economicamente mais viáveis, práticos e sustentáveis de controle de parasitos que evitem ou minimizem o uso de AHs convencionais. Uma possível alternativa disponível é o emprego de plantas, seus extratos e substâncias, podendo ser administrados como fitoterápicos ou alimento funcional (nutracêuticos) (HOSTE et al., 2006; HOSTE et al., 2011).

Certamente, as plantas são utilizadas no tratamento de doenças em humanos e animais desde a antiguidade. Estima-se que mais de 30% dos medicamentos recentemente introduzidos são direta ou indiretamente obtidos de origens naturais, incluindo plantas, fungos e animais (VAN WYK; WINK, 2017). Salienta-se ainda que o crescente interesse dos países desenvolvidos por produtos agropecuários isentos de substâncias nocivas conduz a uma procura por substâncias naturais bioativas. Havendo, na última década, um aumento significativo no número de estudos focados na farmacologia veterinária, particularmente para as plantas ricas em compostos fenólicos (SPIEGLER; LIEBAU; HENSEL, 2017).

De fato existem diversos estudos *in vitro* e *in vivo* de espécies leguminosas de clima temperado, as quais contêm taninos condensados, o que sugere que estas substâncias possam ter atividade anti-helmíntica (BRUNET & HOSTE, 2006; BRUNET; JACKSON; HOSTE, 2008). Bem como, existem várias pesquisas visando determinar os efeitos de leguminosas sobre os NGIs, e a incorporação destas na dieta animal em regiões tropicais, mas ainda são escassas as pesquisas objetivando determinar as substâncias responsáveis pela atividade anti-helmíntica (HOSTE et al., 2008).

Em vista disso, uma leguminosa arbórea amplamente distribuída pelo Brasil, *Pterogyne nitens*, popularmente chamada de “bálsamo”, “cocal”, “amendoim-bravo”, “madeira-nova” ou “yvi-raró, apresenta indicações populares do uso de suas cascas de caule para o combate de *Ascaris lumbricoides* em humanos (CRIVOS et al., 2007). Bem como, *P. nitens* apresenta diversas classes de metabólitos secundários, tais como ácidos fenólicos (REGASINI et al., 2008a), flavonoides (SANGALLI-LEITE et al., 2016; SHIMIZU et al., 2017), alcaloides guanidínicos (OLIVEIRA et al., 2018), terpenos e esteróis (REGASINI et al., 2009a; LIMA et al., 2016), os quais apresentaram diversas bioatividades, incluindo citotóxica, antitumoral, antimicrobiana e antioxidante.

Dessa forma, objetivou-se com o presente estudo investigar os efeitos de diferentes extratos e substâncias de *P. nitens* em diferentes estágios do ciclo de vida de duas espécies de NGIs, importantes na criação de pequenos ruminantes em áreas temperadas e tropicais, *Haemonchus contortus* e *Trichostrongylus colubriformis*.

Em suma, esta tese está dividida em cinco capítulos (1–5). Primeiramente, o Capítulo I abordou uma breve introdução seguida de uma revisão de literatura pontuando a importância da ovinocultura e caprinocultura, bem como retratou um dos maiores problemas sanitários encontrados pelos pecuaristas, o parasitismo causado por nematoides gastrintestinais. Além de relatar métodos convencionais e alternativos de controle aos nematoides de pequenos ruminantes. Neste capítulo foi destacado o uso de plantas medicinais, seus extratos e substâncias naturais para o combate dos NGIs, dando ênfase em uma leguminosa nativa do Brasil, *Pterogyne nitens*, a qual tem indicações etnofarmacológicas como vermífugo.

O Capítulo II descreveu o estudo da atividade ovicida e larvicida dos extratos de *P. nitens* e flavonoides relacionados contra um isolado susceptível de *T. colubriformis* originário da França, com base em três ensaios *in vitro*; o ensaio de eclosão dos ovos (TIEO), ensaio de desenvolvimento larval (TIDL) e ensaio de desembainhamento larval ($TIDL_3$). Paralelamente, foi feita uma análise de múltipla correspondência, para avaliar se o número e a posição dos grupos hidroxila presentes na estrutura química dos flavonoides alteraram a atividade anti-helmíntica nos três ensaios biológicos.

No Capítulo III foi avaliada e comparada a capacidade dos extratos e substâncias isoladas de *P. nitens* em inibir o processo de desembainhamento larval de dois isolados susceptíveis de *H. contortus* originários da França e do Brasil. O potencial efeito larvicida dos extratos e substâncias foi avaliado pela concentração que induziu metade do efeito máximo (EC_{50}) e porcentagem de inibição.

O Capítulo IV é a avaliação dos efeitos ovicida e larvicida dos diferentes extratos e substâncias fenólicas de *P. nitens* sobre o isolado susceptível de *H. contortus* originário do Brasil. Foi verificado a inibição de eclosão de ovos e desenvolvimento larval em diferentes concentrações, além de constatar possíveis alternativas viáveis para o controle de fases de vida livre dos NGIs.

No Capítulo V foi compilado as conclusões gerais discutindo e comparando os resultados obtidos nos capítulos anteriores (II–IV).

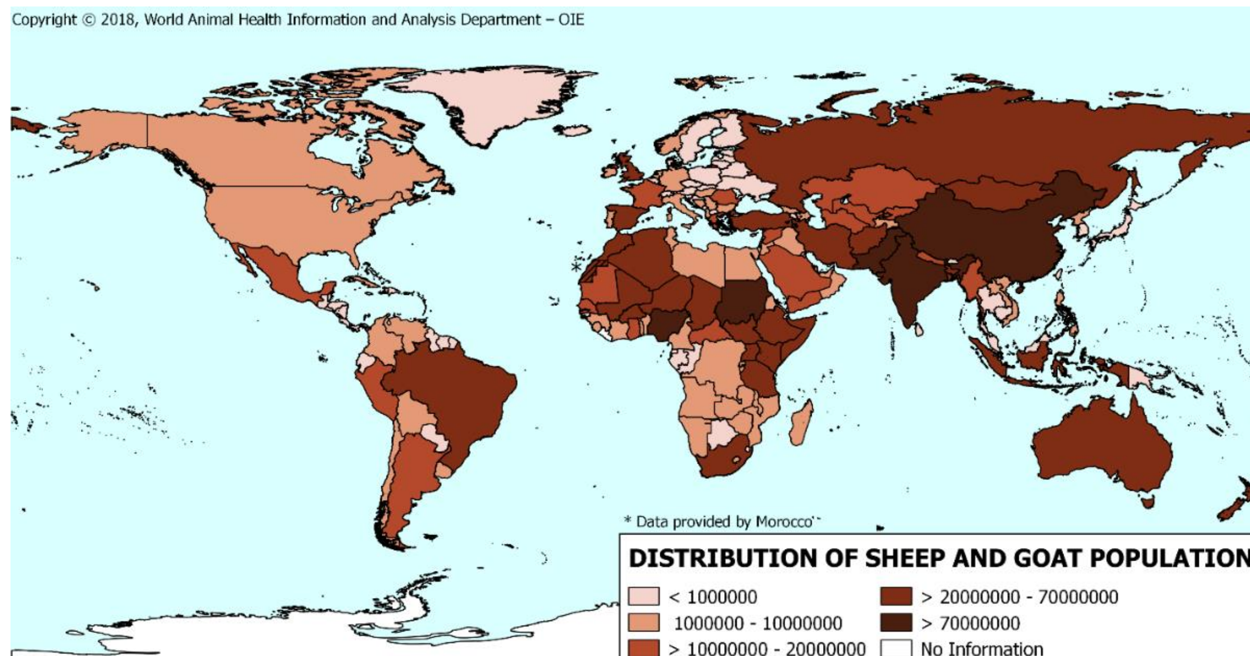
REVISÃO DE LITERATURA

1. Ovinocultura e Caprinocultura

A pecuária desempenha papel importante no agronegócio mundial e contribui, em média, com 40% do PIB de diversos países. Criações de pequenos ruminantes, como ovinos e caprinos, apresentam números significativos na pecuária mundial, estando presentes em todos os continentes, sobretudo nos países em desenvolvimento (OIE & FAO, 2015).

Em 2019, o rebanho mundial de caprinos era da ordem de 1,06 bilhão de cabeças, estando distribuídos por todos os continentes. No caso dos ovinos, o rebanho era da ordem de 1,2 bilhão de cabeças, também presente em todos os continentes (Figura 1). Nos últimos anos, observou-se uma taxa de crescimento anual de 1% e 1,5% do rebanho caprino e ovino mundial, respectivamente (FAO, 2019).

Figura 1. Densidade populacional mundial de ovinos e caprinos (número de cabeças por km^2), em 2018.

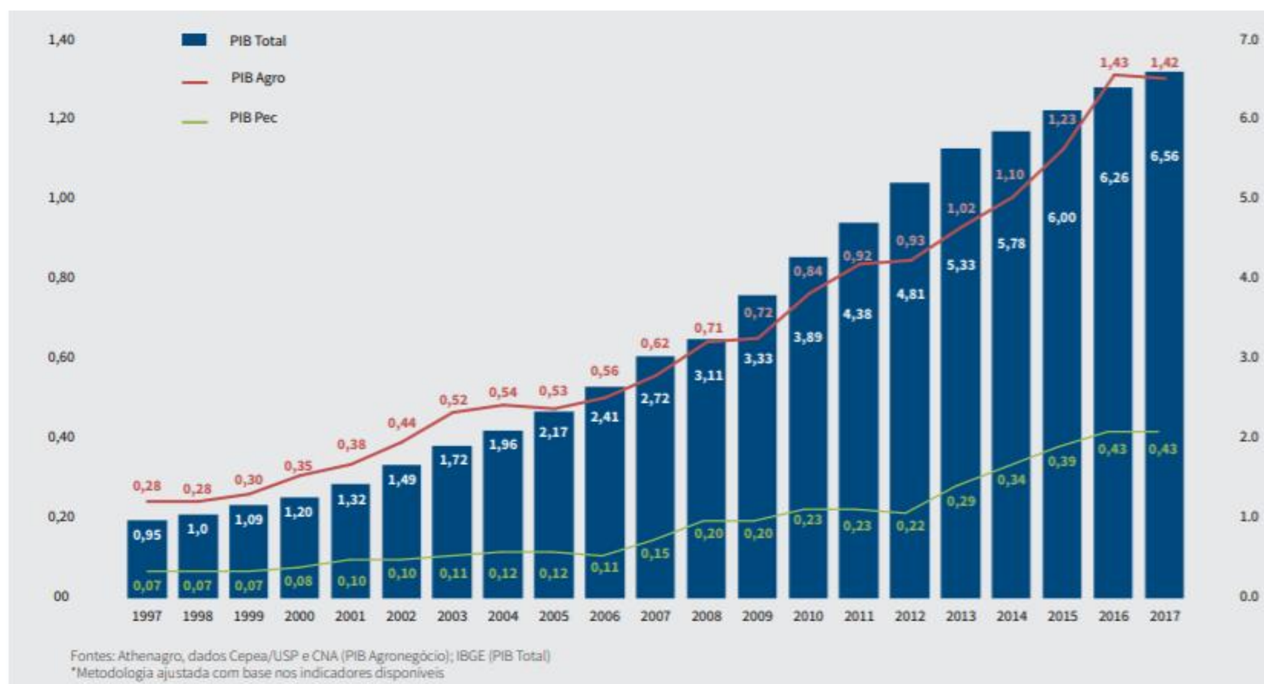


Fonte: OIE (2018).

O Brasil encerrou o ano de 2018 registrando crescimento no PIB, que atingiu R$ 6,83 trilhões. No mesmo período, o PIB da pecuária somou R$ 597,22 bilhões, 8,3% acima dos R$ 551,41 bilhões apurados em 2017. Com isso, o PIB da pecuária elevou para 8,7% sua participação no PIB total brasileiro (Figura 2). As receitas geradas em 2018 variaram em torno de R$ 1,38 bilhões, movimentando a economia de diversas cidades em todo o país e em seus vários setores produtivos (CEPEA; CNA; FEALQ, 2019).

Em 2018, o rebanho ovino brasileiro apresentou 19 milhões de cabeças e foi considerado o décimo oitavo rebanho no mundo. Além disso, o Brasil concentra o vigésimo segundo rebanho mundial de caprinos, com aproximadamente 10,7 milhões de cabeças (IBGE, 2018).

Figura 2. Valores correntes e reais (em trilhões de R$) do PIB total, PIB do agronegócio e PIB da pecuária entre os anos 1997 e 2017.



Fonte: ABIEC (2018).

2. Nematoides gastrintestinais (NGIs) de ruminantes

2.1 Informações gerais

Os maiores problemas sanitários encontrados nos rebanhos de ovinos e caprinos, incluem as verminoses gastrintestinais, as quais têm como agentes etiológicos, espécies de NGIs pertencentes à ordem Strongylida e a duas famílias distintas: Trichostrongyloidae (principais gêneros: *Haemonchus, Trichostrongylus, Teladorsagia* e *Cooperia*) e Strongyloidea (gênero: *Oesophagostomum*) (URQUHART, 1996).

Os NGIs podem apresentar distribuição global, mas com predomínio de espécies variáveis de acordo com as principais zonas climáticas. Em zonas temperadas, as espécies mais frequentemente encontradas são *Teladorsagia circumcincta* (espécie abomasal) e *Trichostrongylus colubriformis* (espécie intestinal) (O'CONNOR; WALKDEN-BROWN; KAHN, 2006). *Haemonchus contortus*, outra espécie com nicho no abomaso, é menos comum em regiões temperadas, mas amplamente distribuído e altamente patogênico nas zonas tropicais e subtropicais (URQUHART, 1996). *T. colubriformis* também é frequentemente encontrado em regiões tropicais (O'CONNOR; WALKDEN-BROWN; KAHN, 2006).

Embora essas espécies apresentem estreitas relações evolutivas e semelhanças morfológicas, existem diferenças distintas nos seus respectivos ciclos de vida parasitária, que impactam na imunidade ou imunopatologia dos hospedeiros. Essas diferenças estão principalmente relacionadas aos locais de infecção e pelos estádios larvais, bem como ao modo de alimentação das formas adultas (BALIC; BOWLES; MEEUSEN, 2000).

Cada espécie de nematoide possui uma fase larval em um tecido e uma fase adulta no lúmen gastrintestinal ou epitélio da mucosa, podendo resultar em suscetibilidade variável de cada estádio de desenvolvimento ao ataque imune mediado. Além disso, um mecanismo de ação que é eficaz contra uma espécie de nematoide pode ser ineficaz contra outro estádio de desenvolvimento da mesma espécie ou contra o mesmo estádio de outra espécie de nematoide que ocupa um nicho diferente (BALIC; BOWLES; MEEUSEN, 2000). Na tabela 1 pode-se observar o tipo de órgão e os

diferentes nichos dentro de cada órgão que os estádios larvais e adultos dos NGIs habitam.

Tabela 1. Classificação e ciclo de vida das principais espécies de NGIs em ruminantes.

Espécie de nematoide	Ordem/ Superfamília	Hospedeiro	Nicho da fase larval L_3	Nicho da fase larval L_4	Nicho da forma adulta
Haemonchus contortus	Strongylida/ Trichostrongyloidae	Ovinos Caprinos	Mucosa/ Lúmen do Abomaso	Mucosa/ Lúmen do Abomaso	Lúmen do Abomaso
Trichostrongylus colubriformis	Strongylida/ Trichostrongyloidae	Ovinos Caprinos Bovinos	Epitélio do intestino delgado	Epitélio do intestino delgado	Epitélio/ Lúmen do intestino delgado
Trichostrongylus axei	Strongylida/ Trichostrongyloidae	Ruminantes Suínos Equinos Primatas	Epitélio do abomaso	Epitélio do abomaso	Epitélio/ Lúmen do abomaso
Teladorsagia circumcincta	Strongylida/ Trichostrongyloidae	Ovinos Caprinos	Mucosa/ Lúmen do Abomaso	Mucosa do Abomaso	Lúmen do Abomaso
Cooperia spp.	Strongylida/ Trichostrongyloidae	Bovinos	Mucosa/ Lúmen Rúmen	Criptas do intestino delgado	Epitélio/ Lúmen do intestino delgado
Ostertagia ostertagi	Strongylida/ Trichostrongyloidae	Bovinos	Mucosa/ Lúmen do Abomaso	Mucosa do Abomaso	Lúmen do Abomaso
Nematodirus battus	Strongylida/ Trichostrongyloidae	Ovinos	Lúmen do intestino delgado	Submucosa/ Lúmen do intestino delgado	Lúmen do intestino delgado
Oesophagostomum spp.	Strongylida/ Strongyloidea	Ruminantes Suínos Primatas	Submucosa do intestino delgado e grosso	Submucosa/ Lúmen do intestino delgado e grosso	Colón/ Lúmen do intestino grosso

Tabela baseada em (SOULSBY, 1984); (BALIC; BOWLES; MEEUSEN, 2000).

2.1.1 *Haemonchus contortus*

Trata-se da principal espécie de nematoide que parasita ovinos e caprinos em regiões com clima tropical e subtropical, devido à alta prevalência e a grande patogenicidade (AMARANTE; RAGOZO; SILVA, 2014). As formas adultas podem medir de 1 a 3 cm de comprimento, sendo facilmente observados a olho nu. As fêmeas podem liberar diariamente entre 5.000 e 10.000 ovos por dia (ROMERO; BOERO, 2001) e seu período pré-patente, período entre a ingestão da L_3 até a deposição de ovos junto às fezes, é de 18 a 22 dias (SANTOS et al., 2014). *H. contortus* é uma espécie hematófaga que possui como local de infecção o abomaso dos ruminantes (AMARANTE, 2014). Podendo ingerir de 0,05 a 0,08 mL de sangue por dia e, por isso, quando o animal está com uma carga parasitária elevada, pode apresentar anemia e edema submandibular, podendo vir a óbito. (AMARANTE; SALES, 2007; AMARANTE; RAGOZO; SILVA, 2014).

2.1.2 *Trichostrongylus* spp.

T. colubriformis e *T. axei* são as principais espécies do gênero *Trichostrongylus* que parasitam ovelhas e cabras, sendo *T. colubriformis* a mais relevante, tendo como nicho o intestino delgado. Os adultos vivem em túneis na superfície da mucosa, onde se alimentam de líquido mucoso, comida digerida e detritos celulares. Esses NGIs geralmente têm menos de 7 mm de comprimento e têm um período pré-patente que pode durar de duas a três semanas (URQUHART, 1996). Além disso, as fêmeas adultas do gênero liberam de 100 a 200 ovos diariamente, sendo resistentes à seca, pois são capazes de se tornarem anidrobióticos, reidratarem-se e retomarem o desenvolvimento após o restabelecimento da umidade ambiental (URQUHART, 1996; ROMERO; BOERO, 2001). Além de perda de apetite, as infecções intensas por *T. colubriformis* estão associadas a graves enterites, com atrofia das vilosidades, espessamento e erosão das mucosas, provocando baixa absorção de nutrientes (HOLMES, 1985).

2.2 *Ciclo biológico dos NGIs*

O ciclo de vida dos NGIs de ruminantes (Figura 3) é monoxênico (hospedeiro único), sendo constituído de duas fases: uma fase livre no ambiente (também chamada fase exógena) e uma fase parasitária (ou fase endógena) (URQUHART, 1996).

2.2.1 Fase livre (fase exógena)

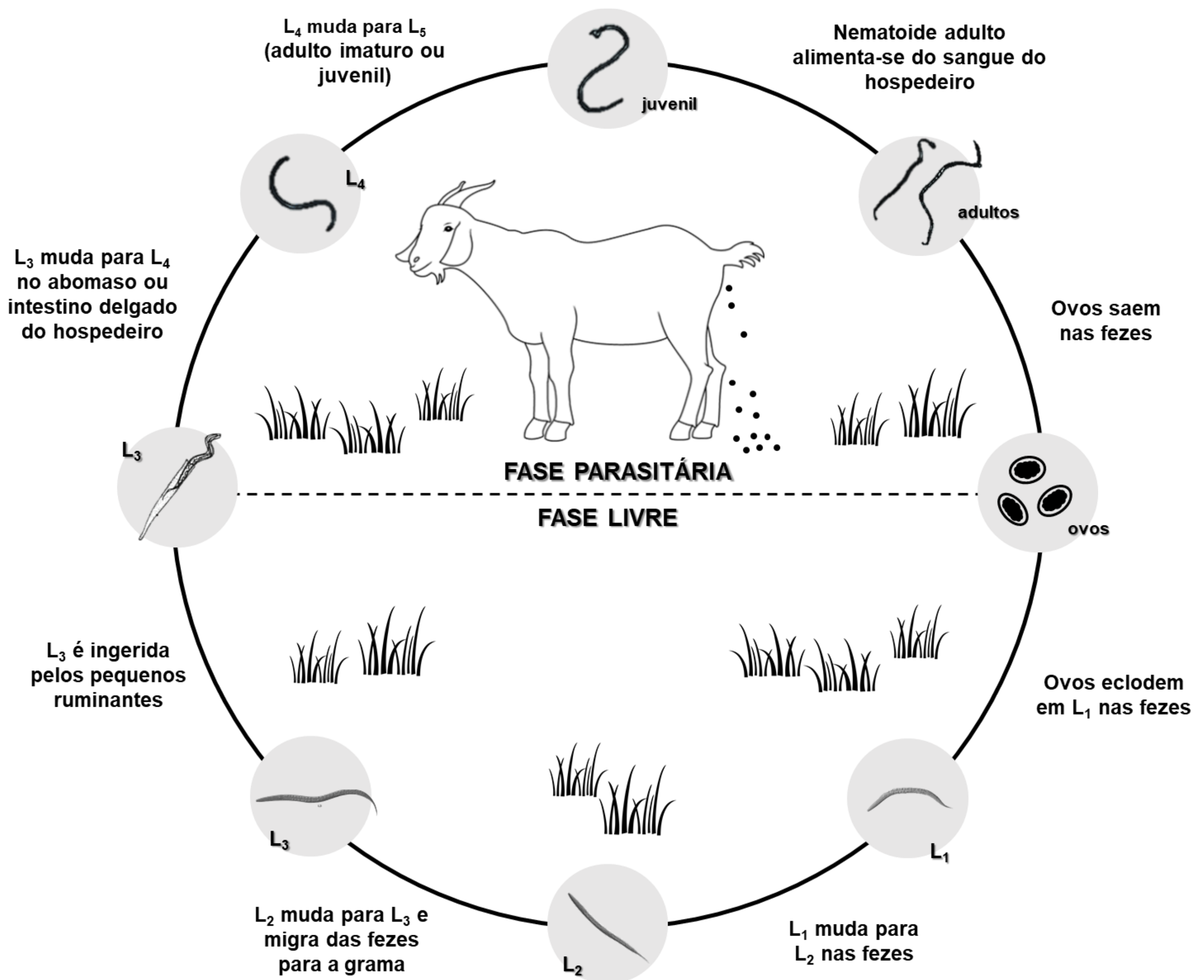
A fase exógena do ciclo biológico inicia-se com a liberação de ovos nas fezes do hospedeiro, os quais foram postos pelas fêmeas dos NGIs. Quando as condições ambientais são favoráveis (temperatura mínima de 10 °C e umidade de 60%), ocorre a eclosão dos ovos embrionados, libertando as larvas no estádio L_1 (SMITH; SHERMAN, 2009; URQUHART, 1996). Após o período de incubação as larvas L_1 evoluem por meio de duas mudas e se tornam larvas infectantes (L_3). Os estádios intermediários L_1 e L_2 são pouco resistentes no ambiente, ao contrário dos ovos e L_3. Dependendo das condições ambientais, L_3 pode sobreviver no ambiente por meio de suas reservas de gordura por vários meses em zonas temperadas, e poucas semanas em áreas tropicais ou subtropicais (URQUHART, 1996; O'CONNOR; WALKDEN-BROWN; KAHN, 2006).

2.2.2 Fase parasitária (fase endógena)

A fase parasitária inicia-se com a ingestão de larvas infectantes L_3 pelo hospedeiro durante o pastejo. Uma vez ingeridas, as L_3 no trato gastrintestinal do hospedeiro, são primeiramente liberadas de sua bainha, marcando a transição entre a vida livre para a vida parasitária (ROGERS; SOMMERVILLE, 1968). As L_3 desembainhadas penetram então na mucosa digestiva, transformando-se em larvas 4 (L_4), normalmente 24–96 horas após a infecção. L_4 mudam para o estádio 5, também chamado de adultos jovens (juvenil ou imaturo), ocorrendo de 8–16 dias após a infecção, dependendo da espécie (HERTZBERG et al., 2002). A transição para o estágio adulto corresponde à aquisição da maturidade sexual. Após a fertilização pelo macho, as fêmeas põem ovos, os quais são excretados nas fezes do hospedeiro. O tempo decorrido entre a ingestão de L_3 pelo hospedeiro e a primeira postura pelos NGIs é chamado de período pré-patente (SOULSBY, 1984; URQUHART, 1996).

No inverno ou durante um longo período de seca, é comum que L_4 encistem na mucosa digestiva, retardando assim o seu desenvolvimento (fenômeno nomeado de hipobiose larval) e retomando sua evolução na primavera ou na próxima estação chuvosa (SMITH; SMITH; MURRAY, 1994). Na ausência de hipobiose larval, o período pré-patente dura geralmente 2 a 3 semanas para a maioria das espécies de parasitos em ovelhas e cabras, enquanto pode durar até 5 semanas para algumas espécies parasitos de bovinos, tais como: *Oesophagostomum radiatum* (URQUHART, 1996; BALIC; BOWLES; MEEUSEN, 2000).

Figura 3. Ciclo biológico geral de nematoides gastrintestinais (NGIs) de ruminantes.



Fonte: autoria própria (2019).

2.3 Epidemiologia

Segundo Echevarria e colaboradores (1996), 95% das populações de NGIs se encontram no meio externo, em diferentes estratos da pastagem, e aproximadamente 5% se encontram nos hospedeiros (ECHEVARRIA et al., 1996). Na fase de vida livre, as condições ambientais, tais como: área de pastejo, vegetação com boa cobertura do solo e os inimigos naturais do estágio larval, como fungos, bactérias e coleópteros são os principais fatores relacionados ao desenvolvimento e sobrevivência dos parasitos. Na fase parasitária, os aspectos relativos à genética, nutrição, estados fisiológicos, manejo do rebanho, taxa de lotação, regime de criação e aspectos relativos ao bem-estar animal repercutem no desenvolvimento dos nematódeos (VIEIRA, 2007).

2.3.1 Fatores ambientais

Os fatores ambientais podem estar relacionados às condições climáticas, tais como: temperatura, precipitação pluviométrica, umidade relativa do ar, temperatura do solo e radiação ultravioleta (UV), os quais são fundamentais e determinam decisivamente na contaminação do ambiente (BARGER, 1999; ROCHA et al., 2012). Dentre esses, o fator mais decisivo é o índice pluviométrico, sendo que a transmissão da maioria dos nematódeos só ocorre quando o índice médio mensal for superior a 50 mm^3 (COSTA; SIMÕES; RIET-CORREA, 2011). No entanto, havendo o mínimo de precipitação, por volta de 12 mm^3, é possível promover uma intensa migração das larvas das fezes para o pasto, fechando o ciclo biológico dos parasitos. (AMARANTE, 2014; BRESCIANI et al., 2017).

Tanto a temperatura quanto a umidade são fatores climáticos que afetam o desenvolvimento de ovo até L_3 (O'CONNOR; WALKDEN-BROWN; KAHN, 2006). A umidade das fezes é fator determinante para a migração da L_3 dos cíbalas fecais (WANG et al., 2014). Dependendo da espécie de NGIs, a temperatura ideal é entre 23 e 28 °C e a porcentagem ótima de umidade entre 60 e 70%. Além disso, os ovos de *T. colubriformis* parecem mais resistentes ao frio do que os de *H. contortus* (SMITH; SMITH; MURRAY, 1994). Quando as fezes de são mantidas em estufa com temperatura controlada de

25°C, o desenvolvimento de ovo à L_3 leva em média 7 dias para a maioria das espécies de NGIs (AMARANTE, 2004).

O tempo de sobrevivência da larva na pastagem pode variar de algumas semanas em zonas tropicais a 12 meses em zonas temperadas, bem como varia segundo o estágio de desenvolvimento do parasita (LEVINE; TODD, 1975). Larvas de estágio inicial são relativamente vulneráveis a valores extremos de temperatura, enquanto L_3 são capazes de suportar condições muito mais severas (MORGAN; VAN DIJK, 2012). As larvas infectantes também são mais resistentes aos fatores ambientais que o ovo (LEVINE; TODD, 1975).

A geada ou dessecação acelera a morte de L_3. No entanto, o enterro de algumas L_3 no solo ou a formação de microclimas favoráveis no interior das fezes e da superfície do solo, formam excelentes reservatórios de parasitos, quando as condições ambientais são prejudiciais ao fechamento do ciclo biológico (LEVINE; ANDERSEN, 1973; CALLINAN; WESTCOTT, 1986). Outro fator que influencia a sobrevivência das larvas é a radiação UV. Os resultados obtidos por DIJK et al., (2009) evidenciam que os níveis naturais de radiação UV aumentam a taxa de mortalidade das larvas, havendo declínio na contaminação da pastagem na primavera, onde a radiação solar sobe rapidamente e a temperatura ainda é baixa.

2.3.2 Fatores relacionados ao hospedeiro e manejo do rebanho

Na pecuária, a restrição da atividade de pastejo em dois momentos favoráveis de infecções poderia resultar na diminuição da contaminação por NGIs. Ao amanhecer e ao anoitecer, quando o teor de umidade no extrato superior da forragem é elevado e a luz do sol é reduzida (SMITH; SMITH; MURRAY, 1994). Outra prática comumente utilizada para diminuir as populações de larvas é a rotação de pastagem (RODA et al., 1995), entretanto, essas práticas de manejo não apresentam resultados significativos na maior parte do Brasil, onde a sobrevivência de L_3 na pastagem é prolongada .

A taxa de lotação das áreas pastoris e o tempo de operação das parcelas são outros fatores que podem interferir nos níveis de parasitos, causando riscos para a saúde animal, contribuindo para intensificação das helmintoses e a ocorrência de outras

doenças (HOSTE; LE FRILEUX; POMMARET, 2001). O maior número de animais por área permite uma maior contaminação da pastagem. Portanto, a taxa de lotação adequada da pastagem favorece o controle de populações parasitárias, além de determinar uma altura de pastejo que impede a ingestão de larvas pelos animais (RODA et al., 1995).

Diferenças na preferência alimentar entre espécies de ruminantes influenciam o nível de infecção. De fato, devido ao comportamento de L_3, o risco de contaminação pelos hospedeiros durante a exploração de arbustos é reduzido. Por outro lado, o consumo de pastagem é propício ao contato com L_3. Esta preferência alimentar pode explicar, em parte, que cabras com comportamento de colheita são geralmente menos infectadas do que ovelhas, que apresentam comportamento de pastoreio, as quais podem se alimentar de qualquer porção das plantas forrageiras, sejam folhas, brotos e ramos, e de espécies dos variados portes, sejam herbáceas, arbustivas ou arbóreas (HOSTE; LEVEQUE; DORCHIES, 2001). Inclusive, até mesmo animais descritos como pastoreios, como ovelhas, podem apresentar comportamentos adquiridos, a fim de evitar áreas ricas em fezes (HUTCHINGS et al., 2003).

Além disso, a resistência e imunidade do hospedeiro podem limitar a instalação parasitária (DOUCH et al., 1996; HOSTE et al., 2006). Há dois tipos de respostas imunes que podem explicar a resistência do hospedeiro a infecções pelos nematoides. Na resposta imunológica inata, o hospedeiro é capaz de regular as populações de NGIs por mecanismos fisiológicos não específicos, tais como: peristaltismo, pH gástrico, secreção de muco e elevada resposta inflamatória. A resposta imunológica adquirida é uma resposta a infecções anteriores, sendo expressa de maneira mais específica ao NGI em questão, mediadas por linfócitos Th2 CD4+, o aumento do número de mastócitos na mucosa, a eosinofilia, a produção de anticorpos específicos, presença de substâncias inibidos no muco, bem como o aumento da produção de muco (RAHMAN; COLLINS, 1990; AMARANTE, 2004)

Em geral, os animais jovens são os mais sensíveis às infecções. No entanto, o fator "etário" é mais discriminatório em ovinos do que em cabras (HUTCHINGS et al., 2003). Estas diferenças etárias são principalmente relacionadas com a aquisição de

imunidade após infecções repetidas. Outros fatores como espécie hospedeira, raça e linhagem são determinantes para os níveis de infecções (SMITH; SMITH; MURRAY, 1994; HOSTE et al., 1999).

3. Anti-helmínticos (AH) convencionais

O controle dos NGIs é convencionalmente realizado com base na administração repetida de AHs convencionais. Um AH ideal pode ser definido como uma substância nematicida que causa a expulsão do parasito, sem causar danos significativos ao hospedeiro, apresentando um tratamento multivalente, não tóxico e rapidamente eliminado, fácil de administrar e economicamente viável (MARTIN; ROBERTSON; BJORN, 1997). Por muitos anos, os AHs convencionais demonstraram ser eficazes. No entanto, com o passar dos anos essas substâncias atingiram seus limites de eficácia (AMARANTE, 2014).

3.1 Classificação dos AHs convencionais

A classificação dos AHs ocorre de acordo com a estrutura química e modo de ação. Atualmente, existem oito grandes classes químicas de amplo espectro utilizadas em ruminantes (AMARANTE, 2014). Embora exista uma alta prevalência de NGIs parasitos no mundo, o desenvolvimento de novos fármacos AHs tem sido lento ao longo dos anos, devido a maioria dos infectados por helmintos viverem em países em desenvolvimento, que não possuem investimentos para descobrir medicamentos inovadores (PINK et al., 2005). Sendo que o custo para a confecção de um novo medicamento AHs é estimado em US $ 400 milhões para uso pecuário, e mais de US $ 800 milhões para uso humano (MORGAN et al., 2011). A Tabela 2 aborda os grupos de AHs convencionais utilizados no tratamento das helmintoses de ruminantes e seus respectivos mecanismos de ação.

Tabela 2. Anti-helmínticos (AHs) utilizados no tratamento das helmintoses de ruminantes e seus respectivos mecanismos de ação.

Classe	Fármacos	Mecanismo de ação
Benzimidazóis e pró-benzimidazóis e praziquantel	oxfendazol fenbendazol albendazol triclabendazol	Ligam-se seletivamente com alta afinidade à β-tubulina do parasito e inibem a polimerização e filamentação dos microtúbulos no tegumento e nas células intestinais dos NGIs. Interrompendo os processos celulares vitais, uma vez que microtúbulos são unidades estruturais essenciais de muitas organelas e necessários para inúmeros processos celulares. Consequentemente, provocam déficit energético por destruição das células intestinais e inibição da produção de ovos.
Imidazotiazóis	levamisol	Atuam como agonistas do receptor nicotínico de acetilcolina (nAChR), induzindo a abertura dos canais iônicos, o que aumenta a condução de sódio e provoca a despolarização da membrana. Desta forma, são considerados agonistas colinérgicos, provocando contração muscular e paralisia espástica dos parasitas, que são posteriormente eliminados do hospedeiro.
Lactonas macrocíclicas	ivermectina abamectina doramectina moxidectina	Atuam como agonistas seletivas de canais de cloreto controlados por glutamato (GluCls) que estão presentes nos neurônios e músculos faríngeos de nematoides. Estes AHs penetram nos parasitos supostamente por absorção transcuticular ou ainda, por via oral, nos helmintos hematófagos, provocando paralisia seletiva dos parasitas, pelo aumento da permeabilidade de íons cloro (Cl^-) no músculo, potencializando os canais iônicos mediados pelo glutamato (GluCl). Em baixas concentrações, potencializa o efeito do glutamato, e em altas concentrações, abrem os canais mediados diretamente pelo glutamato.
Derivados de Amino acetonitrila	monepantel	Nova classe de AH com amplo espectro contra NGIs resistentes aos benzimidazóis, imidazotiazóis e lactonas macrocíclicas. Atuam nas subunidades Cel-acr-23 (ou ACR-23) e Hco-mptl-1 (MPTL-1), membros da subfamília DEG-3 de genes do receptor de acetilcolina (nAChR), que é encontrado apenas em nematódeos. Este grupo de AHs atua, portanto, como agonista dos canais iônicos, causando hipercontração e consequente paralisia espástica do nematódeo.
Spiroindoles	derquantel*	Age como um antagonista dos nAChRs, o que causa paralisia flácida, resultando na expulsão de parasitos do hospedeiro.
Salicilanilidas/fenóis substituídos	closantel** disofenol** rafoxanide** niclosamida nitroxynil	Paralisia flácida e redução dos níveis de ATP (desacoplador da fosforilação oxidativa).
Benzenosulfonamida	clorsulon	Recomendado para o tratamento e controle de vermes hepáticos adultos (*Fasciola hepatica* e *Fasciola gigantica*) em

		bovinos. Normalmente é administrado em associação com a ivermectina. Este AH inibe as enzimas implicadas na via glicolítica, sendo um inibidor competitivo da 8-fosfoglicerato quinase e fosfogliceromutase, e bloqueia a oxidação da glicose em acetato e propinato, diminuindo os níveis de ATP (fasciolose).
Organofosforado	triclorfon	Ligam-se ao sítio ativo da acetilcolinesterase (AChE), impossibilitando-a de exercer sua função de hidrolisar o neurotransmissor acetilcolina em colina e ácido acético.

*Derquantel está sendo comercializado associado a abamectina; **Atuam em nematódeos hematófagos, como *Haemonchus*; Tabela baseada em AMARANTE, 2014 e SPINOSA; GÓRNIAK; BERNARDI, 2014.

3.2 Resistência aos AHs convencionais

3.2.1 Definição de resistência anti-helmíntica

Em termos gerais, uma população de NGIs resistentes aos AHs é aquela que adquiriu geneticamente a capacidade de resistir a níveis de fármacos habitualmente efetivos para indivíduos da mesma espécie (PRICHARD, 2017; SANGSTER; GILL, 1999). Como a resistência é baseada no determinismo genético, esse traço é transmitido hereditariamente (COLES, 2002; WOLSTENHOLME et al., 2004). Por ser um fenômeno evolutivo os parasitos que se tornaram resistentes por mutação genética são inicialmente poucos, mas seu desenvolvimento e abundância são favorecidos pela pressão de seleção, devido ao uso repetido dos AHs (JACKSON, 1993; WALLER, 2006).

3.2.2 Mecanismos de resistência anti-helmíntica

O uso repetido e inadequado de AHs convencionais levou ao desenvolvimento de resistência em numerosas espécies de parasitos veterinários em todo o mundo, com preocupações crescentes de que isso possa se estender aos parasitos humanos (ROSE et al., 2015; ABONGWA; MARTIN; ROBERTSON, 2017). Além disso, uma vez que os AHs pertencentes a mesma classe química podem agir de forma semelhante, a resistência a uma determinada substância é provavelmente acompanhada por resistência a outros AHs dessa mesma classe química (resistência lateral) ou com mecanismo de ação semelhante (WHITTAKER et al., 2017). Existe também a probabilidade de desenvolvimento de resistência cruzada a uma ou mais classes de AHs, uma vez que estes compartilham alvos moleculares semelhantes, gerando resistência generalizada à maioria das classes de fármacos AHs (WOLSTENHOLME et al., 2004).

Os mecanismos de resistência aos AHs incluem: (i) mutação ou deleção de um ou mais nucleotídeos nos genes-alvo, (ii) redução no número de receptores, (iii) diminuição da afinidade dos receptores para os fármacos e (iv) ausência de enzimas ativadoras. Sendo que a causa da resistência dos NGIs é muitas vezes complexa, em que dentro de uma espécie de nematoide, diferentes mutações podem levar à resistência contra o mesmo AH (GILLEARD, 2006; (SANGSTER; COWLING; WOODGATE, 2018).

3.2.3 Mecanismos de controle de resistência aos AHs

A fim de preservar a eficácia dos AHs, e retardar o surgimento e a difusão da resistência, tornou-se necessário o gerenciamento desses fármacos de forma mais racional, baseado no entendimento de fatores extrínsecos que favorecem o desenvolvimento de resistência (HOSTE; LE FRILEUX; POMMARET, 2002). A prevenção da resistência baseia-se em soluções que neutralizam os erros de comportamento identificados como conducentes à difusão da resistência (WALLER, 2004).

A resistência anti-helmíntica pode ser retardada ou superada por meio de diversas práticas, tais como: (i) identificar novos alvos de fármacos com diferentes perfis farmacológicos daqueles existentes, (ii) introduzir novos AHs com diferentes modos de ação daqueles já existentes, (iii) terapia combinada, membros da combinação de diferentes classes de medicamentos, (iv) fármacos rotativos com diferentes modos de ação entre as estações de dosagem (COLES, 2002; LEATHWICK, 2012).

4. Métodos alternativos aos AHs convencionais

Devido às crescentes limitações dos AHs convencionais, há uma crescente necessidade na utilização de métodos alternativos para controlar as infecções por NGIs. Estes métodos são baseados em três princípios de controle, os quais visam: esgotar a fonte de contaminação dos hospedeiros, aumentar a resistência do hospedeiro e eliminar NGIs do hospedeiro (MIN et al., 2017).

No entanto, raramente é possível evitar o uso de AHs convencionais. Uma situação mais comum é combinar técnicas alternativas com o uso estratégico de

substâncias, diminuindo, mas não eliminando, a necessidade destes. Este princípio é usado no sistema FARMACHA© para o controle de *H. contortus* em ovinos, o que permite aos pecuaristas avaliar o grau de anemia causada pelo parasito, comparando a cor das membranas mucosas do animal com um gráfico padrão. Os animais mais afetados podem ser identificados para tratamento, evitando o tratamento desnecessário de animais saudáveis (VAN WYK; BATH, 2002).

Os métodos utilizados para esgotar ou zerar a fonte de contaminação animal são baseados em três princípios: prevenir, evitar e diluir (BARGER, 1999; MIN et al., 2017). No caso dos dois primeiros princípios, o método de rotação de pastagem é bastante difundido e utilizado, o qual depende da disponibilidade de áreas limpas ou com baixos níveis de infecção (HOSTE et al., 2004; LEGARTO; LECLERC, 2007).

O princípio da diluição pode ser alcançado com a diminuição de densidade dos animais (ETTER et al., 2000), e/ ou com a mistura de animais sensíveis ao parasitismo com animais resistentes ou entre animais de diferentes idades; ou ainda por pastoreio misto, alternado ou simultâneo, entre duas espécies de hospedeiros (por exemplo, bovino/ ovino ou cavalos/ pequenos ruminantes) (ROCHA et al., 2008; MIN et al., 2017).

Outro método que visa zerar a fonte de infecções é a descontaminação ativa das pastagens, o qual visa reduzir ou mesmo exterminar larvas L_3 de nematoides por métodos químicos, utilizando cal ou a cianamida de cálcio nas pastagens (HOSTE et al., 2004), métodos físicos (práticas culturais) com reversão por pastagens aradas (PIZA, 2017) ou queima controlada de pastagens (TARIQ, 2017), e métodos de controle biológico, em que se utilizam fungos nematófagos ou bactérias predadoras que infectam os estádios livres de nematoides (BALOYI; LAING; YOBO, 2012; XUE et al., 2018).

O segundo tipo de método alternativo visa melhorar a resistência do hospedeiro, podendo ser baseada em três princípios, o primeiro propõe selecionar animais hospedeiros mais resistentes aos NGIs (WINDON, 1996; MIN et al., 2017). O segundo utiliza a vacinação, visando contatar de forma proativa o hospedeiro com doses muito baixas de antígenos do parasito, a fim de estimular o sistema imunológico e, portanto, para proteger contra futuros ataques por estes agentes patogênicos (BASSETTO et al.,

2014). E o terceiro princípio objetiva a melhoria da ingestão de alimentos do hospedeiro, por meio da adição de ração e consumo de proteína na ração, ajudando na melhora da resposta imune do hospedeiro ao parasitismo (TOSCAN et al., 2017).

O último princípio tem por objetivo eliminar os nematoides gastrintestinais, por meio da combinação de tratamentos convencionais com AHs, usados racionalmente, e tratamentos alternativos. Estes métodos alternativos baseiam-se na administração de novas substâncias com propriedades anti-helmínticas, tais como partículas de óxido de cobre (CHARTIER et al., 2000) ou a exploração de plantas medicinais com propriedades de anti-helmínticas (FRENCH, 2018).

4.1 Uso de plantas medicinais e suas substâncias como método alternativo

As plantas são utilizadas para tratar doenças em humanos e animais desde a antiguidade. Estima-se que mais de 30% dos fármacos são direta ou indiretamente baseados em estruturas químicas de produtos naturais (VAN WYK; WINK, 2017). Além disso, o crescente interesse dos países pela agricultura livre de substâncias nocivas em produtos de consumo está resultando no interesse em produtos naturais bioativos. Durante a última década, houve um aumento nos números de estudos focados na farmacologia veterinária, particularmente para as plantas portadoras de compostos fenólicos (HOSTE et al., 2016; SPIEGLER; LIEBAU; HENSEL, 2017).

O objetivo da fitoterapia veterinária é tratar animais parasitados com plantas inteiras ou preparações medicinais com propriedades anti-helmínticas. Há duas maneiras de preparo e uso das plantas, a primeira é na forma de fitoterápico, o qual é uma preparação cujo o ativo é originário de plantas, constituindo uma mistura complexa de substâncias que podem ser usadas medicamentosamente (HOSTE et al., 2006; HOSTE et al., 2011). A segunda forma de administração do material vegetal é como um alimento funcional (nutracêutico), que é definido como uma planta consumida por animais, visando aproveitar além das propriedades benéficas para a saúde o seu valor nutricional (ANDLAUER; FÜRST, 2002). Sua incorporação na ração poderia ser para fins de prevenção a longo prazo, ou em uma ação curativa (HOSTE et al., 2015).

Um levantamento sobre diversos estudos utilizando diferentes métodos *in vitro*, tais como: teste de inibição da eclosão de ovos (TIEO), teste de inibição do desenvolvimento larval de L_1 para L_3 (TIDL), teste de inibição da migração larval (TIML), teste de inibição da alimentação larval (TIAL), teste de inibição do desembainhamento de larvas L_3 (TIDL$_3$) e teste de inibição da motilidade de adultos (TIMA), os quais demonstraram que a maioria das plantas utilizadas apresentou uma atividade anti-helmíntica devido a uma quantidade substancial de compostos fenólicos, destacando-se os taninos condensados e hidrolisáveis (SPIEGLER; LIEBAU; HENSEL, 2017).

Diversos extratos de plantas e frações purificadas demonstram efeitos AHs *in vitro* e *in vivo* contra várias espécies de NGIs. Nos ensaios *in vitro*, certos estágios do ciclo de vida parecem ser mais sensíveis aos extratos (por exemplo, adultos e larvas L_3) do que outros (ovos). Além disso, alguns ensaios, como o TIDL$_3$, parecem detectar mais facilmente os efeitos AHs de taninos e compostos fenólicos quando comparado com outros testes realizados com o mesmo estádio de desenvolvimento (TIML) (SPIEGLER; LIEBAU; HENSEL, 2017).

Além disso, sob condições *in vivo*, algumas diferenças, tais como a suscetibilidade dos NGIs, diferenças entre espécies que residem no abomaso ou no intestino delgado, bem como diferenças nos hospedeiros podem interferir na atividade anti-helmíntica de extratos, frações e substâncias (SPIEGLER; LIEBAU; HENSEL, 2017). Estudos *in vivo* utilizando extratos de plantas, frações enriquecidas com polifenóis ou compostos puros de quebracho (*Schinopsis* spp.) mostraram efeitos AHs contra vários NGIs, sendo que na maioria dos casos, uma redução na contagem de ovos fecais de NGI por grama de fezes (OPG) foi alcançada em ovinos contra os parasitos ruminantes comuns *T. colubriformis*, *H. contortus* e *T. circumcincta* (ATHANASIADOU et al., 2000; 2001; MAX et al., 2009).

Outro estudo avaliou as alterações ultraestruturais causadas em adultos de *H. contortus* obtidos de cabras alimentadas com forragens à base de plantas ricas em polifenóis, *Lysiloma latisiliquum*, *Onobrychis viciifolia* (sainfoin) e quebracho. Foi observado a vacuolização das células intestinais, musculares e hipodérmicas dos NGIs,

podendo afetar a motilidade, nutrição e, eventualmente, o sucesso reprodutivo (MARTÍNEZ-ORTIZ-DE-MONTELLANO et al., 2019).

PAOLINI et al., (2003a; 2003b) observaram uma diminuição na excreção de ovos e fecundidade de NGIs fêmeas de *T. colubriformis* e *T. circumcincta* em cabras jovens, mas não em cabras adultas quando tratadas com sainfoin, apontando para variações dos efeitos AHs dependentes do organismo hospedeiro. Em relação ao ovos por grama (OPG), alguns estudos sugeriram que espécies de nematoides residentes no abomaso de ruminantes (por exemplo, *Haemonchus*) sejam mais suscetíveis a taninos do que intestinais (por exemplo, *Trichostrongylus*) (MAX et al., 2009), enquanto outros revelaram uma redução tanto no OPG quanto na carga de NGIs por taninos de *Acacia molissima* ocorreu para *H. contortus* e *T. colubriformis* (MINHO et al., 2008).

Em relação à redução da carga parasitária, as diferenças entre as espécies de parasitos são ligeiramente mais claras: diferentes extratos enriquecidos com taninos são mais eficazes contra as espécies intestinais (*T. colubriformis* e *Nematodirus battus*) do que contra as espécies abomasais (*H. contortus* e *T. circumcincta*) em ovinos (ATHANASIADOU et al., 2001; CENCI et al., 2007), bem como em cabras (PAOLINI et al., 2003a; 2003b).

Dentre as diferentes classes de produtos naturais com atividade anti-helmíntica, destacam-se alcaloides (GITHIORI; ATHANASIADOU; THAMSBORG, 2006), saponinas (DEEPAK et al., 2002; HOSTE et al., 2006) e taninos condensados (PAOLINI et al., 2003b; ALONSO-DÍAZ et al., 2008a; 2008b; HOSTE et al., 2012). Assim, numerosos estudos têm mostrado que o uso de plantas de biocontrole, e em particular aquelas contendo polifenóis, representa uma alternativa ao uso de AHs convencionais (GITHIORI; ATHANASIADOU; THAMSBORG, 2006; HOSTE et al., 2006).

Na última década, estudos abordando plantas ricas em compostos fenólicos, principalmente taninos, como potenciais AHs, tornou-se um tópico de intensa discussão, especialmente na medicina veterinária no que diz respeito ao tratamento de pequenos ruminantes (SPIEGLER; LIEBAU; HENSEL, 2017). No entanto, investigações utilizando compostos isolados ou frações caracterizadas quimicamente são essenciais, mas ainda escassos, dificultando afirmar correlações entre os efeitos AHs aos polifenóis.

Estudos com taninos condensados (TC) demonstraram que, tanto os conjuntos oligo- e poliméricos, bem como os blocos de construção monoméricos (flavan-3-óis), têm exercido atividade anti-helmíntica Catequina e epicatequina não exerceram ou apenas apresentaram uma fraca atividade contra *Ascaris suum* (WILLIAMS et al., 2014a) e contra diferentes parasitos de ruminantes (BRUNET; HOSTE, 2006; BRUNET; JACKSON; HOSTE, 2008; DESRUES et al., 2016), com exceção de um estudo mostrando uma inibição do desenvolvimento larval de *T. colubriformis* (MOLAN et al., 2003).

Os mesmos estudos citados acima demonstraram que a tri-hidroxilação do anel B (galocatequina e epigalocatequina) dos monômeros flavan-3-óis tiveram um efeito anti-helmíntico aumentado em comparação com os respectivos compostos não-substituídos, devido a presença de uma terceira hidroxila no anel B, inibindo a motilidade e a migração de larvas de *A. suum* (L_3) (WILLIAMS et al., 2014a), alimentação de larvas de *Ostertagia ostertagi* e *Cooperia oncophora* (L_1) (DESRUES et al., 2016) e o desembainhamento larval de *H. contortus* e *T. colubriformis*. (BRUNET; HOSTE, 2006). Isso também foi observado em relação à migração, desenvolvimento larval e eclosão de ovos de *T. colubriformis e T. circumcincta* (MOLAN et al., 2003; 2014).

Em geral, um aumento no número de grupos hidroxila pode estar associado a uma maior atividade anti-helmíntica (BRUNET; HOSTE, 2006; BRUNET; JACKSON; HOSTE, 2008), enquanto a estereoquímica dos flavan-3-óis (anel C) não parece ter influência significativa (WILLIAMS et al., 2014; DESRUES et al., 2016).

A maioria das investigações em relação aos polímeros dos TCs (proantocianidinas oligoméricas, PCO) concentrou-se no teor destes, no grau médio de polimerização (mDP), na razão prodelfinidina/ procianidina (PD/ PC) e na estereoquímica relativa em C_2 e C_3 como características estruturais, afetando a atividade anti-helmíntica, em que verificou-se que uma maior concentração de TCs está quase sempre associado a um aumento na atividade anti-helmíntica (NOVOBILSKÝ; MUELLER-HARVEY; THAMSBORG, 2011; NOVOBILSKÝ et al., 2013; QUIJADA et al., 2015).

Grande parte dos estudos publicados relata uma atividade superior contra nematoides de frações contendo TCs de alta massa molecular quando comparado a

frações consistindo de TCs de baixa massa molecular juntamente com outros compostos fenólicos ou não fenólicos (WILLIAMS et al., 2014a; 2014b; QUIJADA et al., 2015; KLONGSIRIWET et al., 2015). Contudo, um estudo utilizando o ensaio de migração larval (TIML) não encontrou correlação entre a massa molecular dos TC e a bioatividade, no entanto, os experimentos foram realizados em líquido ruminal, uma matriz que não é constante em sua composição (NAUMANN et al., 2014).

Outros dois estudos demostraram que a massa molecular teve um impacto negativo na alimentação de larvas L_1 de *O. ostertagi* e *C. oncophora*, (NOVOBILSKÝ et al., 2013) e, em menor grau, na motilidade adulta de *C. oncophora* (DESRUES et al., 2016). Além disso, verificou-se que o mDP não tem efeito sobre a inibição da migração e motilidade em L_3 de *A. suum* (WILLIAMS et al., 2014a), L_3 de *T. colubriformis* (QUIJADA et al., 2015) e apenas uma fraca influência na capacidade de migração de L_3 de *H. contortus* (NAUMANN et al., 2014).

Como os TCs são frequentemente modificados por esterificação com resíduos de ácido gálico, a influência da esterificação na atividade anti-helmíntica também foi investigada. Três frações contendo TCs de *Vitellaria paradoxa* mostraram um efeito inibitório igual na migração larval (L_3) e na motilidade larval (L_3, L_4) de *A. suum* (WILLIAMS et al., 2014a). Vale ressaltar que uma grande quantidade de TCs substituídos por galato foi encontrada em todas as frações, o que poderia explicar o efeito similar (RAMSAY et al., 2016). Assim como o efeito da inserção de grupos galato foi mostrado para melhorar a atividade de flavan-3-óis monoméricos (BRUNET; HOSTE, 2006).

Como discutido para os monômeros de flavan-3-óis, o padrão de hidroxilação do anel B de oligômeros (PD vs. PC) é uma característica estrutural importante que influencia a atividade *in vitro.* Geralmente, o impacto da relação entre prodelfinidina/ procianidina parece variar muito dependendo de diferentes parasitos e dos respectivos sistemas de teste aplicados, em que uma quantidade maior de PD inibiu o desembainhamento larval em *H. contortus* e *T. colubriformis* (QUIJADA et al., 2015; KLONGSIRIWET et al., 2015).

Escareño-Diaz et al avaliaram a atividade *in vitro* de substâncias naturais obtidas comercialmente: quercetina, ácido caféico, rutina e cumarina e suas combinações contra

a eclosão dos ovos (TIEO) e desembainhamento larval ($TIDL_3$) de *C. punctata*. Foi constatado que a rutina e quercetina não demonstraram bioatividade contra ovos ou larvas. No entanto, quando avaliados em combinação com o ácido caféico e cumarina, observou-se uma interação sinérgica contra o desembainhamento larval e eclosão de ovos, podendo haver uma relação linear entre a baixa massa molecular e a atividade ovicida; em que moléculas com maior massa molecular apresentaram concentrações inibitórias de 50% (CI_{50}) menores para atividade contra a eclosão de ovos (ESCAREÑO-DÍAZ et al., 2019).

Há menos relatos sobre as atividades anti-helmíntica dos taninos hidrolisáveis (TH). Carboidratos, principalmente glicose, esterificados com ácidos hidroxibenzóicos, particularmente ácido gálico e seus dímeros, são os principais elementos estruturais dos THs, que também podem ocorrer como oligômeros. Engström e colaboradores demonstraram que o ácido gálico não possui atividade anti-helmíntica contra ovos e larvas de *H. contortus* (ENGSTRÖM et al., 2016).

Investigações de galotaninos indicaram que estes ésteres foram ativos se pelo menos quatro grupos galatos estavam ligados à subunidade glicosídica, enquanto a atividade aumentou com o aumento do número de substituição por galatos (KIUCHI et al., 1988). Uma correlação similar foi observada para os dímeros do ácido gálico que se tornaram cada vez mais ativos com um maior grau de substituição por unidades de galatos (YAMASAKI et al., 2002; ENGSTRÖM et al., 2016). Portanto, novamente o número de grupos hidroxila parece ser uma importante característica relacionada a atividade anti-helmíntica.

Estudos descrevendo a atividade anti-helmíntica de flavonoides e seus glicosídeos, nicotiflorina (kaempferol-3-*O*-rutinosídeo), rutina (quercetina-3-*O*-rutinosídeo) e a narcisina (isorametina-3-*O*-rutinosídeo), demonstraram ser esses os principais compostos ativos em um extrato acetona: água (7:3) de sainfoin, causando uma redução significativa da motilidade de *H. contortus* (BARRAU et al., 2005). O extrato etanólico das partes aéreas de *Artemisia campestris* inibiram a eclosão de ovos e causou paralisia e morte de adultos de *H. contortus,* bem como mostrou conter

predominantemente derivados de quercetina e da apigenina (MAKKAR; FRANCIS; BECKER, 2007).

Além disso, a fração flavonoídica purificada de um extrato de *Agave sisalana* inibiu fortemente a eclosão de ovos em cultura mista de nematoides (*H. contortus*, *O. dentatum* e *T. colubriformis*) obtidos de cabras naturalmente infectadas, mas não influenciaram na migração larval (ENGEMANN et al., 2012). Luteolina-7-*O*-glicosídeo e quercetina-3-*O*-glicosídeo foram identificados como os compostos ativos de *Vicia pannonica* e mostraram uma eficácia comparável na inibição da motilidade das larvas de *T. colubriformis* no líquido ruminal (KOZAN; ANUL; TATLI, 2013). Além disso, a luteolina e a quercetina foram capazes de inibir o desembainhamento larval em *H. contortus*, bem como aumentaram a atividade dos TCs de maneira sinérgica, quando comparados com a atividade mais fraca do PC sozinho (KLONGSIRIWET et al., 2015).

Diversos estudos verificaram a atividade anti-helmíntica de glicosídeos flavonoídicos ou suas agliconas, demonstrando a importância da glicosilação para a biodisponibilidade no nematoide. Um estudo realizado por Duenas e colaboradores (2013) demonstrou que a quercetina-3-*O*-glicosídeo é melhor absorvida do intestino via transporte ativo do que a quercetina (DUEÑAS et al., 2013).

Ayres e colaboradores apontaram os efeitos inibitórios no desenvolvimento larval em *H. contortus* de flavonas metoxiladas isoladas do extrato metanólico de *Struthiola argentea*, bem como tentaram determinar a relação estrutura-atividade das flavonas, no entanto, nenhum padrão claro emergiu (AYERS et al., 2008).

Um estudo realizado por Borges e colaboradores verificou que a quercetina combinada com a ivermectina em ensaios *in vitro* aumentou o efeito do AH convencional contra larvas L_3 (TIML) de *H. contortus*, mas não teve efeito em adultos do mesmo parasita (TIMA). Além disso, eles observaram que a ação combinada (quercetina + ivermectina) foi influenciada pelo grau de resistência e estágio de desenvolvimento do parasita. Ao ser administrada por via intra-abomasal, a combinação não foi eficaz na redução de OPG e da carga parasitária de ovelhas naturalmente infectadas (BORGES et al., 2020).

A alta diversidade estrutural dos flavonoides e os diferentes sistemas de teste usados dificultam a atribuição de uma característica estrutural responsável pela atividade anti-helmíntica. Mesmo as investigações que visavam principalmente detectar as relações estrutura-atividade no mesmo bioensaio não levaram a resultados conclusivos. Nesse contexto, estudos com flavonoides focados nessas questões são escassos em comparação àqueles com TCs, e mais pesquisas são necessárias para obter dados conclusivos.

5. *Pterogyne nitens* e suas substâncias

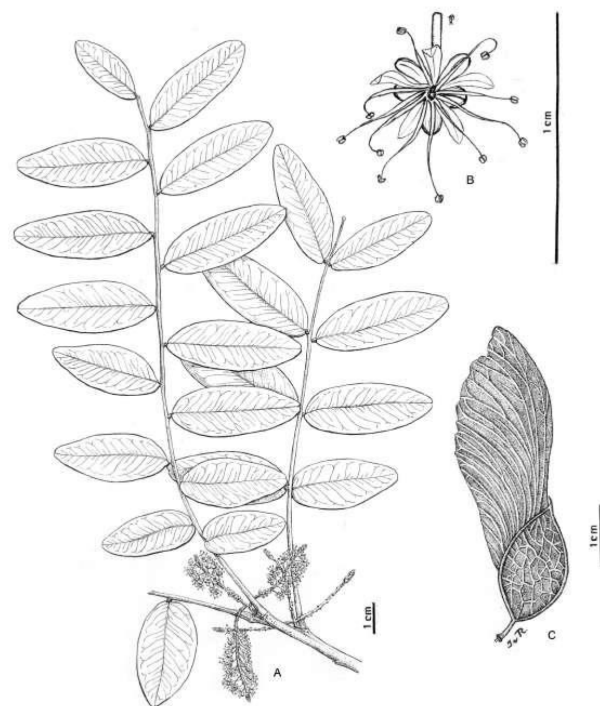
Pterogyne nitens Tulasne (Figura 4) é uma espécie arbórea pertencente a um gênero monoespecífico da família Fabaceae, sendo popularmente conhecida como "amendoim-do-campo", "amendoim-bravo" ou "bálsamo" (LORENZI, 2002). Na América Latina essa espécie ocorre na Argentina, Bolívia, Paraguai e Brasil. No território brasileiro ocorre com frequência na Caatinga, Cerrado e Mata Atlântica (LIMA, 2013) (Figura 5).

P. nitens é uma planta rústica, adaptada à grande parte do território nacional, pouco exigente em fertilidade do solo. Constituindo uma boa opção para a silvicultural, sendo amplamente empregada na construção civil devido sua madeira ser densa e resistente (CARVALHO, 2003). Esta planta também é conhecida pela beleza e odor de suas flores, bem como pela intensidade de suas folhagens, que possui valor ornamental, sendo recomendada para a arborização de vias urbanas e rodovias e na reposição de mata ciliar em locais com inundações periódicas para recuperação de áreas degradadas (PAULA; ALVES, 2007).

Figura 4. Indivíduo de *Pterogyne nitens* utilizado para coleta do material botânico. Detalhe dos órgãos vegetativos (folhas) e reprodutivos (flores e frutos).

Fonte: autoria própria (2014);

Fonte: kew's herbarium (1991);



Fonte: A. UlibarrI (2008).

Figura 5. Mapa da ocorrência e lista da distribuição geográfica de *Pterogyne nitens* no Brasil.

Fonte: Flora do Brasil (2020).

Em um estudo etnofarmacológico em comunidades indígenas *Izoceño-Guaraní,* estabelecidas no sudeste da Bolívia, foi constatado que as folhas desta árvore são utilizadas para alimentação dos animais nas comunidades. Além disso, é usada para tratar problemas de anúria (diminuição ou suspensão da secreção urinária), em que folhas secas e moídas são misturadas a bebida "mate" ou café e administradas até que o enfermo volte a urinar. Folhas e caules também são queimados, e suas cinzas são lavadas e adicionadas à gordura animal ou são preparadas por meio de decocções, para serem utilizadas no banho ou aplicação local, respectivamente, para o tratamento da sarna (*Sarcoptes scabiei*) (BOURDY; CHAVES DE MICHEL; ROCA-COULTHARD, 2004). Em outras duas comunidades indígenas *Mbay-Guaraní* no nordeste da Argentina, pesquisadores descreveram o uso de preparações aquosas das cascas dos caules de *P. nitens* para o tratamento de infecções parasitárias por *Ascaris lumbricoides*, conhecida na comunidade por *tacho* (CRIVOS et al., 2007).

P. nitens apresenta inúmeros extratos e substâncias bioativas avaliadas em diversas atividades biológicas. Na tabela 3 estão listados os trabalhos com extratos e substâncias isoladas, bem como os ensaios biológicos realizados durante os últimos anos. As estruturas químicas das substâncias foram representadas nas diferentes figuras ao longo do texto (Figura 6–8).

Tabela 3. Levantamento bibliográfico dos trabalhos realizados com extratos e substâncias de *Pterogyne nitens*, abrangendo diferentes atividades biológicas.

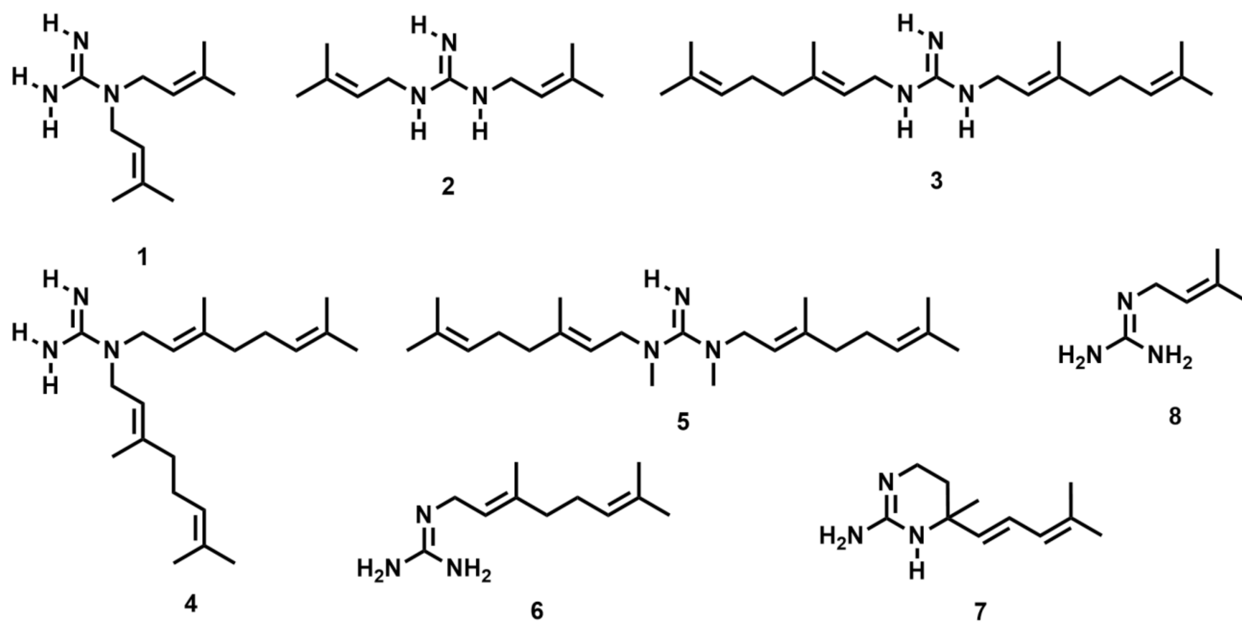
Atividade Biológica	Extrato, fração, substância	Classe	Parte da planta	Referência Bibliográfica
n.d.	pteroginina (**1**)	alcaloide guanidínico	galhos, caule	(CORRAL; ORAZI; PETRUCCI, 1969)
n.d.	pteroginidina (**2**)	alcaloide guanidínico	galhos, caule	(CORRAL; ORAZI; PETRUCCI, 1970)
atividade anti-hiperglicêmica in vivo	extrato etanólico	-	folhas	(SOUZA et al., 2009) (SOUZA et al., 2010)
atividade mutagênica em Tradescantia pallida	extrato etanólico	-	folhas	(OLIVEIRA et al., 2007)
mutagênico ou antimutagênico	extrato etanólico, fração butanólica, hidroalcoólica, fração acetato de etila, pteroginina, pteroginidina	alcaloides guanidínicos	folhas	(FERREIRA et al., 2009)
citotóxica	pteroginina, pteroginidina, nitensidina A–C (**3**–**5**)	alcaloides guanidínicos	folhas	(BOLZANI; GUNATILAKA; KINGSTON, 1995) (DUARTE et al., 2010)
citotóxica	nitensidina D (**6**), nitensidina E (**7**), galegina (**8**)	alcaloides guanidínicos	folhas	(REGASINI et al., 2009b)
atividade tóxica contra osteoclastos	galegina, pteroginina, pteroginidina, nitensidina A	alcaloides guanidínicos	s.i.	(TAJIMA et al., 2015)
citotóxica (resistência de células tumorais)	pteroginina, pteroginidina, galegina, nitensidina A	alcaloides guanidínicos	folhas, caules	(SATAKE et al., 2015)
substrato de transportadores do tipo ABCB1	nitensidina A	alcaloide guanidínico	s.i.	(TAJIMA et al., 2014)

apoptose em células de carcinoma cervical	nitensidina B		folhas	(OLIVEIRA et al., 2018)
antifúngica	extratos e frações	-	raízes, caules, frutos verdes, cascas de caule	(REGASINI et al., 2010)
antibacteriana	extrato metanólico	-	caules	(SALVAT et al., 2004)
antibacteriana	pteroginina, pteroginidina, nitensidina A–E, galegina	alcaloides guanidínicos	s.i.	(COQUEIRO et al., 2014)
n.d.	apigenina (**9**), pedalina (**10**)	flavonoides	folhas	(BOLZANI; GUNATILAKA; KINGSTON, 1995)
Inibitória de MPO	pteroginina, pteroginidina, taxifolina (**11**), quercetina 3-*O*-soforosídeo (**12**), astilbina (**13**), ourateacatequina (**14**), ácido caféico (**15**), ácido ferúlico (**16**), ácido sinápico (**17**), ácido clorogênico (**18**), ácido gálico (**19**)	alcaloides guanidínicos flavonoides ácidos fenólicos	flores	(REGASINI et al., 2008a)
antiproliferativa (células de melanoma)	extrato hexânico, extrato etanólico, fração n-butanol, fração hidroalcoólica, fração AcOEt quercetina, isoquercetrina (**20**)	flavonoides	cascas de caule	(REGASINI et al., 2007)
antioxidante e citotóxico	kaempferol (**21**), quercetina (**22**), isoquercetrina	flavonoides	s.i.	(VELLOSA et al., 2011)
antioxidante	extrato etanólico	-	folhas	(DOS SANTOS et al., 2009)
antioxidante, anti e pró-hemolítica	extrato etanólico	-	folhas	(PASQUINI-NETTO et al., 2012)
antioxidante	fração AcOEt, miricetina (**23**), quercitrina (**24**), miricitrina (**25)**	flavonoides	caule	(REGASINI et al., 2008b)
antioxidante	pteroginosídeo (**26**), kaempferitrina (**27**), afzelina (**28**)	flavonoides	frutos	(VELLOSA et al., 2015)
antioxidante	pedalitina (**29**), isoquercetrina	flavonoide	folhas	(OKUMURA et al., 2012)

inibitória de MPO e antioxidante	pedalitin 6-O-β-glicopiranosídeo (**30**), sorbifolina (**31**), sorbifolina 6-*O*-β-glicopiranosídeo (**32**), nitensosídeo A (**33**) nitensosídeo B (**34**), pedalitina	flavonoides	folhas	(FERNANDES et al., 2008)
inibitória de MPO	pterogynosídeo, kaempferol, afzelina, kaempferitrina, quercetina, isoquercitrina, rutina (**35**)	flavonoides	frutos	(REGASINI et al., 2008c)
antifúngico	sorbifolina, pedalina, nitensosídeo B, quercetina, isoquercitrina, quercetina 3-*O*-soforosídeo, rutina, ourateacatequina, ácido caféico, ácido ferúlico, ácido sinápico, ácido clorogênico, ácido gálico, ácido oleanônico (**36**), ácido betulínico (**37**)	flavonoides ácidos fenólicos triterpenos	folhas, frutos, flores	(LIMA et al., 2016)
antifúngico in vitro e in vivo	pedalitina	flavonoide	s.i.	(SANGALLI-LEITE et al., 2016)
anti-HCV	pedalitina, sorbifolina	flavonoides	folhas	(SHIMIZU et al., 2017)
n.d.	β-amirina (**38**), acetato de taraxerol (**39**), lupenona (**40**), β-amirrenona (**41**), germanicona (**42**), campesterol (**43**), estigmasterol (**44**), β-esitosterol (**45**)	triterpenos esteroides	folhas, frutos, flores, caules	(REGASINI et al., 2009a)

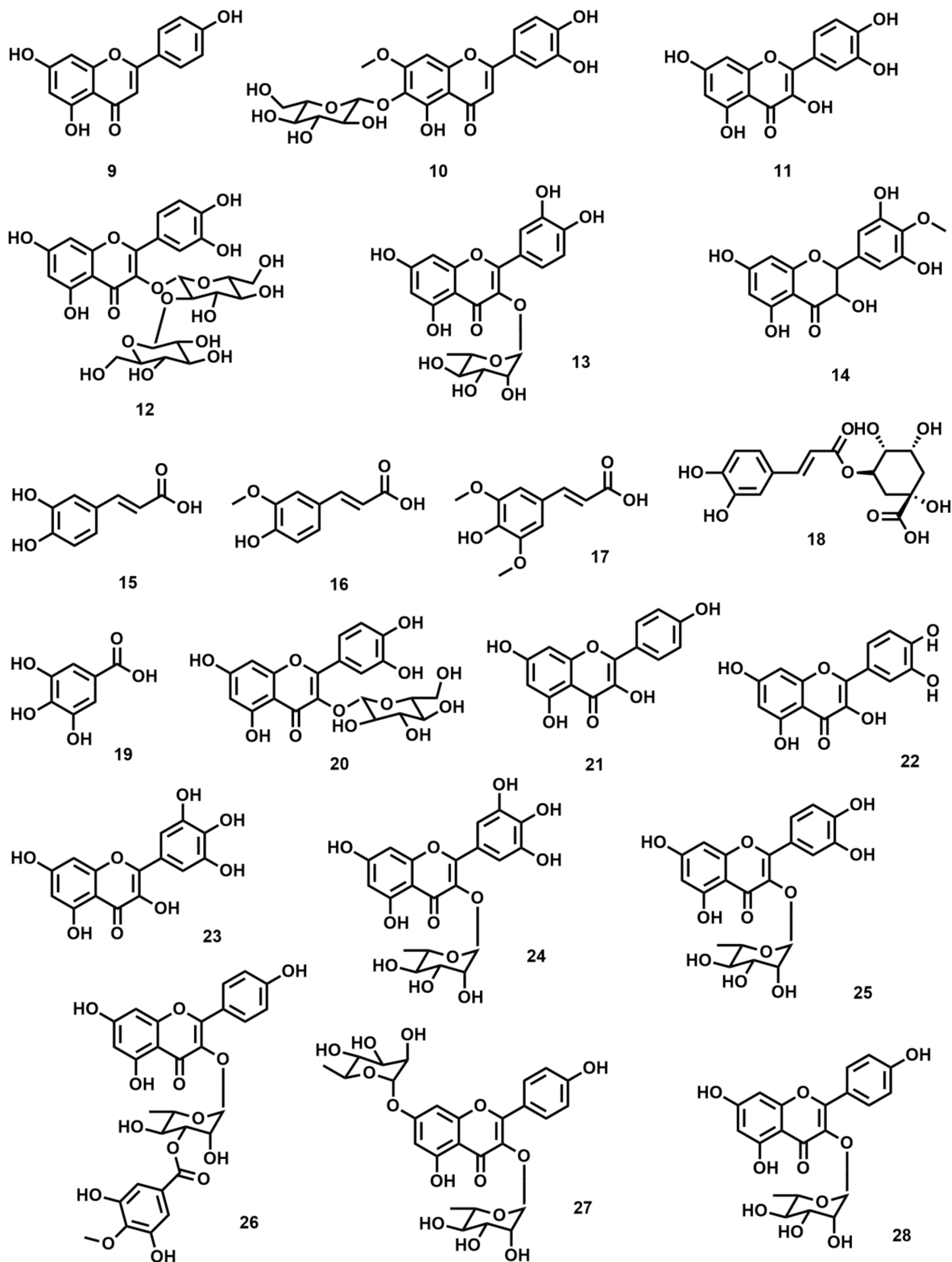
n.d. = não determinado; s.i. = sem isolamento

Figura 6. Estrutura química de alcalóides guanidínicos: pteroginina (**1**), pteroginidina (**2**), nitensidinas A–E (**3**–**7**) e galegina (**8**).



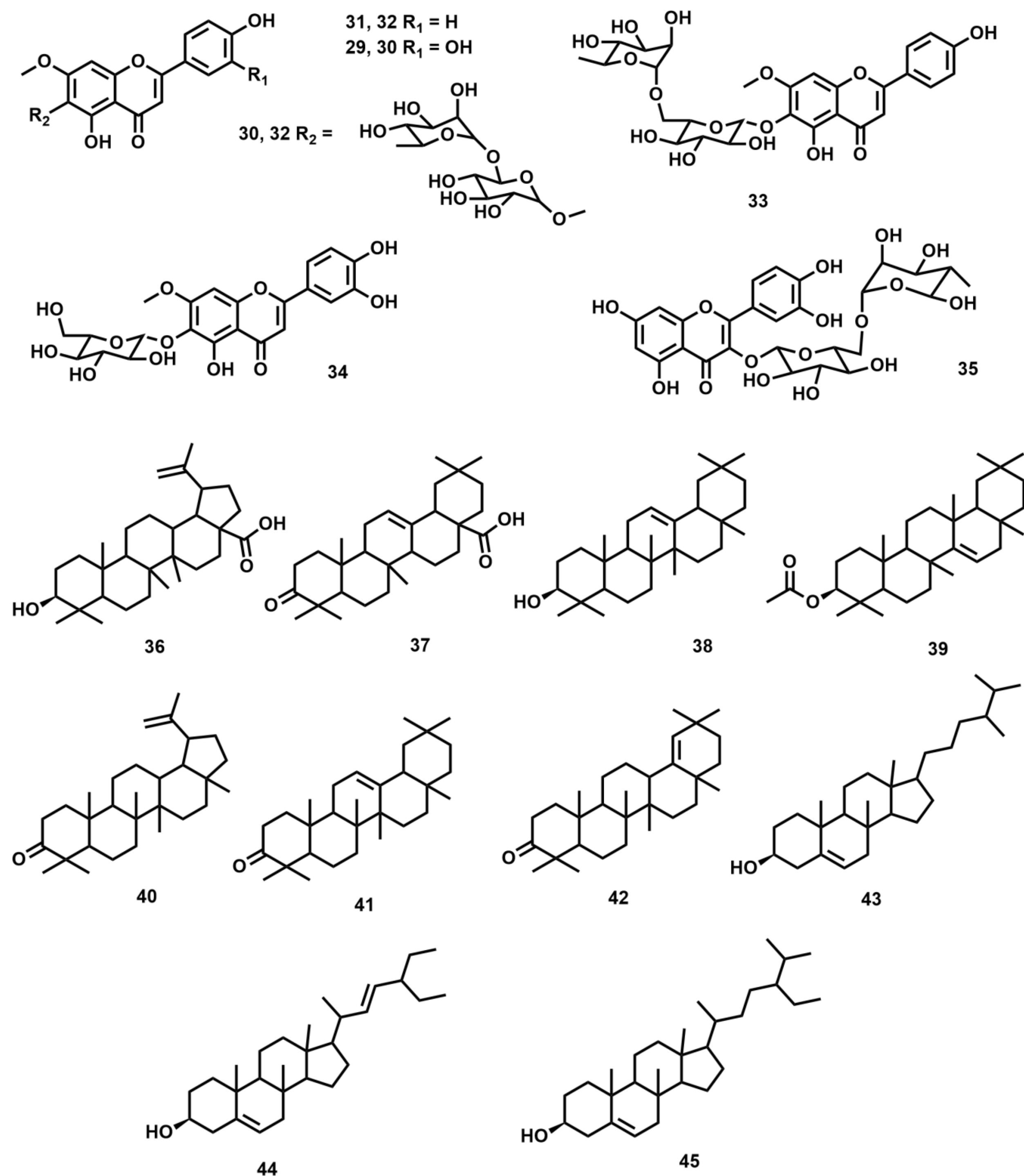
Fonte: autoria própria (2019).

Figura 7. Estrutura química de flavonoides (**9**–**14** e **20**–**28**) e ácidos fenólicos (**15**–**19**).



Fonte: autoria própria (2019).

Figura 8. Estrutura química dos compostos fenólicos (**29**–**35**), triterpenos e esteroides (**36**–**45**).



Fonte: autoria própria (2019).

OBJETIVOS

Objetivo Geral

Avaliar a atividade anti-helmíntica *in vitro* de extratos e substâncias de *Pterogyne nitens* contra duas espécies de nematoides gastrintestinais (NGIs) de pequenos ruminantes, *Haemonchus contortus* e *Trichostrongylus colubriformis*.

Objetivos Específicos

A) Preparar extratos etanólico das folhas, frutos e flores de *P. nitens*;

B) Isolar e identificar substâncias dos extratos etanólicos de *P. nitens*;

C) Avaliar a atividade anti-helmíntica dos extratos etanólicos e substâncias contra isolados susceptíveis de *T. colubriformis* e *H. contortus* originários da França e do Brasil;

D) Avaliar *in vitro* a eclodibilidade de ovos de NGIs por meio do teste de inibição de eclosão de ovos (TIEO);

E) Avaliar *in vitro* o desenvolvimento larval de formas imaturas de NGIs utilizando o teste de inibição do desenvolvimento larval (TIDL);

F) Avaliar *in vitro* o desembainhamento de larvas infectantes de NGIs por meio do teste de inibição do desembainhamento ($TIDL_3$).

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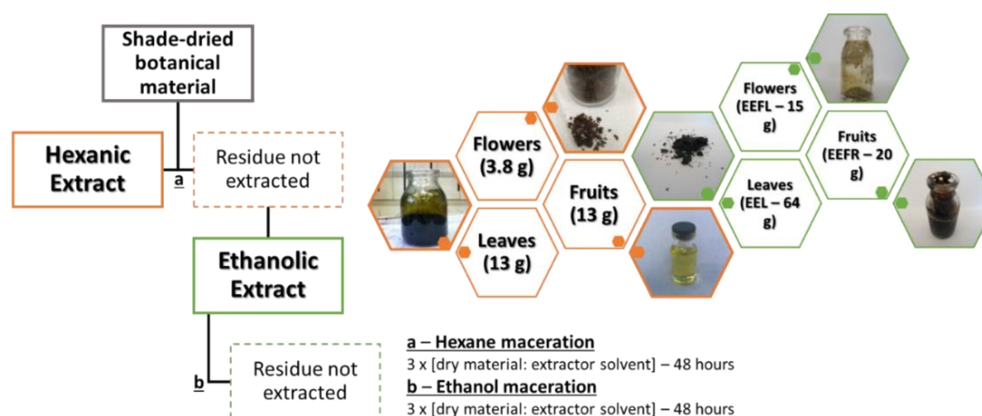
APÊNDICE A – MATERIAL SUPLEMENTAR

Fonte: autoria própria (2020).

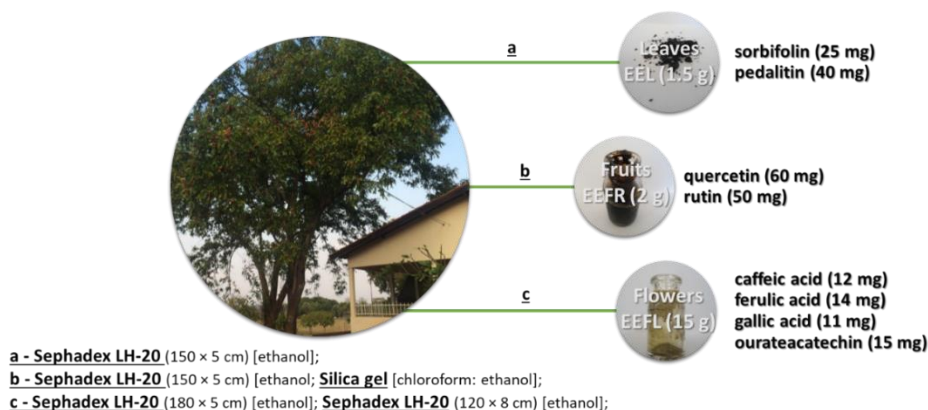
Figura 1. Material vegetal de *Pterogyne nitens* foi coletado no campus IBILCE da Universidade Estadual de São Paulo (Unesp) em São José do Rio Preto (Brasil), em julho de 2014. Uma exsicata (HISA 10291) foi depositada no Herbário de Ilha Solteira (HISA). O número de acesso à biodiversidade brasileiro A85B7D5 foi registrado no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado.



Fonte: autoria própria (2020).

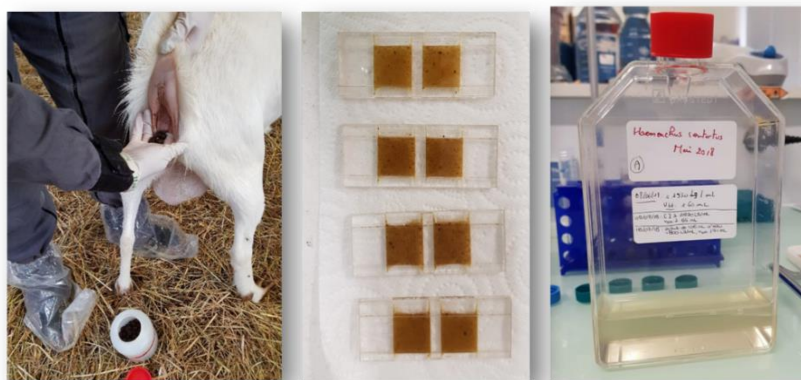
Figura 2. O material vegetal [folhas (630 g), frutas (200g) e flores (1.500 g)] foi seco à sombra e moído em um triturador de facas. O pó obtido foi primeiro macerado com hexano (1L × 3) por 48 horas, para remover os compostos apolares, o material vegetal foi separado da solução de extração por filtração simples e o filtrado foi seco sob

evaporação rotativa. Posteriormente, o resíduo não extraído foi macerado com etanol (1L × 3) por 48 horas e o mesmo procedimento foi repetido. Três extratos foram obtidos; extrato etanólico das folhas (EEL, 64 g); extrato etanólico de frutos (EEFR, 20 g); e extrato etanólico de flores (EEFL, 15 g).



Fonte: autoria própria (2020).

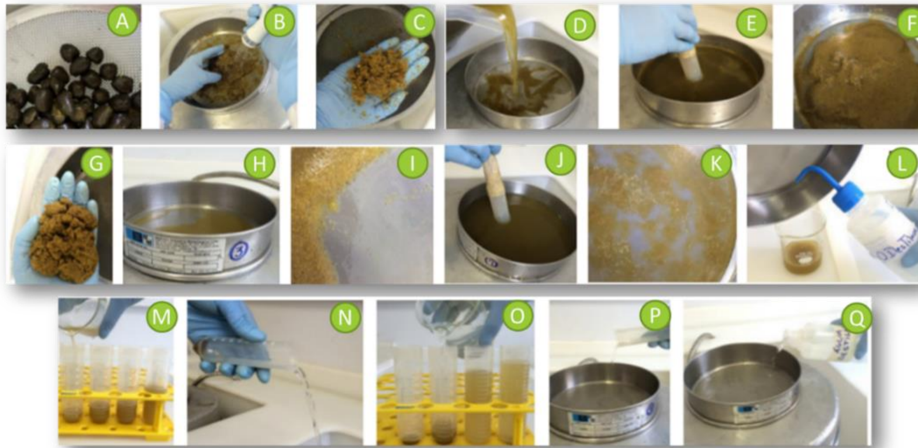
Figura 3. Flavonoides (derivados de flavona, flavonol e catequina) e ácidos fenólicos foram isolados utilizando procedimentos fitoquímicos. Flavonas (sorbifolin, pedalitina) foram isolados das folhas. Flavonóis (quercetina e rutina) foram obtidos de frutos. E o flavan-3-ol (ourateacatechin) e ácidos fenólicos (ácido cafeico, ácido ferúlico e ácido gálico) foram isolados das flores.



Fonte: autoria própria (2020).

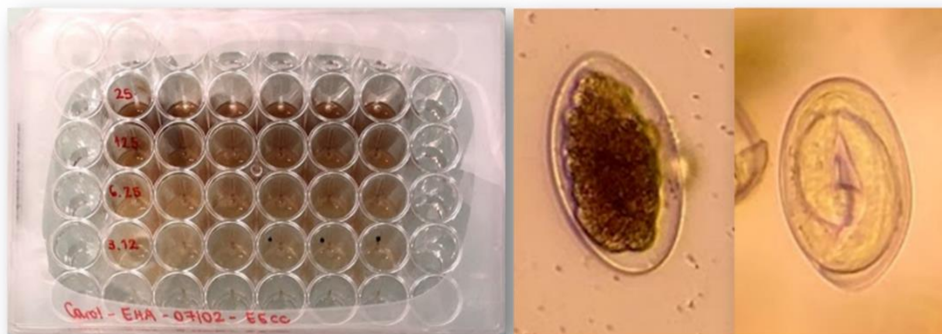
Figura 4. Os isolados suscetíveis de *Haemonchus contortus* e *Trichostrongylus colubriformis* foram utilizados para infecção monoespecífica e cada animal foi infectado via oral com aproximadamente 4000 larvas L_3. Após 28 dias de incubação, a infecção foi confirmada através da verificação da contagem do OPG. Os animais com contagem

acima de 1500 foram considerados parasitologicamente competentes como doadores de fezes para os TIEO e TIDL. As larvas infectantes L_3 foram mantidas em frascos e condições ideais de sobrevivência em laboratório pelo menos por um mês antes de serem utilizadas o $TIDL_3$.



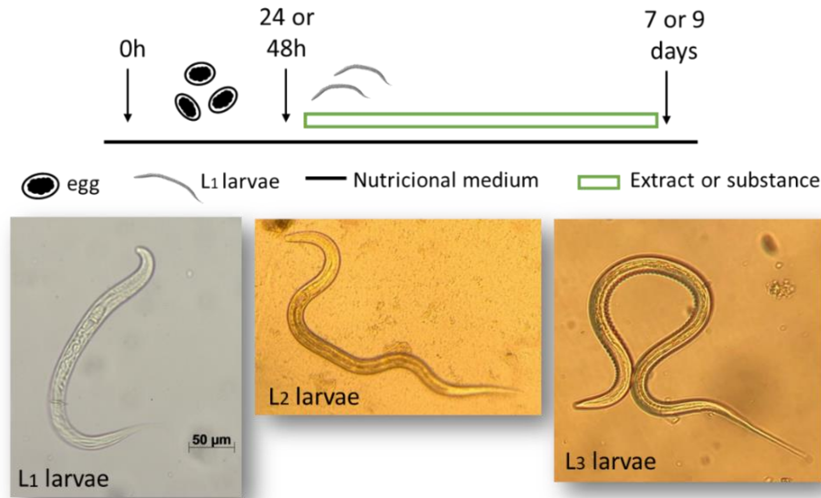
Fonte: adaptado de I. Agnolon (2015).

Figura 5. Recuperação e preparação de ovos para os TIEO e TIDL foram realizados de acordo com o protocolo descrito por Chagas et al. (2011) com pequenas modificações.



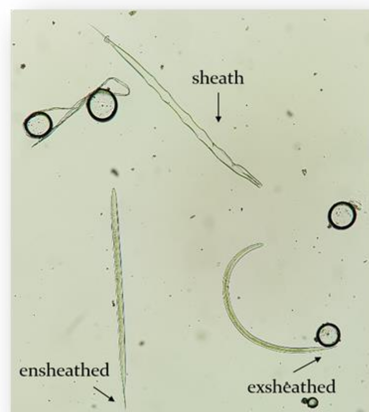
Fonte: autoria própria (2020).

Figura 6. Aproximadamente, 100 ovos foram adicionados a cada poço de uma microplaca de 48 poços. Tratamentos e controles foram testados em seis repetições. Os ovos e as larvas de L_1 foram contados em um microscópio invertido para calcular a inibição da eclosão dos ovos.



Fonte: autoria própria (2020).

Figura 7. Aproximadamente, 100 ovos foram adicionados a cada poço de uma microplaca de 48 poços com meio nutritivo e anfotericina B. As placas foram incubadas por 24 ou 48 horas para obter larvas de L_1. Após esse período as soluções foram adicionadas. As placas foram incubadas por mais 7 ou 9 dias e cada poço foi analisado em um microscópio invertido para contar as larvas L_3 e larvas não desenvolvidas (L_1 e L_2) para estimar a inibição do desenvolvimento larval.



Fonte: M. Oliveira (2019).

Figura 8. A cinética do desembainhamento foi medida sob um microscópio óptico com ampliação de 200x para identificar a proporção entre larvas com e sem bainha.

Capítulo II

In vitro ovicidal and larvicidal activities of flavonoids from Pterogyne nitens against Trichostrongylus colubriformis: an explanation of the structure-activity relationships

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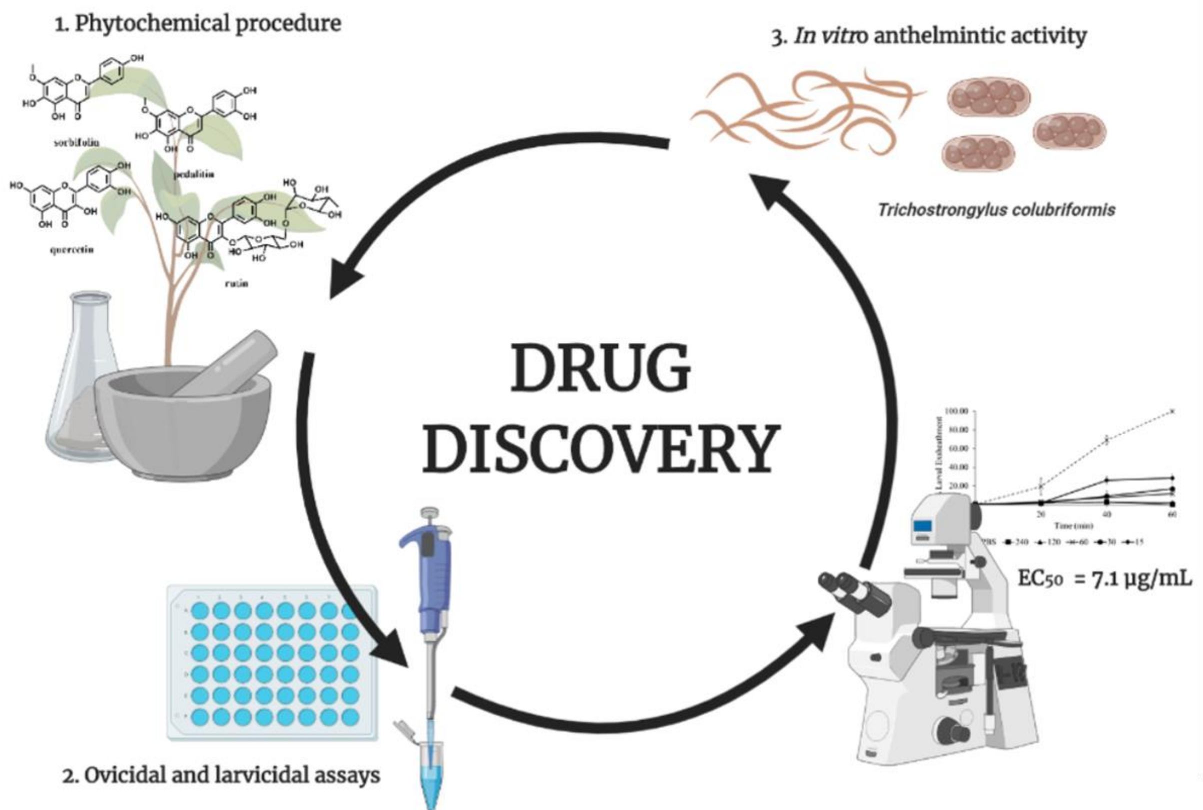
HIGHLIGHTS

- Extracts and flavonoids of *P. nitens* showed potent *in vitro* AH activity.
- Extracts were more active to inhibit larval development than the flavonoids.
- Among the 4 natural flavonoids, quercetin was the most active in the 3 assays.
- Overall, the number and position of hydroxyl groups modulate the AH activity.
- This is the first investigation AH activity of sorbifolin, pedalitin, and chrysin.

GRAPHICAL ABSTRACT



ABSTRACT: Because of the worldwide diffusion of resistance to synthetic anthelmintics (AH) in gastrointestinal nematodes (GINs) of ruminants, there has been a strong impetus to seek alternative solutions. The exploitation of bioactive plant compounds (particularly secondary metabolites) with AH properties has been identified as the main option to achieve more sustainable control of GINs, used either as phytotherapeutics and/or nutraceuticals. This study aimed to ascertain the ovicidal and larvicidal activities of *Pterogyne nitens* extracts and related flavonoids against *Trichostrongylus colubriformis*, based on three *in vitro* assays; the egg hatch assay (EHA), larval development assay (LDA) and larval L_3 stage exsheathment assay (LEA). The AH activity was expressed as an effective concentration having 50% of the maximal response against the parasites (EC_{50}) and respective 95% confidence interval (95% CI). Multivariate analysis (MCA) was performed using XLSTAT-Premium 2019.3.2. Fresh leaves and/or dried fruits of *P. nitens* were collected, shade-dried, ground, defatted with hexane and subjected to extraction by maceration with ethanol. Ethanolic extracts of leaves (EEL) and fruits (EEF) were repeatedly passed through chromatography columns. Four flavonoids (sorbifolin, pedalitin, quercetin, and rutin) were purified from the extracts and two commercial flavonoids [chrysin (C80105) and morin (M4008)] were obtained from Merck®, to derive chemical structure and AH activity relationship studies. Flavonoid structures were characterized mainly using nuclear magnetic resonance (NMR) spectral analyses, including ^{1}H NMR and ^{13}C NMR. The results showed that all extracts and compounds were active in the EHA, LDA, and LEA. Egg hatch and larval development inhibitory effects of EEL (EC_{50} = 2.9 mg/mL and 8.9 µg/mL) were twice as potent as EEF (EC_{50} = 5.9 mg/mL and 20 µg/mL). Among the compounds, quercetin was the most potent anthelmintic, presenting EC_{50} of 0.4 mg/mL in the EHA, 100 µg/mL in the LDA and 7.1 µg/mL in the LEA. Morin was the most active flavonoid against *T. colubriformis* L_3 (EC_{50} = 2.9 µg/mL), suggesting its potential *in vivo* therapeutic effect. The number and position of flavonoid hydroxyl groups changed the AH activity in the three biological assays, confirmed by the MCA. Altogether, our results corroborate the findings of other studies of AH compounds based on traditional knowledge, evidencing the AH effect of *P. nitens* and its bioactive flavonoids.

KEYWORDS: ruminants' parasitic nematodes, polyphenols, *in vitro* assays, anthelmintic.

1. Introduction

Parasitism by gastrointestinal nematodes (GINs) is one of the major health problems of small ruminant breeding, causing severe weight loss, lower production and death (Zajac, 2006; Velde et al., 2018). Small ruminants are affected by GINs of the Trichostrongyloidae superfamily, with a wide range of different species, such as *Haemonchus contortus* and *Teladorsagia circumcincta* in the abomasum; and *Trichostrongylus* spp., *Cooperia* spp. and *Oesophagostomum* spp. in the intestine. These different parasitic species usually occur as co-infections (Hoste et al., 2006).

Trichostrongylus colubriformis which inhabits the small intestine is a ubiquitous species present worldwide. It is one of the most important causes of parasitic enteritis, inducing protracted diarrhea, weakness, loss of production and death in lambs and kids. The adult worms live in tunnels on the mucosa surface, where they feed on mucous fluid, digested food, and cellular debris. Its infective larvae (L_3 stage) have a high capacity to survive even in adverse weather conditions (Velde et al., 2018).

In flocks where *H. contortus* is the GIN species prevalent, the FAMACHA© system is considered one of the best criteria used and was developed to diagnose anemia in small ruminants. However, it should not be used as a selection criterion in the diagnosis of non-hematophagous parasites, as *T. colubriformis* (Mahieu et al., 2007). Wherein, the diarrhea score and body condition score, as well as declines in productivity, can be used to diagnose both hematophagous and nonhematophagous parasites (Bath and van Wyk, 2009).

In the last 50 years, the control of GIN infections in ruminants has been generally carried out by “broad spectrum” AH drugs, which have been considered the exclusive mode of controlling GINs. However, a few numbers of limitations of these chemical compounds have now been identified. One issue is the need to manage the possible consequences of residues in the environment or in food products (Bártíková et al., 2016). Furthermore, their repeated use have led to the development of AH resistance in nematode populations which has become a global problem of increasing concern, mainly to control GINs in small ruminants (Rose et al., 2015; Abongwa et al., 2017).

To preserve small ruminant's health and reduce resistance emergence and diffusion, accessible and sustainable alternatives for parasite control are necessary to avoid or minimize AH drug use (Hoste et al., 2006; Salgado and Santos, 2016). In this context, several recent studies have confirmed that medicinal plants and their bioactive compounds represent promising alternatives to control GINs (Hoste et al., 2015; Hoste et al., 2016).

Pterogyne nitens Tulasne (Fabaceae) is popularly called as "bálsamo", "cocal", "amendoim-bravo", "madeira-nova" or "yvi-raró" in Brazil, and is the only member of the *Pterogyne* genus within the Fabaceae family (Lorenzi, 2002). Ethnopharmacological studies in Guarani-indigenous communities of Argentina indicated that cold aqueous preparations from its stem bark are used to treat helminthic infections in humans, mainly against *Ascaris lumbricoides* (Crivos et al., 2007). In Bolivia, *P. nitens* leaves and bark are used to treat anuria and scabies infections, as well as, leaves are used to feed the animals in Guarani-indigenous communities (Bourdy et al., 2004).

Thus, the first objective of this study was to evaluate *P. nitens* extracts either from leaves or fruits against *T. colubriformis* based on three *in vitro* assays, to investigate the effect on three key processes of the GIN life cycle, namely, i) egg hatching, ii) larval development and iii) exsheathment of third-stage infective larvae (L_3). In a second step, we investigated the role of pure compounds isolated from the ethanolic extracts in this AH activity. This involved four bioactive flavonoids: sorbifolin, pedalitin, quercetin, and rutin. The third objective was to explore the relationships between the structure of the flavonoids present in *P. nitens* extracts and the AH efficacy. Based on a previous study (Brunet and Hoste, 2006), we examined the hypothesis that the number and/or the position of the hydroxyl groups could influence the AH effect. Furthermore, to better explain this hypothesis, two commercial flavonoids (chrysin and morin) were added in *in vitro* assays because of their structure.

2. Material and methods

2.1. Plant materials

Fresh leaves and dried fruits of *P. nitens* were collected at the campus of the Institute of Biosciences, Humanities and Exact Sciences of São Paulo State University (Unesp), São José do Rio Preto, SP, Brazil (20°47′02.4″S 49°21′36.0″W), in July 2014. A voucher specimen (HISA 10291) was deposited in the Ilha Solteira Herbarium (HISA) at the Faculty of Engineering, Unesp in Ilha Solteira, SP. Brazilian biodiversity access number A85B7D5 was registered on National System for the Management of Genetic Heritage and Associated Traditional Knowledge.

2.2. Phytochemical procedures and chemicals

Leaves (630 g) were shade-dried, ground, defatted with hexane at room temperature (1 L × 3) and submitted to extraction by maceration with ethanol (1 L × 3). The ethanolic solution of leaves was concentrated under reduced pressure to yield 64 g of ethanol extract of leaves (EEL). EEL (1.5 g) was submitted to chromatography by gel permeation on Sephadex LH-20 (150 × 5 cm) and eluted with ethanol to afford two pure compounds, sorbifolin (25 mg) and pedalitin (40 mg). Naturally dried fruits (200 g) were ground, defatted with hexane at room temperature (400 mL × 3) and submitted to extraction by maceration with ethanol (400 mL × 3). The ethanolic solution of fruits was concentrated under reduced pressure to yield 20 g of ethanol fruit extract (EEF). EEF (2.0 g) was subjected to gel permeation on Sephadex LH-20 (150 × 5 cm) and eluted with ethanol. Fractions were purified by repeated column chromatography with silica gel and eluted with mixtures of chloroform and ethanol to furnish two pure compounds, quercetin (60 mg) and rutin (50 mg). The structures of sorbifolin, pedalitin, quercetin and rutin were characterized by comparison with literature data, mainly using nuclear magnetic resonance (NMR) spectra analysis, including ^{1}H NMR and ^{13}C NMR. Commercial samples of chrysin (C80105) and morin (M4008) were purchased from Merck®.

2.3. In vitro anthelmintic assays

2.3.1. *Trichostrongylus colubriformis* isolate

The isolate of *Trichostrongylus colubriformis* INRAE – INMD (Institut National de Recherche pour l’agriculture, l’alimentation et l’environnement – Interractions Nématode Milieu Digestif) was obtained from monospecifically infected goats and the larvae had

been maintained in the laboratory for at least for one month before use in a batch with more than 95% sheathed larvae. The susceptible isolate was used in all bioassays with the extracts, flavonoids from *P. nitens* and commercial flavonoids. The housing of the animals and trials were performed according to French ethical and welfare rules (agreement number C 31 555 27 of 19 August 2010).

2.3.2. Recovery and preparation of eggs

Recovery, preparation of eggs, egg hatch assay (EHA) and larval development assay (LDA) were performed according to the protocol described by Chagas et al. (2011) with minor modifications. Eggs were recovered from 100 g of fresh feces by mixing with 500 mL of distilled water. The suspension was filtered through 100 µm and 25 µm mesh sieves. Eggs were washed from the 25 µm sieve and centrifuged at 3000 rpm for 5 min to form pellets. The supernatant was removed and a saturated NaCl solution was added to the pellet, centrifuged at 3000 rpm for 5 min. Floating eggs were collected using a 25 µm sieve and washed with phosphate-buffered saline (PBS, 0.1 M phosphate, 0.05 M NaCl, pH 7.2). Eggs were separated, quantified and used within two hours for the EHA and LDA.

2.3.3. Egg hatch assay (EHA)

Approximately, one hundred eggs were added to each well of a 48-well microplate. PBS (P5368) and thiabendazole (T8904) obtained from Merck® were used as negative and positive controls, respectively. Extract solutions at 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5 and 25 mg/mL and flavonoid solutions at 0.03, 0.06, 0.12, 0.25, 0.5 and 1 mg/mL were evaluated. Treatments, positive and negative controls were tested in six repetitions, using six wells for each treatment and approximately 600 eggs. Plates were sealed with PVC film and incubated at 27 °C with relative humidity ≥ 80% for 48 h. Then eggs and L_1 larvae were counted with an inverted microscope to calculate egg hatch inhibition. The percent inhibition was calculated by the following equation: $\% \text{ inhibition} = A/(A + B) \times 100$, where A is the number of unhatched eggs and B is the number of L_1 larvae.

2.3.4. Larval development assay (LDA)

Approximately, one hundred eggs were added to each well of a 48-well microplate with PBS, *Escherichia coli* (Strain B lyophilized, EC11303) nutritive medium and 0.5 mg/mL of amphotericin B (A2942) obtained from Merck®, reaching a total volume of 125 µL. Plates were incubated for 48 h at 27 °C and ≥ 80% relative humidity to obtain L_1 larvae when the solutions were added. PBS and ivermectin (Sigma-Aldrich) were used as negative and positive controls, respectively. Extract solutions at 19, 39, 78, 156, 312, 625, 1250, 2500 and 5000 µg/mL and flavonoid solutions at 31.2, 62.5, 125, 250, 500 µg/mL were evaluated. Treatments, positive and negative controls were tested in six repetitions, using six wells for each treatment and approximately 600 eggs. Plates were incubated for 7 days, and each well was analyzed with an inverted microscope to count all L_3 and undeveloped larvae to estimate the larval development inhibition. The percent inhibition was calculated using the following equation: % inhibition = (A/B) × 100, where A is the number of L_1 + L_2 larvae and B is the total number of larvae (L_1 + L_2 +L_3).

2.3.5. Third-stage larvae (L_3) exsheathment assay (LEA)

The larval exsheathment inhibition assay was performed as described by Bahuaud et al. (2006) with minor modifications. The third-stage larvae batch of one-month-old of *T. colubriformis* was used in this assay. Briefly, approximately 800–1000 sheathed L_3 larvae were incubated for 3 h at 25 °C with extracts at 75, 150, 300, 600 and 1200 µg/mL and flavonoids at 15, 30, 60, 120 and 240 µg/mL, which were diluted in PBS. PBS was also used as the negative control. After incubation, larvae were washed and centrifuged three times in PBS and submitted to exsheathment. L_3 larvae were submitted to a solution of sodium hypochlorite solution (2% w/v) and sodium chloride solution (16.5% w/v), which was diluted from 1 to 200 in PBS. Exsheathment kinetics were measured under an optical microscope at 200x magnification to identify the proportion between exsheathed and unsheathed larvae. Consecutive examinations were performed for 0, 20, 40, and 60 minutes after exposure to the exsheathment solution. Four replicates were carried out per concentration and per observation time. The percent inhibition was calculated by the following equation: % inhibition = A/ (A + B) × 100, where A is the number of sheathed L_3 larvae and B is the number of exsheathed L_3.

2.3.6. Statistical analyses

The concentration that caused 50% egg hatching inhibition, larval development inhibition and exsheathment inhibition (EC_{50}) of the parasites was calculated using SPSS IBM Statistics® v. 20. Data are presented as the mean percentage ± standard error (SEM). The t-test or one-way ANOVA followed by the Tukey test was used to detect significance among groups and concentrations. $P \leq 0.05$ was considered to be statistically significant.

In addition, multivariate analysis (multiple correspondence analysis, MCA) was performed using the XLSTAT-Premium 2019.3.2 statistical and data analysis solution (Addinsoft, Boston, USA) to obtain a synthetic description of the relationships among the biological effects on egg hatching, larval development from L_1 to L_3 or larval exsheathment of *T. colubriformis,* and also the main characteristics of the flavonoid structures. The eight variables composing the column of the matrix used for the MCA were categorical. They included the total number of free hydroxyl groups (Tot2, Tot3, Tot4, or Tot5), the possible structural variations in radicals: R_1 (H, OH or Glucose), R_2 (H or OH), R_3 (OH or OCH_3), R_4 (H or OH), R_5 (H or OH), or R_6 (H or OH), and the observed effects (EC_{50}) measured and classified at different group (potent, moderate or low activity) from each assay.

The classification was generated based on the literature as well as on the EC_{50} values obtained for the six compounds in the three *in vitro* experiments. The values were grouped according to the experiment (EHA, LDA, and LEA). In the case of EHA, values between 0 and 1 were considered potent, values between 2 and 10 moderate and above 11 low. For LDA, EC_{50} values between 0 and 400 were considered potent and values above 400 low. In the LEA, values between 0 and 10 were deemed to be potent, 11 to 30 moderate and above 31 low. The six rows (individual data) of the matrix corresponded to the six different flavonoids assayed.

3. Results

3.1. Ethanol extracts of plant leaves and fruits

Ethanolic extracts, flavonoids of *P. nitens*, as well as the commercial flavonoids were submitted to three anthelmintic assays: egg hatch inhibition (EHA), larval

development inhibition (LDA) and larval exsheathment inhibition (LEA). EC_{50} values and confidence intervals (95% CI) of extracts and flavonoids are summarized in Table 1. The ovicidal activity is indicated in mg/mL and larvicidal activity in µg/mL.

Table 1. *In vitro* ovicidal and larvicidal activities of extracts of *Pterogyne nitens* and flavonoids against *Trichostrongylus colubriformis*.

Extracts and Flavonoids	EHA EC_{50} mg/mL	EHA 95% CI	LDA EC_{50} µg/mL	LDA 95% CI	LEA EC_{50} µg/mL	LEA 95% CI
ethanolic extract leaves	2.9	2.6–3.2	8.9	8.3–9.4	118	85.4–150
ethanolic extract fruits	5.9	5.4–6.5	20.0	18.2–22.1	158	140–178
sorbifolin	3.5	2.5–5.8	841	675–1110	64.0	43.7–94.6
pedalitin	3.0	2.4–4.1	468	425–524	26.8	22.8–31.0
quercetin	0.4	0.3–0.4	100	93–109	7.1	2.3–12.0
rutin	0.4	0.3–0.4	459	413–518	34.6	30.0–39.6
chrysin	3.3	2.2–7.8	221	207–237	24.9	18.5–31.2
morin	0.7	0.6–0.9	451	412–502	2.9	0.2–8.0
thiabendazole*	0.002	0.001–0.004	nt	nt	nt	nt
ivermectin*	nt	nt	11	7–15	nt	nt

EHA = Egg hatch assay; LDA = Larval development assay; LEA = Larval exsheathment assay; EC_{50} = Fifty-percent effective concentration; 95% CI = 95% confidence interval; nt = not tested. *Reference anthelmintic drugs.

The inhibition percentages in egg hatch, larval development from L_1 to L_3 and larval exsheathment mediated by EEL and EEF are displayed in Table 2. At the two highest concentrations, both extracts presented high efficacy, inhibiting the larval development and larval exsheathment of *T. colubriformis* by more than 90%. The ethanolic extracts of both leaves (EEL) and fruits (EEF) demonstrated potent ovicidal and larvicidal activities.

Based on the EC_{50} values of the inhibitory effects according to the EHA and LDA, the EEL (for EHA =2.9 mg/mL and 8.9 µg/mL for larval development) was twice as potent as the EEF (EHA values EC_{50} = 5.9 mg/mL and LDA values = 20 µg/mL) ($p \leq 0.05$). Furthermore, in the LDA, the EEL had statistically equal efficacy as the positive control. The larval exsheathment inhibitory activities of EEL (EC_{50} = 118.43 µg/mL) and EEF (EC_{50} = 158.08 µg/mL) were close ($p \geq 0.05$).

Table 2. Inhibition efficacy (mean percentage ± SEM) of ethanolic extracts (EEL and EEF) of *Pterogyne nitens* in the egg hatch assay (EHA, mg/mL), larval development assay (LDA, µg/mL) and larval exsheathment assay (LEA, µg/mL) against *Trichostrongylus colubriformis.* Also shown are controls containing PBS, thiabendazole and ivermectin.

Conc.	EHA*		Conc.	LDA*		Conc.	LEA*	
(mg/mL)	EEL	EEF	(µg/mL)	EEL	EEF	(µg/mL)	EEL	EEF
25	100 ± 0.00[a]	97.36 ± 0.53[a]	**312**	100 ± 0.00[a]	93.32 ± 1.08[a]	**1200**	100 ± 0.00[a]	100 ± 0.00[a]
12.5	94.76 ± 1.03[a]	79.78 ± 1.80[b]	**156**	100 ± 0.00[a]	91.75 ± 1.82[a]	**600**	95.56 ± 2.72[ab]	100 ± 0.00[a]
6.25	78.20 ± 2.92[b]	45.25 ± 7.79[c]	**78**	98.83 ± 0.45[a]	80.68 ± 0.92[b]	**300**	91.67 ± 5.32[ab]	95.66 ± 2.70[a]
3.12	47.67 ± 2.56[c]	21.13 ± 2.36[d]	**39**	98.06 ± 0.67[a]	78.79 ± 1.73[b]	**150**	74.10 ± 6.98[b]	36.67 ± 4.51[b]
1.56	19.33 ± 3.24[d]	8.65 ± 0.97[de]	**19**	79.51 ± 3.18[b]	58.12 ± 1.54[c]	**75**	10.72 ± 6.84[c]	6.25 ± 4.42[c]
0.78	9.87 ± 1.56[e]	2.73 ± 0.41[e]	**9.7**	64.74 ± 4.97[c]	27.17 ± 0.88[d]			
0.39	4.72 ± 0.48[e]	nt	**4.8**	14.92 ± 1.81[d]	12.48 ± 0.90[e]			
0.19	1.28 ± 0.49[e]	nt	**2.4**	5.31 ± 0.84[e]	3.36 ± 0.38[f]			
PBS	1.09 ± 0.52[e]	1.32 ± 0.38[e]	**PBS**	0.34 ± 0.34[e]	0.34 ± 0.34[f]	**PBS**	0.00 ± 0.00[d]	0.00 ± 0.00[c]
thiabendazole	100 ± 0.00[a]	100 ± 0.00[a]	**ivermectin**	100 ± 0.00[a]	100 ± 0.00[a]			

nt = not tested. *Different lowercase letters in the same column correspond to the different concentrations tested indicate significantly different values by ANOVA and the Tukey test (p≤0.05).

3.2. Natural and commercial flavonoids

These results encouraged us to investigate the compounds of EEL and EEF that could be responsible for their AH activity. Phytochemical fractionation of EEL and EEF furnished two flavones (sorbifolin and pedalitin) and two flavonols (quercetin and rutin), respectively (Figure 1).

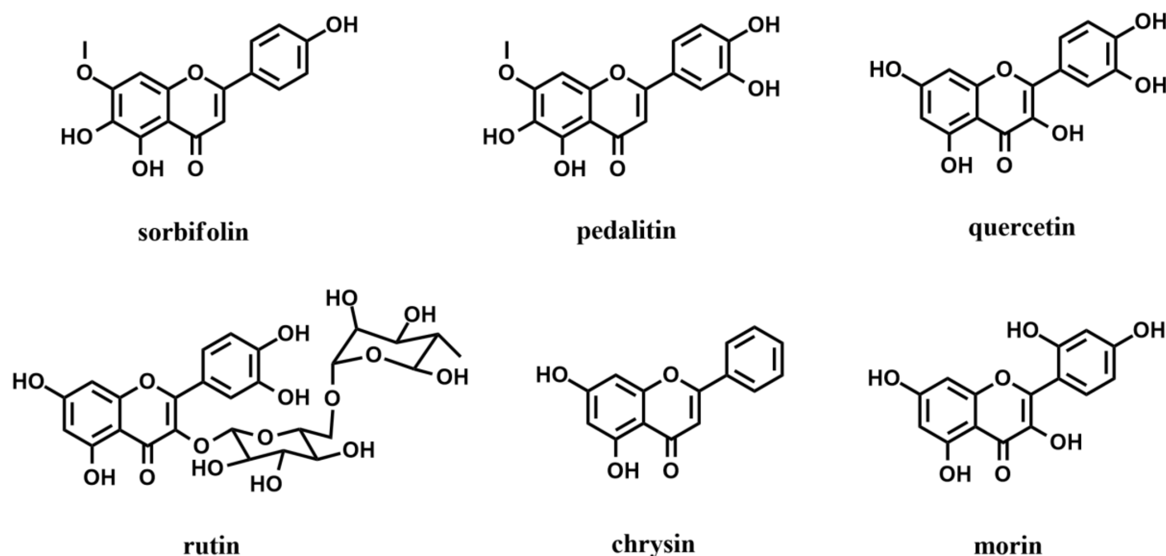


Figure 1. Structure of sorbifolin, pedalitin, quercetin, rutin (obtained from *Pterogyne nitens*) and two commercial flavonoids [chrysin (C80105) and morin (M4008)], supplied by Merck®.

The inhibition percentages mediated by flavonoids in the EHA, LDA and LEA are displayed in Table 3–5. A dose-response relation was observed for all four compounds in all assays. In the EHA and LDA, sorbifolin and pedalitin presented the lowest efficacy among the compounds (Table 3 and 4). In the EHA, only the concentration of 1 mg/mL was statistically different from PBS for both compounds. Ovicidal activity of sorbifolin (EC_{50} = 3.5 mg/mL) was similar to that of pedalitin (EC_{50} = 3.0 mg/mL). In contrast, larvicidal activity evaluated by the LDA and LEA experiments indicated pedalitin was more active than sorbifolin, although both reached 100% inhibition at 240 µg/mL (Table 4 and 5). Quercetin presented the best biological activity in all bioassays.

Quercetin and rutin were able to inhibit egg hatching at EC_{50} values of 0.4 mg/mL. The larvicidal activities of quercetin (EC_{50} = 100 μg/mL and 7.1 μg/mL in the LDA and LEA, respectively) were higher than those of rutin (EC_{50} = 459 μg/mL and 34.6 μg/mL in the LDA and LEA, respectively). In order to understand the preliminary structural features related to the potent AH activity of quercetin, we evaluated its isomer morin. In the EHA and LDA experiments, morin had lower inhibition percentages than quercetin (Tables 3 and 4), with EC_{50} values of 0.7 mg/mL and 451 μg/mL, respectively. On the other hand, in the LEA experiment, morin caused up to 90% inhibition at 30 μg/m, more potent than quercetin with an EC_{50} value of 2.9 μg/mL.

Table 3. Inhibition efficacy (mean percentage ± SEM) of flavonoids on *in vitro* hatching of eggs against *Trichostrongylus colubriformis* in the egg hatch assay (EHA, mg/mL). Also shown are controls containing PBS and thiabendazole.

	EHA*					
Conc. (mg/mL)	**sorbifolin**	**pedalitin**	**quercetin**	**rutin**	**chrysin**	**morin**
1	13.98 ± 1.31^{b}	16.08 ± 1.35^{b}	85.13 ± 2.12^{b}	74.02 ± 1.96^{b}	12.48 ± 1.83^{b}	68.48 ± 2.83^{b}
0.5	3.85 ± 0.63^{c}	9.27 ± 1.55^{c}	59.65 ± 1.20^{c}	59.56 ± 1.75^{c}	3.33 ± 0.46^{c}	30.11 ± 2.42^{c}
0.25	1.28 ± 0.36^{c}	6.09 ± 0.96^{c}	30.77 ± 2.06^{d}	36.89 ± 0.95^{d}	nt	15.68 ± 0.80^{d}
0.12	Nt	nt	10.26 ± 0.52^{e}	14.30 ± 1.27^{e}	nt	8.06 ± 1.07^{de}
0.06	nt	nt	4.78 ± 0.29^{e}	8.14 ± 1.11^{f}	nt	5.38 ± 1.17^{e}
PBS	1.25 ± 0.35^{c}	2.32 ± 0.52^{c}	2.10 ± 0.25^{e}	2.10 ± 0.25^{f}	2.10 ± 0.25^{c}	2.10 ± 0.25^{e}
Thiabendazole	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}

nt = not tested. *Different lowercase letters in the same column correspond to the different concentrations tested indicate significantly different values by ANOVA and the Tukey test ($p \leq 0.05$).

Table 4. Inhibition efficacy (mean percentage ± SEM) of flavonoids on *in vitro* larval development against *Trichostrongylus colubriformis* in the larval development assay (LDA, µg/mL). Also shown are controls containing PBS and ivermectin.

Conc. (µg/mL)	LDA*					
	sorbifolin	**pedalitin**	**quercetin**	**rutin**	**chrysin**	**morin**
500	40.05 ± 1.10[b]	54.48 ± 0.96[b]	83.19 ± 0.97[b]	49.87 ± 1.77[b]	78.89 ± 1.96[b]	56.73 ± 2.34[b]
250	28.54 ± 1.35[c]	26.63 ± 1.33[c]	68.18 ± 0.66[c]	35.18 ± 1.27[c]	51.14 ± 2.83[c]	26.12 ± 1.44[c]
125	18.94 ± 1.52[d]	13.15 ± 1.05[d]	55.82 ± 1.28[d]	17.56 ± 1.91[d]	28.55± 1.24[d]	13.51 ± 1.02[d]
62.5	11.81 ± 0.66[e]	5.63 ± 0.37[e]	37.27 ± 0.70[e]	4.98 ± 0.85[e]	15.22 ± 1.32[e]	5.53 ± 0.52[e]
31.2	5.67 ± 0.33[f]	nt	26.87 ± 1.28[f]	nt	5.57 ± 0.44[f]	nt
15.6	nt	nt	15.73 ± 0.89[g]	nt	nt	nt
PBS	4.39 ± 0.67[f]	4.39 ± 0.67[e]	4.39 ± 0.67[h]	4.39 ± 0.67[e]	4.39 ± 0.67[f]	4.39 ± 0.67[e]
Ivermectin	100 ± 0.00[a]	100 ± 0.00[a]	100 ± 0.00[a]	100 ± 0.00[a]	100 ± 0.00[a]	100 ± 0.00[a]

nt = not tested. *Different lowercase letters in the same column correspond to the different concentrations tested indicate significantly different values by ANOVA and the Tukey test ($p \leq 0.05$).

Table 5. Inhibition efficacy (mean percentage ± SEM) of flavonoids (µg/mL) on *in vitro* larval exsheathment of *Trichostrongylus colubriformis* susceptible isolate in the larval exsheathment assay (LEA). Also shown is the control PBS.

Conc. (µg/mL)	LEA*					
	sorbifolin	**pedalitin**	**quercetin**	**rutin**	**chrysin**	**morin**
240	100 ± 0.00[a]	100 ± 0.00[a]	100 ± 0.00[a]	98.69 ± 1.67[a]	96.88 ± 3.13[a]	98.53 ± 1.79[a]
120	75.67 ± 4.83[b]	98.44 ± 1.56[b]	97.92 ± 2.08[a]	98.33 ± 1.32[a]	86.34 ± 2.08[a]	98.22 ± 1.47[a]
60	31.12 ± 9.66[c]	88.44 ± 5.67[c]	89.73 ± 3.45[a]	50.41 ± 9.60[b]	77.47 ± 7.25[ab]	98.08 ± 1.92[a]
30	12.07 ± 2.33[d]	67.67 ± 5.92[d]	80.91 ± 5.41[ab]	33 ± 5.74[bc]	55.73 ± 3.93[bc]	92.97 ± 2.76[a]
15	10.30 ± 10.29[e]	13.18 ± 2.93[e]	72.39 ± 4.63[b]	14.34 ± 1.68[c]	34.06 ± 7.67[c]	79.67 ± 5.40[b]
PBS	0.00 ± 0.00[f]	0.00 ± 0.00[f]	0.00 ± 0.00[c]	0.00 ± 0.00[d]	0.00 ± 0.00[d]	0.00 ± 0.00[c]

* Different lowercase letters in the same column correspond to the different concentrations tested indicate significantly different values by ANOVA and the Tukey test ($p \leq 0.05$).

A graph was generated by the MCA, containing the observed variables, projected on the plane defined by two-dimensional combination (DIM1 and DIM2), representing 55.83% of the variance (Figure 2). Considering all variables as "inputs" of the MCA and analyzing the visualization represented by the two-dimensional plane indicated that each dimension corresponded to a linear combination of variables of the matrix. The main variables contributing to create DIM1 were the "effect", "total number of OH group", and "structure of the R_1 and R_2 group". In contrast, the four major variables contributing to axis 2 were the "structure of R_3 to R_6". The main objective was to analyze the overall relationships of the different categories of effects on nematode life stages with other variables. By this method, variables that are positively related are located in the same area of the plane. We observed that the efficacy of flavonoids in the test was in proximity with the presence of five or ten OH groups, as well as the presence of a hydroxyl group in R_1 (R_1OH), and to a lesser extent, the presence of a glucose group (R_1G) in the molecule. These findings confirm the potent AH activity of the flavonols. The moderate effect in the three different processes was associated with the low number of total OH groups (Tot2) and the absence of an OH group in R_5 (R_5H). Finally, the low activity was related to the presence of the methoxy group in R_3 (R_3OCH_3), the number of OH groups equal 3 or 4 (Tot3 or Tot 4).

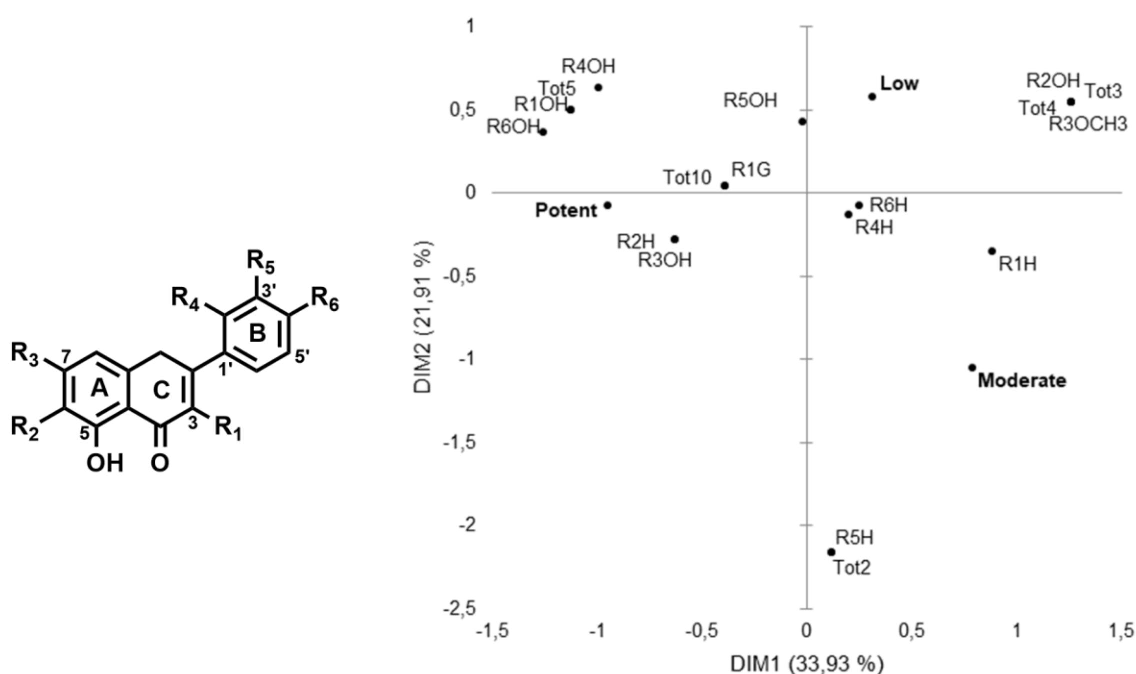


Figure 2. Principal plane of interactions (total variability represented = approx. 56%) obtained from the MCA applied to a matrix composed of eight variables describing the structure of flavonoids and the AH effects on egg hatching, larval development from L_1 to L_3 and L_3 exsheathment of *Trichostrongylus colubriformis*. Abbreviations: H = hydrogen; OH = hydroxyl group; G = Glucose; Tot = Total; R = radical. Level of AH activity of flavonoid groups (Potent, Moderate, and Low); total number of OH groups in the biochemical structure (Tot2, Tot3, Tot4, Tot5, or Tot10); structural variations of R_1 group (R_1H, R_1OH, or R_1G); structural variations of R_2 group (R_2H or R_2OH); structural variations of R_3 group (R_3H, R_3OH or R_3OCH_3), structural variations of R_4 group (R_4H or R_4OH); structural variations of R_5 group (R_5H or R_5OH); structural variations of R_6 group (R_6H, R_6OH). The classification was generated based on the EC_{50} values obtained for the six compounds in the three experiments. The values were grouped according to the experiment (EHA, LDA, and LEA). In the case of the EHA, values between 0 and 1 were considered potent, values between 2 and 10 moderate and above 11 low. For the LDA, EC_{50} values between 0 and 400 were considered potent and values above 400 low. In the LEA, values between 0 and 10 were deemed potent, 11 to 30 moderate and above 31 low.

4. Discussion

The anthelmintic activity of *P. nitens* extracts observed in this study corroborates the traditional use of *P. nitens* as an antiparasitic plant. Previous studies have reported that extracts of *P. nitens* leaves and fruits demonstrated several bioactivities, including antiproliferative, antimutagenic (Regasini et al., 2007), antioxidant (Pasquini-Netto et al., 2012), and antifungal (Lima et al., 2016). The results of the present study encouraged us to investigate compounds of the EEL and EEF that might be responsible for their AH activity.

Pedalitin was more active against larvae than sorbifolin. The structural difference between sorbifolin and pedalitin is the number of hydroxyl groups on ring B, and this group seems to orchestrate the ovicidal and larvicidal activity of flavones. In order to confirm the relevance of hydroxyl groups for AH activity, a commercial sample of chrysin was tested. Structurally, chrysin is a flavone with unsubstituted ring B and ring A substituted by two

hydroxyls. This compound was more active than natural flavones, indicating the reduced number of hydroxyls in the flavone framework increased the AH activity, mainly the ovicidal effect. The different anthelmintic effects of flavones may be associated with the structure of the eggshell membrane and larval cuticle, mainly regarding permeation by passive diffusion through chitin and collagen, respectively (Hagerman et al., 1998; Poncet-Legrand et al., 2006).

Our result is the first showing AH activity of sorbifolin, pedalitin, and chrysin. However, Ayers and collaborators investigated the activity of flavones from *Struthiola argentea*, which are structurally related to flavones of *P. nitens*. The pentamethoxylated flavone of *S. argentea* was active against *H. contortus*, a hematophagous nematode of small ruminants, exhibiting 90% inhibition of larval motility at 3.1 μg/mL (Ayers et al., 2008).

The larvicidal activity of quercetin was higher than that of rutin. These results indicate that sugar subunit (rutinose) significantly reduced the larvicidal effect of flavonols, but it was not relevant to the ovicidal effect. Anthelmintic activity of quercetin and rutin has been described by other authors, who investigated the *in vitro* effects of these flavonols against *H. contortus*. Soldera-Silva et al. (2018) described the anti-*Haemonchus contortus* activity of quercetin and rutin from seeds of *Persea americana* (avocado).

A few studies enumerated hypothetical mechanisms related to egg hatching inhibition by plant compounds, including changes in eggshell permeability (Perry, 2002), inhibition of hydrolases such as proteases, lipases and chitinases (Rogers and Brooks, 1977), and antagonism with hatching factors of the eggshell (Doncaster and Shepherd, 1967; Perry, 2002; Vargas-Magaña et al., 2014). In this context, flavonoids have been recognized as hydrolase inhibitors, which can explain their inhibitory activity against *T. colubriformis* L_1 larvae hatching and egg rupture.

Quercetin and rutin were evaluated by the larval migration test (LMT), exhibiting EC_{50} values of 7.8 μg/mL and 30 μg/mL, respectively (Soldera-Silva et al., 2018). Klongsiriwet and collaborators reported the inhibitory activity of quercetin against the exsheathment of *H. contortus* L_3 larvae, displaying an EC_{50} value of 21.0 μg/mL

(Klongsiriwet et al., 2015). Rutin from *Onobrychis viciifolia* (sainfoin) reduced the percentage of *H. contortus* larval migration at 1,200 µg/mL (Barrau et al., 2005).

The AH activity of quercetin stood out in the EHA and LDA, compared to its isomer morin, which on the other hand was the most active flavonoid against the infective L_3 stage of *T. colubriformis*, suggesting its potential *in vivo* therapeutic effect. This result indicated that the displacement of the hydroxyl group from position 3’ to 2’ (ring B) modified the AH activity. Besides that, this is the first description of morin’s anthelmintic activity against *T. colubriformis*.

Furthermore, the larvicidal activity of plant compounds has been related to their complexation with collagen of the nematode cuticle (Bravo, 1998; Jerónimo et al., 2016). These interactions are usually due to hydrogen bonds and/or hydrophobic interactions (Hagerman et al., 1998; Poncet-Legrand et al., 2006). Some authors have proposed that the interactions of these compounds with the surface proteins of larvae can explain the effects on the exsheathment process (Kahn and Diaz-Hernandez, 1999; Molan et al., 2003).

The MCA results showed there was a positive correlation regarding the AH efficacy in the three *in vitro* assays, with an increase in hydroxyl number as well as the presence of the substituted OH group (R_1OH). It was previously demonstrated that the anthelmintic activity of condensed tannin monomers is associated with the total number of OH groups, as well as the presence of an OH group in the R_3 position (Brunet and Hoste, 2006). In addition, this classification based on the EC_{50} values obtained for the six compounds against egg hatch, larval development, and L_3 exsheathment which is suggested in this work could be used as an AH activity scale based on in flavonoid structure-activity relationships for the future studies with this subject.

Altogether, extracts and flavonoids from *P. nitens* were able to disrupt the life cycle of *T. colubriformis* by inhibition of egg hatching, larval development from L_1 to L_3 stages, and L_3 exsheathment. This is the first report of the anthelmintic action of *P. nitens* extracts and flavonoids against three different stages of this small intestine nematode species. Hence, the results of this study suggest the existence of an alternative within phytotherapy, which can be consolidated with the concept of integrated parasite control.

This would aim to inhibit different phases of the parasite's biological cycle. A blend of natural substances with previously defined concentrations, since the different flavonoids structural diversity influences their absorption by the intestine, and consequently their bioavailability (Hollman, 2004).

In this way, rutin could be administrated as a functional food focusing to reduce the establishment of infection because it presented interesting results against the process of larval exsheathment since the glycoside flavonoids were considered too hydrophilic for absorption by passive diffusion in the small intestine, and consequently, they could act directly in the GIN stage allocated in this organ (Tapas et al., 2008; Hollman, 2004). Subsequently, the liberated aglycone (as quercetin or rutin) can be absorbed across the small intestine, metabolized by the body, or can also be subject to catabolism by the gut microbiota, which could act in the adult nematode forms, and eventually excreted in the feces as phenolic acids, like gallic acid (Almeida et al., 2018).

5. Conclusion

The current study is the first to assess the AH activity of extracts and compounds of *P. nitens*. The results showed clear *in vitro* anthelmintic activities in three stages of *T. colubriformis* and inhibitory activity of L_3 exsheathment. These findings corroborate the traditional use of *P. nitens* against human intestinal parasites. In addition, chromatography fractionation of bioactive ethanolic extracts furnished four flavonoids (sorbifolin, pedalitin, quercetin and rutin) responsible for the ovicidal and larvicidal activity. However detailed investigations are needed to determine the mechanism of action of extracts and flavonoids from *P. nitens*. *In vivo* experiments should also be carried out to explore the therapeutic efficacy and toxicity of *P. nitens*.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES), Brazilian Council for Scientific and Technological Development (CNPq) (Grants 471129/2013-5, 306251/2016-7 and 429322/2018-6), and

São Paulo Research Foundation (FAPESP) (Grants 2014/18330-0 and 2018/15083-2). C. S. L. thanks *CAPES (finance code 001)* for the scholarships and the Doctoral Program Sandwich Abroad at the Institut National de la Recherche Agronomique of Toulouse, France (Grant 88881.187940/2018-01).

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Capítulo III

Potential of extracts and phenolic compounds from Pterogyne nitens (Fabaceae) on the exsheathment process of Haemonchus contortus isolates from Brazil and France

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ABSTRACT: The use of plant secondary metabolites with potential anthelmintic activities against gastrointestinal nematodes (GINs) is an alternative solution to chemical treatments. *Pterogyne nitens* Tul. (Fabaceae) is a Brazilian tree, which presents a variety of bioactive metabolites with reported bioactivities, as guanidine alkaloids, flavonoids, phenolic acids, triterpenes, and sterols. In this field, the aim of this study was to evaluate the anthelmintic activity of extracts and phenolic compounds from *P. nitens* against two susceptible isolates of *Haemonchus contortus* (French Isolate: HcINRAE and Brazilian Isolate: HcEch91). Sorbifolin, pedalitin, quercetin, rutin, ourateacatechin, caffeic acid, ferulic acid, and gallic acid were isolated from leaves, fruits, and flowers ethanolic extracts by successive chromatography columns. Two commercial flavonoids (chrysin and morin) were obtained from Merck®, to derive chemical structure and anthelmintic activity relationship studies. Extracts and natural compounds were tested against third-stage larvae (L_3) by the larval exsheathment assay (LEA). Statistical analyses were performed by SPSS IBM Statistics® v. 20, through probit analysis, for the determination of EC_{50}. All the extracts tested were active against *H. contortus* L_3 of both isolates (EC_{50} ranging between 78.6 to 152.5 µg/mL). Natural compounds presented EC_{50} ranging between 1.6 to 132.4 µg/mL for HcINRAE and 5.8 to 54.6 µg/mL for HcEch91. Quercetin presented the best inhibition activity for both nematode isolates, with EC_{50} = 1.62 µg/mL for HcINRAE and 5.8 µg/mL for HcEch91, suggesting its potential for *in vivo* therapeutic effect. Among the acids, the gallic acid demonstrated the best activity with 28.1 µg/mL for HcINRAE and 44.9 µg/mL for HcEch91. The results showed that the number and position of phenolic compounds hydroxyl groups may change the anthelmintic activity. These data are the first description of anthelmintic activities for some natural compounds, suggesting a sustainable template for discovery and design of novel anthelmintic agents.

KEYWORDS: plant secondary metabolites, anthelmintic, nematode, larval exsheathment inhibition assay.

1. Introduction

Plants have been used to treat diseases in humans and animals since the primordial of civilization. More than 30% of newly introduced drugs are estimated to be directly or indirectly based on chemical structures of natural products (van Wyk and Wink 2017). In addition, countries' growing interest in harmful-free agriculture in consumer products is resulting in a new interest in bioactive natural products. Over the past decade, there has been an increase in the number of studies focusing on veterinary pharmacology, particularly for plants carrying phenolic compounds (Hoste et al. 2016; Spiegler et al. 2017).

The goal of herbal medicine is to treat parasitized animals with whole plants or medicinal preparations with anthelmintic properties. There are two ways for preparing and using plant natural material, the first form is as a nutraceutical, which is defined as a plant consumed by animals, aiming to take advantage of its nutritional value in addition to its beneficial properties for health (Andlauer and Fürst 2002; Hoste et al. 2015). Their incorporation into the feed could have for long term prevention purposes, or a curative action. The second form is as herbal medicine, which is a preparation whose asset originates from plants, constituting a complex mixture of substances that can be used medically (Hoste et al. 2006; Hoste et al. 2011).

There are several *in vitro* and *in vivo* studies focused on species of leguminous plants containing phenolic compounds or condensed tannins in temperate areas (Paolini et al. 2003a; 2003b; Barrau et al. 2005; Brunet and Hoste 2006), and in tropical areas (Athanasiadou et al. 2001; Cenci et al. 2007; Alonso-Díaz et al. 2008; Max et al. 2009; Martínez-Ortiz-De-Montellano et al. 2019), which may also have anthelmintic properties. Some of those plants are already widely incorporated into animal feeding regimes in tropical regions, however, are scarce researches have been done to determine their effects on GINs as well as to determine natural compounds responsible for anthelmintic activity.

Pterogyne nitens (Fabaceae), popularly called as "bálsamo", "cocal", "amendoim-bravo" or "yvi-raró", is a brazilian native tree (Lorenzi 2002). Ethnopharmacological

studies demonstrated that Guarani indigenous communities from Argentina use cold aqueous preparations from its stem barks to treat helminthic infestations, mainly against *Ascaris lumbricoides* (Crivos et al. 2007). As well as, in Bolivia, Guarani indigenous use leaves and barks to treat anuria and scabies infections, and also use the leaves to feed animals (Bourdy et al. 2004). Chemically, *P. nitens* presents a variety of bioactive metabolites, including guanidine alkaloids, triterpenes, steroids and phenolic compounds isolated from leaves, fruits, flowers, branches, and stem barks, which had demonstrated several biological activities, as potent antifungal activity against opportunistic fungi (Lima et al. 2016), antiviral activity (Shimizu et al. 2017), antibacterial multiresistant (Coqueiro et al. 2014).

The objective of the current study was to test the hypothesis that the extracts and phenolic compounds of *P. nitens* might interfere with the very early process of the parasitic life of the *Haemonchus contortus*, i.e. the exsheathment of the third-stage infective larvae (L_3), before invasion of the host mucosal tissues. This question was addressed by examining *in vitro* consequences of incubation with extracts and phenolic compounds on L_3 by the larval exsheathment assay (LEA). Moreover, in order to assess the specificity of the observed effects, comparative data were used to compare *H. contortus* isolates from different continents.

2. Material and methods

2.1. Plant Material

The aerial parts, leaves, fruits, and flowers of *P. nitens* were collected at the campus of the Institute of Biosciences, Letters and Exact Sciences, São Paulo State University (UNESP), São José do Rio Preto, SP, Brazil (20°47′02.4″S 49°21′36.0″W) in July 2014. A voucher specimen (10291) was deposited in the Ilha Solteira Herbarium (HISA) at Faculty of Engineering, Unesp in Ilha Solteira, SP. Brazilian biodiversity access number A85B7D5 was registered on National System for the Management of Genetic Heritage and Associated Traditional Knowledge.

2.2. Preparation of Extracts

The shade-dried leaves (630 g), fruits (200g) and flowers (1,500 g) were ground in a knife grinder. The obtained powder was first macerated with hexane (1 L × 3) for 48 hours, to remove the apolar compounds, the vegetable material was separated from the extraction solution by simple filtration on filter paper and the filtrate was dried under rotary evaporation. Subsequently, the non-extracted residue was macerated with ethanol (1 L × 3) for 48 hours and the same procedure was repeated. Three extracts were obtained; ethanolic extract from leaves (EEL, 64 g); ethanolic extract from fruits (EEFR, 20 g); and ethanolic extract from flowers (EEFL, 15 g). EEFL was not tested on LEA, because as the extracted mass obtained was sufficient only for the isolation of the compounds. As well as it is an element of the plant that is difficult to access once the flowering occurs in a few months of the year.

2.3. Natural compounds

Flavonoids (flavones, flavonols and catechin derivative) and phenolic acids were isolated and identified, using chemical procedures reported previously. Flavone derivatives, sorbifolin, pedalitin, were isolated from leaves (Shimizu et al. 2017). Flavonol derivatives, quercetin, and rutin were obtained from fruits (Regasini et al. 2007; Regasini et al. 2008b). Flavon-3-ol derivative, ourateacatechin and phenolic acids as caffeic acid, ferulic acid, and gallic acid were isolated from flowers (Regasini et al. 2008a). Commercial samples of chrysin (C80105) and morin (M4008) were purchased from Merck®.

2.4. In vitro anthelmintic assay

2.4.1. Nematode strains

The French isolate INRAE (HcINRAE) and the Brazilian isolate (HcEch91) of *H. contortus* were obtained from monospecifically infected small ruminants and the larvae had been maintained in the laboratory at least for four months for HcINRAE isolate and one month for HcEch91 before used in all assay. The batch presented a percentage of sheathed larvae above 95%. These isolates were used to repeat experiments with the

extracts, natural compounds from *P. nitens* and also the two commercial flavonoids (chrysin and morin).

2.4.2. Larval exsheathment assay (LEA)

LEA was performed as described by Bahuaud et al. (2006) with minor modifications. The third-stage larvae (L_3) batch of *H. contortus* was used in this assay, four-month-old for HcINRAE and one-month-old for HcEch91. Briefly, approximately 800–1000 sheathed L_3 larvae were incubated for 3 h at 25 °C with extracts at 75, 150, 300, 600 and 1200 µg/mL and flavonoids at 1.87, 3.25, 7.5, 15, 30, 60, 120 and 240 µg/mL, which were diluted in PBS. PBS was also used as the negative control. After incubation, larvae were washed and centrifuged three times in PBS and submitted to exsheathment. L_3 larvae were submitted to a solution of sodium hypochlorite solution (2% w/v) and sodium chloride solution (16.5% w/v), which was diluted from 1 to 200 in PBS. Exsheathment kinetics were measured under an optical microscope at 200x magnification to identify the proportion between exsheathed and unsheathed larvae. Consecutive examinations were performed for 0, 20, 40, and 60 minutes after exposure to the exsheathment solution. Four replicates were carried out per concentration and per observation time. The percent inhibition was calculated by the following equation: % inhibition = A/ (A + B) × 100, where A is the number of sheathed L_3 larvae and B is the number of exsheathed L_3, using the 60 minutes observed values.

2.4.3. Statistical Analyses

The concentration that causes 50% exsheathment inhibition (EC_{50}) of the parasites was calculated using SPSS IBM Statistics® v. 20. Pearson's correlation coefficient was used to assess potential correlations among the EC_{50} values between two isolates. Data are presented as the mean percentage ± standard error (SEM). The t-test or one-way ANOVA followed by the Tukey test was used to detect significance among groups and concentrations. $P \leq 0.05$ was considered to be statistically significant.

3. Results

3.1 Ethanol extracts of plant leaves and fruits

Ethanolic extracts and phenolic compounds from *P. nitens*, as well as commercial flavonoids were submitted to LEA. The results showed a clear *in vitro* inhibitory activity of L_3 exsheathment process of two isolates from different climate regions.

In general terms, EEL and EEFR inhibited the L_3 exsheathment process in both isolates. The EC_{50} values of EEL [HcINRAE = 78.6 µg/mL (36.55–116.26) and HcEch91 = 83.3 µg/mL (65.82–98.31)] were twice more potent than for EEFR [HcINRAE = 137.1 µg/mL (95.96–178.52) and HcEch91 = 152.5 µg/mL (136.2–170.2)]. Furthermore, both extracts presented a lower EC_{50} against *H. contortus* from Brazil (HcEch91) than comparing with France (HcINRAE).

In negative controls, the exsheathment was > 95% for both isolate larvae after 60 minutes of contact with the solution. The EEL (Figure 1A, 1B) and the EEFR (Figure 1C, 1D) showed a potent effect on the larval exsheathment against both isolates presenting a homogeneous curve and gradual effect on *H. contortus*. The inhibitory percentage ± SEM was obtained for the time 60 minutes on the exsheathment process, and the EEL presented 70–80 % efficacy for HcINRAE and HcEch91 at 150 µg/mL. However, the EEFR was less active at the same concentration, with 40–50 % efficacy (Table 1).

Table 1. Inhibition efficacy (mean percentage ± SEM) of ethanolic extracts (EEL and EEFR) from *Pterogyne nitens* (µg/mL) and PBS control on two isolates of *Haemonchus contortus* (HcINRAE and HcEch91) in the larval exsheathment inhibition assay (LEA).

Concentrations (µg/mL)	**HcINRAE***		**HcEch91***	
	EEL	**EEFR**	**EEL**	**EEFR**
1200	98.44 ± 1.56^{a}	98.81 ± 1.79^{a}	100 ± 0.00^{ab}	100 ± 0.00^{ab}
600	94.28 ± 3.30^{b}	98.22 ± 1.19^{a}	100 ± 0.00^{ab}	100 ± 0.00^{ab}
300	93.80 ± 4.71^{b}	88.15 ± 2.95^{a}	98.22 ± 1.79^{b}	90.83 ± 1.32^{ab}
150	79.92 ± 8.81^{b}	51.24 ± 9.73^{b}	76.27 ± 5.61^{c}	45.44 ± 5.17^{b}
75	34.34 ± 7.09^{c}	18.46 ± 5.64^{c}	43.86± 4.85^{d}	14.4 ± 3.90^{c}
PBS	1.14 ± 1.14^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}

*Different lowercase letters in the same column correspond to the different concentrations tested indicate significantly different values by ANOVA and the Tukey test (p≤0.05).

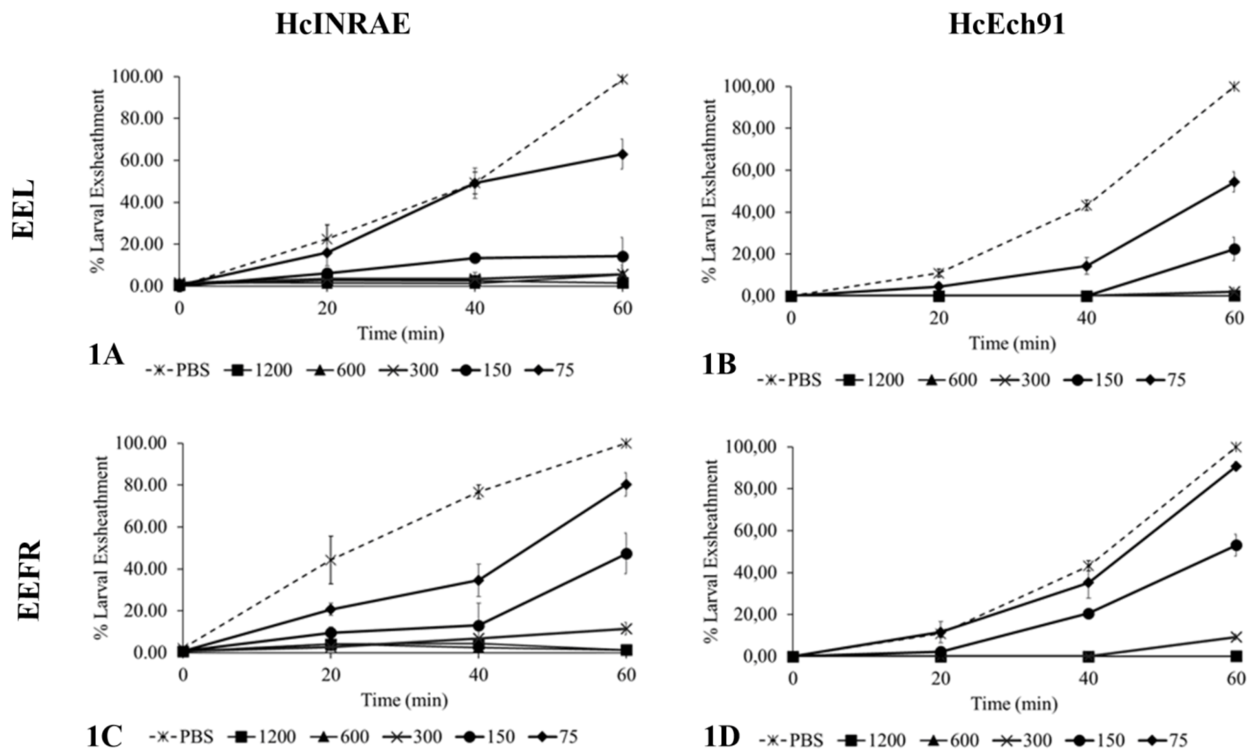


Figure 1. Effect of Ethanolic extract from Leaves (EEL – 1A, 1B) and Ethanolic extract from Fruits (EEFR – 1C, 1D) from *Pterogyne nitens* on *in vitro* exsheathment process on infective larvae (L_3) of two susceptible isolates of *Haemonchus contortus* (HcINRAE and HcEch91).

3.2 Phenolic compounds from P. nitens and commercial flavonoids

Phytochemical fractionation of EEL, EEFR and EEFL furnished two flavones (sorbifolin and pedalitin), two flavonols (quercetin and rutin), a flavon-3-ol (ourateacatechin), and three phenolic acids (ferulic acid, caffeic acid, and gallic acid), respectively (Figure 2). The EC_{50} (µg/mL) and confidence intervals (95% CI) obtained in LEA for the phenolic compounds were presented in Table 2, which the values ranged between 1.6 to 132.4 µg/ml against HcINRAE and 5.8 to 54.6 µg/mL against HcEch91.

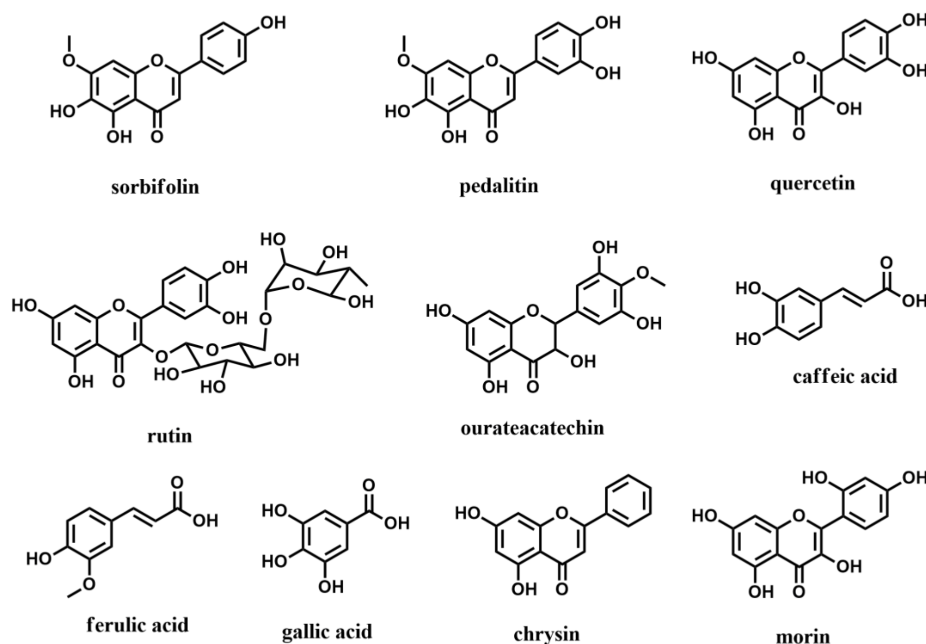


Figure 2. Structure of sorbifolin, pedalitin, quercetin, rutin, ourateacatechin, caffeic acid, ferulic acid, and gallic acid (obtained from *Pterogyne nitens*) and two commercial flavonoids [chrysin (C80105) and morin (M4008)], supplied by Merck®.

Table 2. EC_{50} (µg/mL) and confidence intervals (95% CI) of phenolic compounds from *Pterogyne nitens*, and commercial flavonoids on exsheathment process of infective larvae L_3 of two susceptible isolates of *Haemonchus contortus.*

Phenolic compounds	**HcINRAE**		**HcEch91**	
	EC_{50} µg/mL	**95% CI**	**EC_{50} µg/mL**	**95% CI**
sorbifolin	44.46	35.76–54.51	17.89	14.01–21.34
pedalitin	23.97	17.35–30.37	17.44	14.23–20.20
quercetin	1.62	0.01–5.77	5.83	5.10–6.68
rutin	55.55	41.47–76.37	54.57	49.85–59.86
ourateacatechin	44.72	38.27–52.23	51.71	46.26–57.80
caffeic acid	46.78	39.18–56.45	39.48	35.33–44.00
ferulic acid	132.18	120.52–143.93	44.98	39.82–50.59
gallic acid	28.10	22.23–34.33	44.92	37.94–53.62
chrysin	39.82	31.90–48.89	35.72	32.05–39.63
morin	21.18	18.33–23.98	28.89	25.47–32.46

HcINRAE = French Isolate; HcEch91 = Brazilian Isolate Echevarria 1991.

The inhibition percentages and curves mediated by phenolic compounds in LEA are displayed in Table 3 and Figure 3, respectively. In negative controls, the exsheathment was > 95% for both isolate larvae until 60 minutes of contact with the solution (Figure 3). A dose-response relation was observed for all compounds. Phenolic compounds (Figure 3A–3T) showed a potent effect on the larval exsheathment against both isolates, presenting an anthelmintic effect on *H. contortus*.

Most of the compounds presented more than 90% efficacy against both isolates at 120 µg/mL. However, the ferulic acid (Figure 3M, 3N) was less active at the same concentration, with 40% efficacy against HcINRAE isolate. The EC_{50} values to the flavone (sorbifolin, pedalitin, and chrysin) for the HcEch91 were twice higher than HcINRAE. Quercetin presented the most potent inhibitory effect against both nematode isolates, with 100% of inhibition at 30 µg/mL and presented an EC_{50} five times higher against HcINRAE isolate than HcEch91.

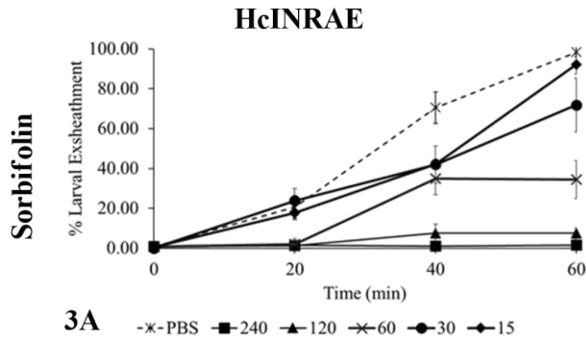
However, morin (quercetin isomer) showed the same values against both isolates, as well as observed for the rutin (quercetin 3-rutinoside). The hydroxycinnamic acid derivatives, caffeic and ferulic acids, presented lower EC_{50} values against HcEch91 (39.5 and 45 µg/mL, respectively) comparing with HcINRAE isolate (46.8 and 132.2 µg/mL, respectively). However, for the gallic acid the value against HcEch91 (44.9 µg/mL) was higher than HcINRAE (28.1 µg/mL).

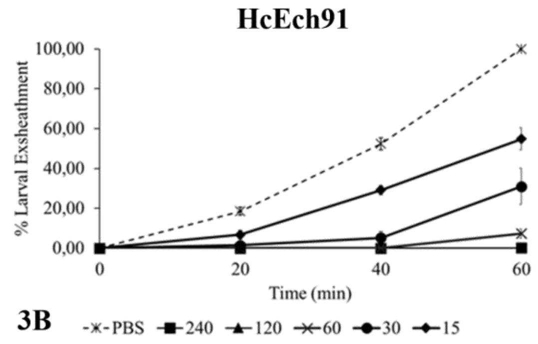
Pearson's correlation coefficient between natural compounds EC_{50} values against two *H. contortus* isolates was r = 0.53, showing a weak correlation between HcINRAE and HcEch91.

Table 3. Inhibition efficacy (mean percentage ± SEM) of phenolic compounds from *Pterogyne nitens,* commercial flavonoids (µg/mL) and PBS as negative control (µg/mL) on larval exsheathment of two isolates of *Haemonchus contortus* (HcINRAE and HcEch91) in the larval exsheathment inhibition assay (LEA).

Compounds/ Conc. (µg/mL) *	*H. contortus* Isolates	240	120	60	30	15	PBS
sorbifolin	INRAE	98.33 ± 1.67^{a}	91.86 ± 3.25^{a}	63 ± 8.34^{b}	39.64 ± 9.27^{bc}	25.10 ± 5.25^{c}	2.27 ± 2.27^{d}
	Ech91	100 ± 0.00^{a}	100 ± 0.00^{a}	93.20 ± 2.32^{a}	68.64 ± 9.11^{b}	43.14 ± 5.59^{c}	0.00 ± 0.00^{d}
pedalitin	INRAE	98.86 ± 1.14^{a}	94.33 ± 2.21^{ab}	86.01 ± 4.81^{ab}	72.38 ± 5.79^{b}	15.08 ± 9.29^{c}	0.00 ± 0.00^{d}
	Ech91	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	74.69 ± 2.78^{b}	45.07 ± 3.18^{c}	0.00 ± 0.00^{d}
quercetin	INRAE	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	83.06 ± 7.87^{b}	1.32 ± 1.32^{c}
	Ech91	100 ± 0.00^{d}	100 ± 0.00^{d}	100 ± 0.00^{d}	100 ± 0.00^{d}	96.69 ± 1.96^{d}	0.00 ± 0.00^{a}
rutin	INRAE	97.73 ± 2.27^{a}	98.33 ± 1.67^{a}	32.29 ± 9.72^{b}	17.08 ± 4.21^{b}	13.08 ± 5.71^{b}	2.58 ± 1.50^{c}
	Ech91	100 ± 0.00^{a}	100 ± 0.00^{a}	53.59 ± 5.00^{b}	9.38 ± 4.38^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}
ouratecatechin	INRAE	100 ± 0.00^{a}	98.33 ± 1.67^{a}	67.33 ± 13.60^{b}	17.76 ± 4.55^{c}	4.92 ± 1.66^{d}	0.00 ± 0.00^{d}
	Ech91	100 ± 0.00^{a}	100 ± 0.00^{a}	46.13 ± 4.93^{b}	27.18 ± 1.90^{c}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
caffeic acid	INRAE	98.44 ± 1.56^{a}	98.33 ± 1.67^{a}	66.21 ± 4.61^{b}	12.04 ± 4.08^{c}	9.23 ± 1.50^{c}	2.20 ± 1.35^{d}
	Ech91	100 ± 0.00^{a}	100 ± 0.00^{a}	74.18 ± 4.70^{b}	30.39 ± 4.27^{c}	3.57 ± 2.06^{d}	0.00 ± 0.00^{d}
ferulic acid	INRAE	97.00 ± 1.79^{a}	38.62 ± 14.53^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
	Ech91	100 ± 0.00^{a}	100 ± 0.00^{a}	59.68 ± 5.69^{a}	28.71 ± 7.59^{b}	2.27 ± 2.27^{c}	0.00 ± 0.00^{c}
gallic acid	INRAE	98.69 ± 1.32^{a}	98.69 ± 1.32^{a}	84.29 ± 4.06^{a}	44.44 ± 7.69^{b}	27.40 ± 8.45^{c}	0.00 ± 0.00^{d}
	Ech91	100 ± 0.00^{a}	100 ± 0.00^{a}	46.53 ± 6.64^{b}	33.36 ± 4.10^{b}	7.18 ± 4.17^{c}	0.00 ± 0.00^{d}
chrysin	INRAE	95.33 ± 2.71^{a}	90.56 ± 2.26^{a}	63.61 ± 10.46^{b}	28.91 ± 2.16^{c}	23.96 ± 3.70^{c}	2.06 ± 1.20^{d}
	Ech91	100 ± 0.00^{a}	98.69 ± 1.32^{a}	81.33 ± 2.40^{b}	42.68 ± 5.88^{c}	1.14 ± 1.14^{d}	0.00 ± 0.00^{d}
morin	INRAE	100 ± 0.00^{a}	100 ± 0.00^{a}	93.17 ± 0.91^{a}	61.47 ± 7.55^{b}	34.17 ± 6.33^{c}	0.00 ± 0.00^{d}
	Ech91	100 ± 0.00^{a}	100 ± 0.00^{a}	97.73 ± 2.27^{b}	45.78 ± 5.84^{c}	11.90 ± 4.52^{d}	0.00 ± 0.00^{e}

*Different lowercase letters in the same column correspond to the different concentrations tested indicate significantly different values by ANOVA and the Tukey test ($p \leq 0.05$).


HcINRAE
Sorbifolin
% Larval Exsheathment
Time (min)
3A
PBS 240 120 60 30 15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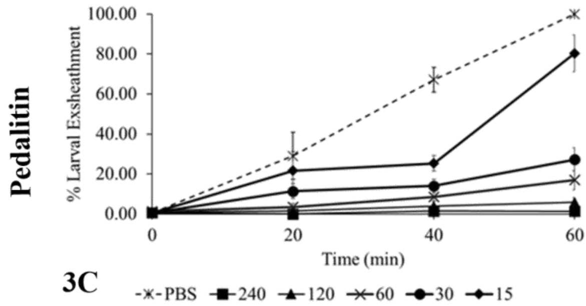
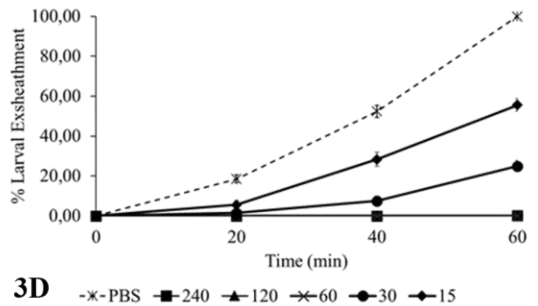
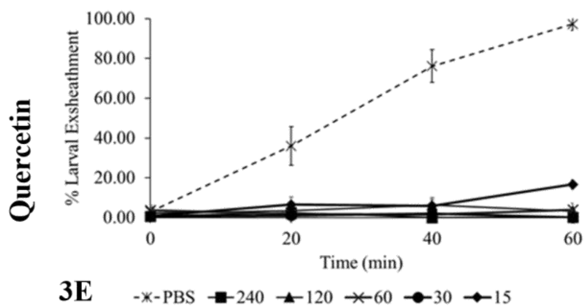
% Larval Exsheathment
Time (min)
3B
PBS 240 120 60 30 15


Pedalitin
% Larval Exsheathment
Time (min)
3C
PBS 240 120 60 30 15


% Larval Exsheathment
Time (min)
3D
PBS 240 120 60 30 15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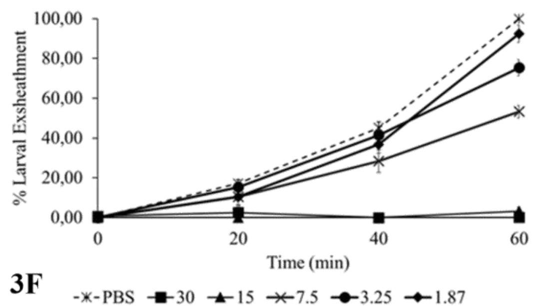
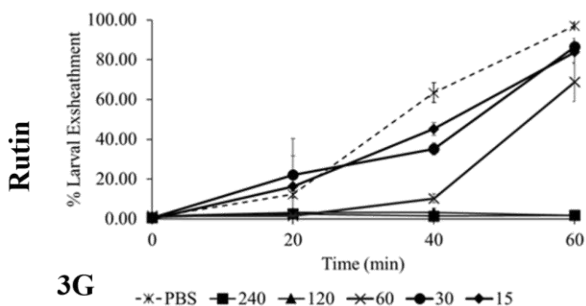
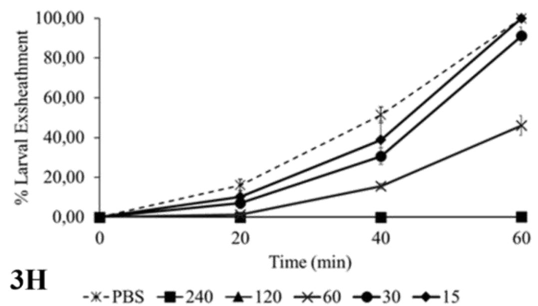
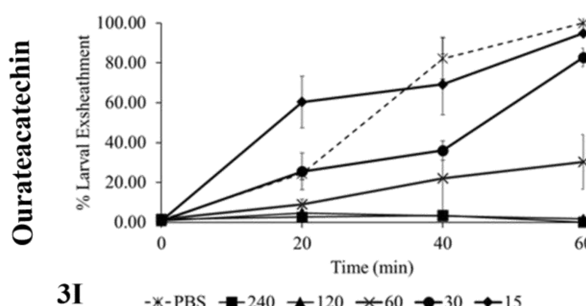
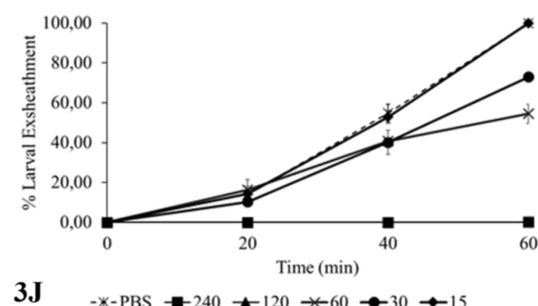
% Larval Exsheathment
Time (min)
3E
PBS 240 120 60 30 15

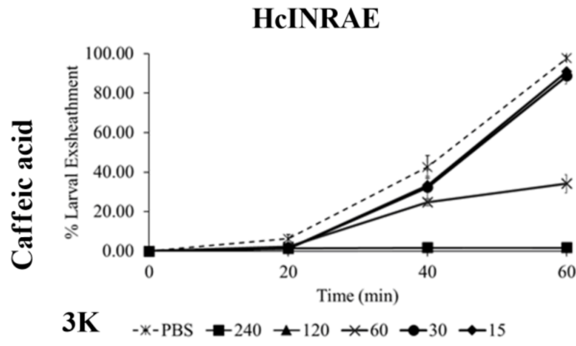

% Larval Exsheathment
Time (min)
3F
PBS 30 15 7.5 3.25 1.87

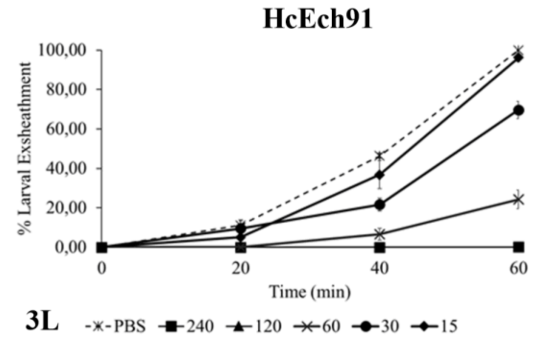

Rutin
% Larval Exsheathment
Time (min)
3G
PBS 240 120 60 30 15

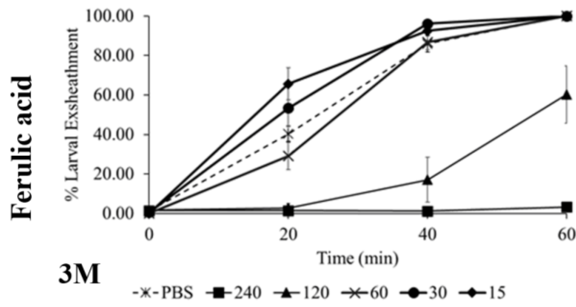

% Larval Exsheathment
Time (min)
3H
PBS 240 120 60 30 15

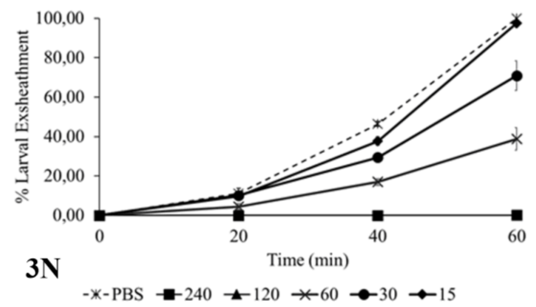

Ourateacatechin
% Larval Exsheathment
Time (min)
3I
PBS 240 120 60 30 15

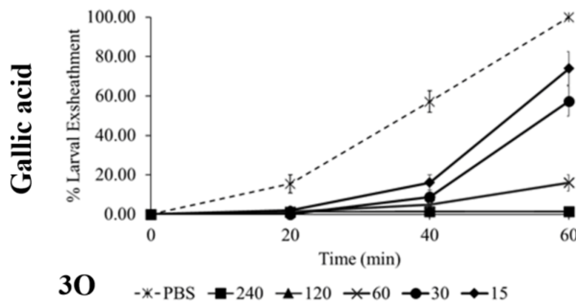

% Larval Exsheathment
Time (min)
3J
PBS 240 120 60 30 15

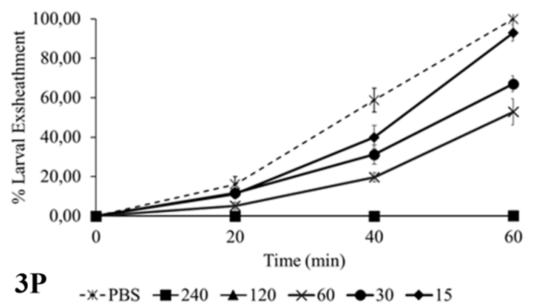

HcINRAE
Caffeic acid
100.00
80.00
60.00
40.00
20.00
0.00
% Larval Exsheathment
0
20
40
60
Time (min)
3K
PBS 240 120 60 30 15

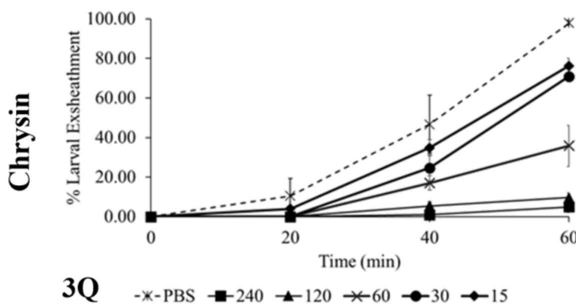

HcEch91
100,00
80,00
60,00
40,00
20,00
0,00
% Larval Exsheathment
0
20
40
60
Time (min)
3L
PBS 240 120 60 30 15

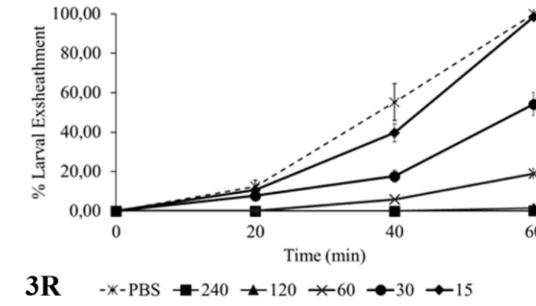

Ferulic acid
100.00
80.00
60.00
40.00
20.00
0.00
% Larval Exsheathment
0
20
40
60
Time (min)
3M
PBS 240 120 60 30 15

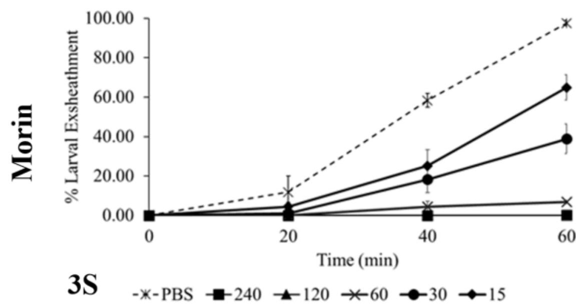

100,00
80,00
60,00
40,00
20,00
0,00
% Larval Exsheathment
0
20
40
60
Time (min)
3N
PBS 240 120 60 30 15


Gallic acid
100.00
80.00
60.00
40.00
20.00
0.00
% Larval Exsheathment
0
20
40
60
Time (min)
3O
PBS 240 120 60 30 15


100,00
80,00
60,00
40,00
20,00
0,00
% Larval Exsheathment
0
20
40
60
Time (min)
3P
PBS 240 120 60 30 15


Chrysin
100.00
80.00
60.00
40.00
20.00
0.00
% Larval Exsheathment
0
20
40
60
Time (min)
3Q
PBS 240 120 60 30 15


100,00
80,00
60,00
40,00
20,00
0,00
% Larval Exsheathment
0
20
40
60
Time (min)
3R
PBS 240 120 60 30 15


Morin
100.00
80.00
60.00
40.00
20.00
0.00
% Larval Exsheathment
0
20
40
60
Time (min)
3S
PBS 240 120 60 30 15

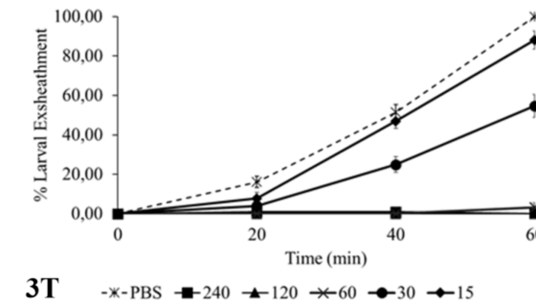

100,00
80,00
60,00
40,00
20,00
0,00
% Larval Exsheathment
0
20
40
60
Time (min)
3T
PBS 240 120 60 30 15

Figure 3. Effect of flavonoids (3A–3J) and phenolic acids (3K–3P) from *Pterogyne nitens* and commercial flavonoids (3Q–3T) on *in vitro* exsheathment process on L_3 larvae of two susceptible isolates of *Haemonchus contortus* (HcINRAE and HcEch91).

4. Discussion

This is the first report, of the anthelmintic effects of *P. nitens* extracts and natural compounds against two different nematode isolates of *H. contortus*. LEA has been developed to artificially measure the ability of extracts or phenolic compounds to delay or inhibit a natural process of the infective larvae. By preventing this process, the larvae may be not able to infect the host (Hertzberg et al. 2002).

LEA has advantages over other bioassays once it is sensitive, reproducible and relevant to processes that occur *in vivo* (Alonso-Díaz et al. 2011; Azando et al. 2011). This assay is usually chosen to evaluate and investigate the effect of plants containing mixtures of phenolic compounds, as tannins and flavonoids. *In vitro* and *in vivo* literature results support the hypothesis that the interference of phenolic compounds, on the larval exsheathment as a possible mechanism of action against GINs (Hoste et al. 2006; Brunet and Hoste 2006; Brunet et al. 2007; 2008).

Overall, the results obtained for both isolates of *H. contortus* showed strong similarities for extracts or phenolic compounds. Only minor variations were observed in the response of the isolate nematodes depending on the phenolic compound concentrations. This modulation was especially illustrated by the results of larval incubation at the 15 and 30 μg/mL concentrations, which induced a higher exsheathment percentage than compared with the negative control (PBS). Possible differences in isolates susceptibility have been previously suggested by comparative *in vitro* results obtained with whole extracts of woody plants or forages (Molan et al. 2003; Paolini et al. 2004), with tannins extracted from plants (Molan et al. 2000) or with commercially available monomers (Brunet and Hoste 2006). However, this is the first description for some flavonoids and phenolic acids.

The possible variations between the EC_{50} values between the natural compounds tested against both GIN isolates may be due to the age of the larvae used in the tests. A

study developed by Castañeda-Ramírez et al. (2017) found that the age of the larvae tested with tannin content extracts influenced the breeding process, suggesting that the lower extract efficacy could be associated with decaying vitality of larvae associated with age. More stable efficacy results were found between two to five weeks of age. Another possible difference between the natural compounds EC_{50} values may be due to the cuticle differentiations in larvae species used in the tests (Bird and Rogers 1956).

Most studies have addressed the anthelmintic properties of tannin rich plant extracts on L_3 to the concentration of condensed tannins. Few studies have explored, other polyphenolic or flavonoid compounds which might contribute to the anthelmintic activity. In the present study different phenolic compounds were evaluated, some of them being reported for the first time with potent anthelmintic activity.

Two condensed tannin basic units, as gallic acid and ourateacatechin, were also tested and presented a relevant anthelmintic activity against both isolates. In previous *in vitro* studies using units of flavan-3-ols and flavan-3-ol gallates had presented an inhibitory activity on egg hatching, larval development and in the viability of *T. colubriformis* L_3 (Molan et al. 2003) and units based in the gallic acid against egg hatching and larval mobility of *H. contortus* (Engström et al. 2016). They observed the quantity of the condensed tannins present might also be an important criterion to explain modulations of effects on nematode larvae (Hagerman et al. 1998; Poncet-Legrand et al. 2006).

Comparing the flavone chemical structures. Chrysin is a flavone with unsubstituted ring B and ring A substituted by two hydroxyls. Adding one (sorbifolin) or two (pedalitin) hydroxyl groups into the chemical structure we are able to observe that the anthelmintic activity was more potent against the Brazilian isolate (HcEch91). Other studies concluded that, in general, an increase in the number of hydroxyl groups may be associated with the increase of anthelmintic activity (Brunet and Hoste 2006).

Nevertheless, against HcINRAE isolate comparing chrysin and sorbifolin, the addiction of one hydroxyl group did not change significantly the EC_{50} values. The different anthelmintic effects of flavones may be associated with the structure of the larval cuticle, mainly regarding permeation by passive diffusion through collagen and other structures with different permeation degrees (Page and Johnstone 2009).

Another study evaluated in LEA the activity of quercetin against HcINRAE isolate, which demonstrated EC_{50} of 21 µg/mL (Klongsiriwet et al. 2015). Comparing with our results with EC_{50} = 1.6 µg/ml for HcINRAE and 5.8 µg/ml for HcEch91, this difference could be explained by the L_3 old age, once older larvae tend to show higher EC_{50} and EC_{90} values ($p \leq 0.05$). Analyzing the quercetin EC_{50} values against the exsheathment process of both *H. contortus* isolates, it can be suggested that this compound could be used as a positive control to *in vitro* assay, since until now there are not a conventional AH used in LEA.

Further experiments will be necessary to elucidate the underlying causes of this difference, such as larval age or donor, as older larvae from sheep were more sensitive than younger larvae from goats (Hoste et al. 1999). Morin is quercetin isomer and both have the same molecular weight. However, it was possible to observe that the change in the position of the hydroxyl group at position 5' to 6' in ring C, had changed significantly the anthelmintic activity against both isolates.

Barrau et al. (2005) demonstrated that rutin inhibits larval migration of *H. contortus* at 1200 µg/mL. In the present study, this flavonol glycoside was active until 60 µg/mL for both GIN isolates. Therefore, we may suggest that the presence of other compounds, such as flavonol glycosides, phenolic acids and/ or flavones might also participate in the modulation of anthelmintic activity.

Thus, it is worth to underline that the similarity of the chemical structure may suggest a similar or close mechanism of action for both types of compounds. Nevertheless, further analyses of the precise mechanisms involved for phenolic compounds, as for flavonol glycosides in addition to condensed tannins, are required to estimate the preventive value of this legume forage for GIN controls in livestock (Hoste et al. 2015).

5. Conclusion

This study showed that the *H. contortus* isolates evaluated present susceptibility towards the extracts and phenolic compounds from *P. nitens*. The results indicated a clear *in vitro* inhibitory activity of L_3 exsheathment process of two isolates from different regions.

As well as polyphenols are also responsible for the anthelmintic activity of different aerial parts extracts tested. Nevertheless, complementary investigations to determine the mechanism of action and interactions of phenolic compounds are needed. *In vivo* experiments should also be carried out to explore the toxicity and therapeutic efficacy of these natural compounds in the future.

Acknowledgments

This work was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES), Brazilian Council for Scientific and Technological Development (CNPq) (Grants 471129/2013-5, 306251/2016-7 and 429322/2018-6), and São Paulo Research Foundation (FAPESP) (Grants 2014/18330-0 and 2018/15083-2). C. S. L. thanks *CAPES (finance code 001)* for the scholarships and the Doctoral Program Sandwich Abroad at the Institut National de la Recherche Agronomique of Toulouse, France (Grant 88881.187940/2018-01).

Compliance with ethical standards

Ethics approval and consent to participate: The present study was approved and conducted in accordance with the guidelines of the French ethical and welfare rules (agreement number C 31 555 27 of 19 August 2010) and in Brazil all procedures were approved by the Embrapa Pecuária Sudeste Ethics Committee on Animal Experimentation (process nº. 04/2017), and are in accordance with national and international principles and guidelines for animal experimentation adopted by the Brazilian College of Experimentation (CONCEA). and Brazilian College of Experimentation (CONCEA).

Consent to publish: Not applicable.

Conflict of interest: The authors declare that there are no conflicts of interest.

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Capítulo IV

***In vitro* anthelmintic effect of *Pterogyne nitens* (Fabaceae) on eggs and larvae of *Haemonchus contortus*: analyses of structure-activity relationships based on phenolic compounds**

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ABSTRACT: Due to high prevalence and large pathogenicity, *Haemonchus contortus* is the main gastrointestinal nematode in tropical and subtropical climate regions. This species is responsible for severe economic losses in sheep and goat breeding in Brazil. However, the control of this parasite is nowadays compromised, mainly, due to anthelmintic resistance. In the search for natural anthelmintic alternatives, *Pterogyne nitens* has been identified. This is a native Brazilian tree with potential ethnopharmacological indication, presenting secondary metabolites with biological activities. The aim of this study was to evaluate the anthelmintic activity of ethanolic extracts and phenolic compounds from *P. nitens*. The ovicidal and larvicidal activity of ethanolic extracts from leaves (EEL) and fruits (EEFR) and natural compounds from *P. nitens* on *H. contortus* was evaluated through egg hatch assay (EHA) and larval development assay (LDA). The anthelmintic activity was expressed as EC_{50} (effective concentration with 50% activity) and respective 95% confidence interval (95% CI). The results showed that all extracts and mostly the phenolic compounds were active in the EHA and LDA. Egg hatch inhibitory effects of EEL (EC_{50} = 316 µg/mL) were more potent than EEFR (EC_{50} = 512 µg/mL). However, larval development inhibitory effects of EEL (EC_{50} = 47 µg/mL) and EEFR (EC_{50} = 35 µg/mL) were similar. Among the compounds, the flavones (sorbifolin, pedalitin, and chrysin) did not show inhibitory effects on egg hatching but presented some activity on larval development of *H. contortus*. In contrast, the flavonols (quercetin, rutin, and morin) showed high activity on the egg hatch, but were inactive on the LDA. The addition of the hydroxyl group and the rutinose group to the flavonoid structure seem to increase the ovicidal and larvicidal activity, respectively. The phenolic acids: caffeic acid, ferulic acid, and gallic acid have the highest anthelmintic effects, presenting EC_{50} of 1.48, 0.56 and 4.93 µg/mL in the EHA; and 31, 22 and 33 µg/mL in the LDA, respectively. These results suggest that *P. nitens* might represent a source of effective alternative to control of *H. contortus*. Further studies are needed i) to better understand the interactions at the molecular level and ii) to validate by *in vivo* experiments the therapeutic efficacy of *P. nitens.*

KEYWORDS: anthelmintic, *in vitro*, egg hatch assay (EHA), larval development assay (LDA), ethanolic extracts, phenolic compounds.

1. Introduction

Haemonchus contortus is a main gastrointestinal nematode that parasites the abomasum of sheep and goats in tropical and subtropical climate regions (Amarante et al., 2014). Compared to other nematodes, *H. contortus* is a highly pathogenic hematophagous species of small ruminants. The female worms are highly prolific from 5,000 to 10,000 eggs per day (Romero and Boero, 2001). Haemonchosis can result in accentuated economic losses by causing appetite depression, damages in gastric function, and alterations in total protein content, energy, and mineral metabolism (Zarlenga et al., 2016). When the animals have high parasitic loads, they present anemia and submandibular edema and may die (Amarante and Sales, 2007; Amarante et al., 2014) capable of causing acute disease (anemia) and high mortality in all classes of small ruminants (Besier et al., 2016).

For more than 50 years, the control of gastrointestinal nematodes, including *H. contortus* has relied on the use of synthetic anthelmintics. However, this mode of control based on chemical molecules is now showing some disadvantages, such as the risk of environmental pollution because of residues and also the increasing development and diffusion of resistant populations of worms (Melo et al., 2003) with reduction of animal production due to reduced efficacy (Wolstenholme et al., 2004; Sangster et al., 2018).

Alternative strategies for the control of nematodes have focused on different options, including improved use of anthelmintic, researches on a vaccine, genetic selection of sheep and goats for resistance to infection, prevention of the build-up of infective larvae on pasture by grazing strategies (Torres-Acosta and Hoste, 2008; Charlier et al., 2018). The last strategy is to explore the anthelmintic properties of plants containing bioactive compounds such as secondary metabolites.

Within this field, a bulk of studies have been dedicated to temperate and tropical plants containing phenolic compounds including condensed tannins and/ or flavonoids, either being integrated into the grazing rotation (Robertson et al., 1995), used as nutraceuticals (Hoste et al., 2015) or by using tannins extracts as a drench to impact the gastrointestinal nematode biology (Minho et al., 2008; Lima et al., 2019).

In this context, *Pterogyne nitens* Tulasne (Fabaceae) has been identified as a possible resource to exploit in Brazil and other tropical countries based on both ethnoveterinary and phytochemical information. *P. nitens* is popularly called as “bálsamo”, “cocal”, “amendoim-bravo”, “madeira-nova” or “yvi-raró” in Brazil, and is the only member of the *Pterogyne* genus within the Fabaceae family (Lorenzi, 2002). Studies in Guarani-indigenous communities of Argentina (Crivos et al., 2007) and Bolivia (Bourdy et al., 2004) indicated the potential ethnopharmacological and botanical uses of this tree.

In addition, *P. nitens* presents a variety of bioactive metabolites that had demonstrated several biological activities, as antiproliferative effect against melanoma cells (Regasini et al., 2007), inhibition of myeloperoxidase (Regasini et al., 2008; Fernandes et al., 2008), radical scavenging properties and antioxidant activities (Okumura et al., 2012; Vellosa et al., 2015), antifungal activity (Lima et al., 2016), antiviral activity (Shimizu et al., 2017), antibacterial multiresistant activity (Coqueiro et al., 2014), cytotoxic and antitumoral activity (Tajima et al., 2015; Satake et al., 2015; Oliveira et al., 2018).

The first objective of the present study was to investigate the potential anthelmintic effects of ethanolic extracts from different parts of *P. nitens* as well as of purified phenolic compounds based on two *in vitro* assays. Our second objective was to analyze the structure-activity relationships between the different natural phenolic compounds. For this purpose, two commercial flavonoids (chrysin and morin) have also been included in the assays.

2. Material and methods

2.1. Plant materials

Fresh leaves and dried fruits of *P. nitens* were collected at the campus of the Institute of Biosciences, Humanities and Exact Sciences of São Paulo State University (Unesp), São José do Rio Preto, SP, Brazil (20°47′02.4″S 49°21′36.0″W), in July 2014. A voucher specimen (HISA 10291) was deposited in the Ilha Solteira Herbarium (HISA) at the Faculty of Engineering, Unesp in Ilha Solteira, SP. Brazilian biodiversity access

number A85B7D5 was registered on National System for the Management of Genetic Heritage and Associated Traditional Knowledge.

2.2. Preparation of extracts

The shade-dried leaves (630 g) and fruits (200g) were ground in a knife grinder. The obtained powder was first macerated with hexane (1 L × 3) for 48 hours, to remove the apolar compounds. Then the vegetal material was separated from the extraction solution by simple filtration on filter paper and the filtrate was dried under rotary evaporation. Subsequently, the non-extracted residue was macerated with ethanol (1 L × 3) for 48 hours and the same procedure was repeated. Two types of extracts were obtained; ethanolic extract from leaves (EEL, 64 g) and ethanolic extract from fruits (EEFR, 20 g).

2.3. Natural compounds

Flavonoids (flavone, flavonol, and catechin derivative) and phenolic acids were isolated and identified, using chemical procedures reported previously. Flavone derivatives, sorbifolin, pedalitin, were isolated from leaves (Shimizu et al., 2017). Flavonol derivatives, quercetin, and rutin were obtained from fruits (Regasini et al. 2007; Regasini et al. 2008b). Flavon-3-ol derivative, ourateacatechin and phenolic acids as caffeic acid, ferulic acid, and gallic acid were isolated from flowers (Regasini et al., 2008a). Commercial samples of chrysin (C80105) and morin (M4008) were purchased from Merck® (Figure 1).

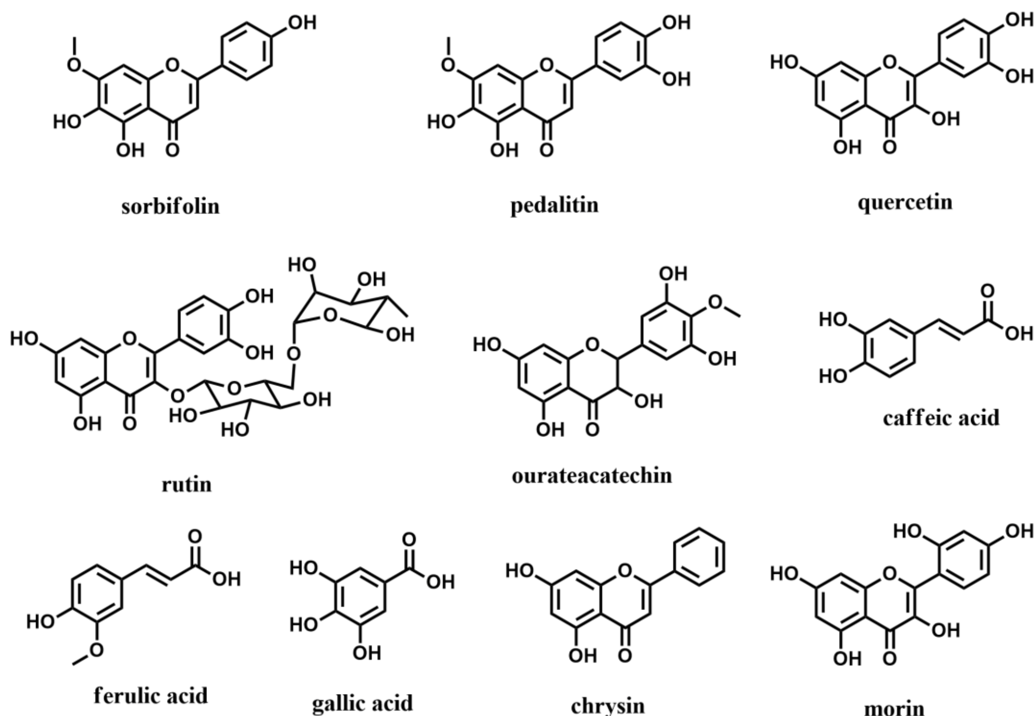


Figure 1. Structure of sorbifolin, pedalitin, quercetin, rutin, ourateacatechin, caffeic acid, ferulic acid, and gallic acid (obtained from *Pterogyne nitens*) and two commercial flavonoids [chrysin (C80105) and morin (M4008)], supplied by Merck®.

2.4. In vitro anthelmintic assays

2.4.1. *Haemonchus contortus* isolate

The susceptible *H. contortus* isolate (Echevarria 1991) was used for monospecific infection and each animal was inoculated orally with approximately 4000 third-stage (L_3) larvae. After 28 days of incubation, the infection was confirmed by checking EPG count. Animals with a count over 1500 were considered parasitological competent as feces donors for the *in vitro* assays.

2.4.2. Recovery and preparation of eggs

Recovery, preparation of eggs, egg hatch assay (EHA) and larval development assay (LDA) were performed according to the protocol described by Chagas et al. (2011) with minor modifications. Eggs were recovered from 100 g of fresh feces by mixing with 500 mL of distilled water. The suspension was filtered through 100 μm and 25 μm mesh

sieves. Eggs were washed from the 25 µm sieve and centrifuged at 3000 rpm for 5 min to form pellets. The supernatant was removed and a saturated NaCl solution was added to the pellet, centrifuged at 3000 rpm for 5 min. Floating eggs were collected using a 25 µm sieve and washed with phosphate-buffered saline (PBS, 0.1 M phosphate, 0.05 M NaCl, pH 7.2). Eggs were separated, quantified and used within two hours for the EHA and LDA.

2.4.3. Egg hatch assay (EHA)

One hundred eggs were added to each well of a 24-well microplate. PBS and thiabendazole (concentrations between 0.024–50 µg/mL; Sigma-Aldrich) were used as negative and positive controls, respectively. Ethanolic extract solutions were evaluated at concentrations of 24, 48, 97, 195, 390, 780, 1560, 3120, 6250, 12500 and 25000 µg/mL, flavonoid solutions at 62.5, 125, 250, 500, 1000 and 2000 µg/mL and phenolic acid solutions at 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 15.6, 31.2, 62.5 and 125 µg/mL were evaluated. Treatments, positive and negative controls were tested in six repetitions, using six wells for each treatment (i.e. approximately a total of 600 eggs). Plates were sealed with PVC film and incubated at 27 °C with relative humidity ≥ 80% for 24 h. Then eggs and L_1 larvae were counted with an inverted microscope to calculate egg hatch inhibition.

2.4.4. Larval development assay (LDA)

Approximately 100 eggs were added to each well of a 24-well microplate with PBS, *Escherichia coli* (Strain B lyophilized) nutritive medium and 0.5 mg/mL of amphotericin B (Sigma-Aldrich), reaching a total volume of 250 µL. Plates were incubated for 24 h at 27 °C and ≥ 80% relative humidity to obtain L_1 larvae when the solutions were added. PBS and ivermectin (concentrations between 0.005–10 µg/mL; Sigma-Aldrich) were used as negative and positive controls, respectively. Ethanolic extract were evaluated at concentrations of 2.4, 4.8, 9.5, 19, 39, 78, 156, 312, 625, 1250, and 2500 µg/mL, and flavonoids and simple phenolic compounds solutions at 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg/mL were evaluated. Treatments, positive and negative controls were tested in six repetitions, using six wells for each treatment and approximately 600 eggs in total. Plates were incubated for 7 days, and each well was analyzed with an inverted

microscope to count all L_3 and undeveloped larvae to estimate larval development inhibition.

2.4.5. Statistical analyses

The percent of inhibition of egg hatch (EHA) was calculated by the following equation: % inhibition = A/ (A + B) × 100, where A is the number of unhatched eggs and B is the number of L_1 larvae. The percent of inhibition of larval development (LDA) was calculated using the following equation: % inhibition = (A/B) × 100, where A is the number of $L_1 + L_2$ larvae and B is the total number of larvae ($L_1 + L_2 + L_3$).

Data are presented as the mean percentage ± standard error (SEM). The one-way ANOVA followed by the Tukey test was used to assess statistical significance depending on two factors: treatments and concentrations. $P \leq 0.05$ was considered to be statistically significant. In order to provide another parameter of comparison between the tested extracts and natural compounds, we calculated the concentration at which occurred 50% of egg hatching inhibition and larval development inhibition (EC_{50}) of the parasites. Analysis was performed by SPSS IBM Statistics® v. 20.

3. Results

3.1. Egg Hatch Assay (EHA)

From the EHA data, the EC_{50} values were calculated for extracts and phenolic compounds from *P. nitens* as well as for commercial flavonoids (Table 1). Because none of the flavones were able to inhibit 50% of the eggs from hatching at the highest concentration, their EC_{50} values were considered ≥ 3000 µg/mL.

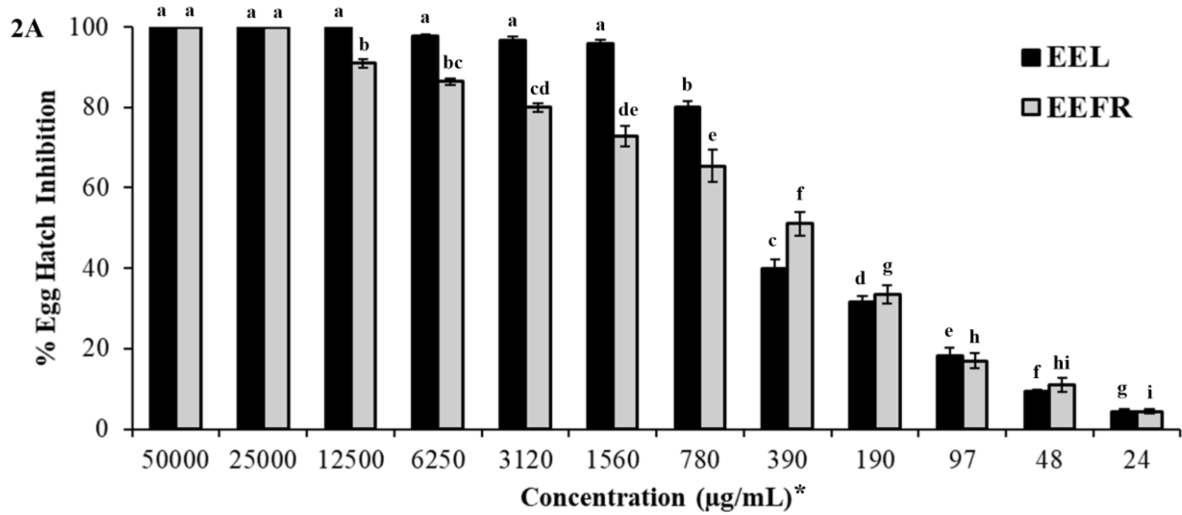
Table 1. EC_{50} (µg/mL) and confidence intervals (95% CI) of extracts, flavonoids and phenolic acids from *Pterogyne nitens* on egg hatch assay (EHA) and larval development assay (LDA) for *Haemonchus contortus.*

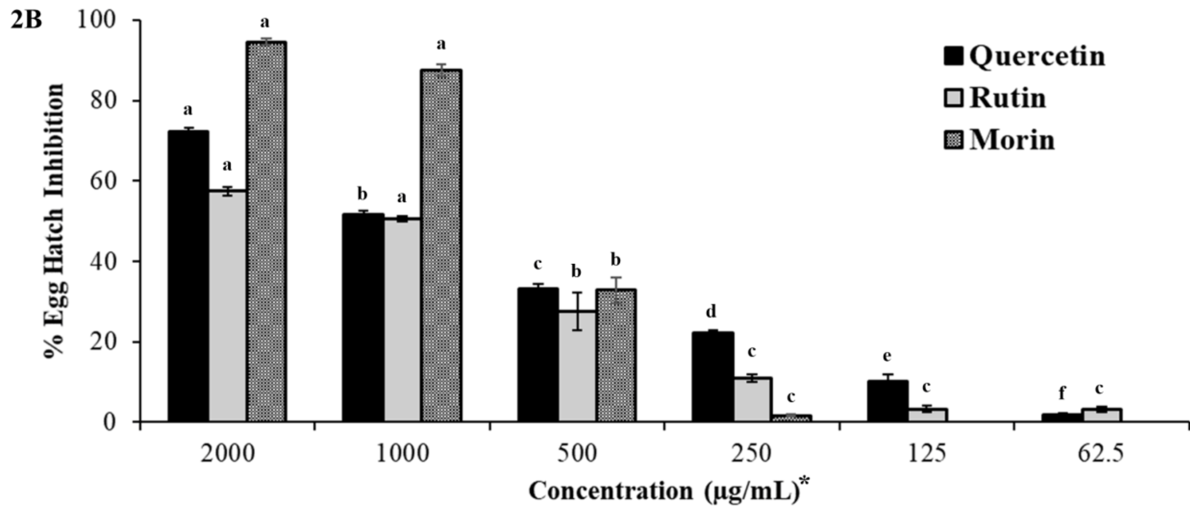
Extracts/natural compound	EHA (µg/mL)	LDA (µg/mL)
EEL	316 (287–348)	47 (31–63)
EEFR	512 (456–573)	35 (24–48)
sorbifolin	≥ 3000	18 (16–19)
pedalitin	≥ 3000	83 (76–90)
quercetin	880 (819–950)	231 (206–259)
rutin	1,260 (1,106–1,461)	104 (93–115)
ourateacatechin	≥ 3000	989 (852–1192)
chrysin*	≥ 3000	58 (54–63)
morin*	663 (591–739)	448 (418–481)
caffeic acid	1.48 (1.34–1.64)	31 (29–33)
ferulic acid	0.56 (0.50–0.63)	22 (21–24)
gallic acid	4.93 (4.47–5.43)	33 (30–36)
thiabendazole	0.01 (0.01–0.01)	nt
ivermectin	nt	0.13 (0.12–0.14)

nt = not tested; EHA = Egg hatch assay; LDA = Larval development assay; *Flavonoids supplied by Merck®.

The ethanolic extracts showed potent trends in their effectiveness at inhibiting the egg hatching. The EEL and EEFR presented statistically similar EC_{50} values ($p \leq 0.05$). Figure 2A shows that the proportion of eggs hatching decreased with the increase in the concentrations of ethanolic extracts. In the negative control wells, 98% of the eggs hatched. The EEL presented a significant reduction of 40% in hatching at low concentration (390 µg/mL) and EEFR exceeded 50% egg hatch inhibition at the same concentration (390 µg/mL).

The flavones (sorbifolin, pedalitin, and chrysin), and flavan-3-ol (ourateacatechin) presented at the highest concentration tested the percentage of egg hatch inhibition statistically equal the negative control ($p \leq 0.05$). At 2000 µg/mL they presented less than 5% of egg hatch inhibition.), so they did not have their EC_{50} and curve of egg hatch inhibition calculated.


2A
% Egg Hatch Inhibition
EEL
EEFR
Concentration (µg/mL)*
50000
25000
12500
6250
3120
1560
780
390
190
97
48
24


2B
% Egg Hatch Inhibition
Quercetin
Rutin
Morin
Concentration (µg/mL)*
2000
1000
500
250
125
62.5

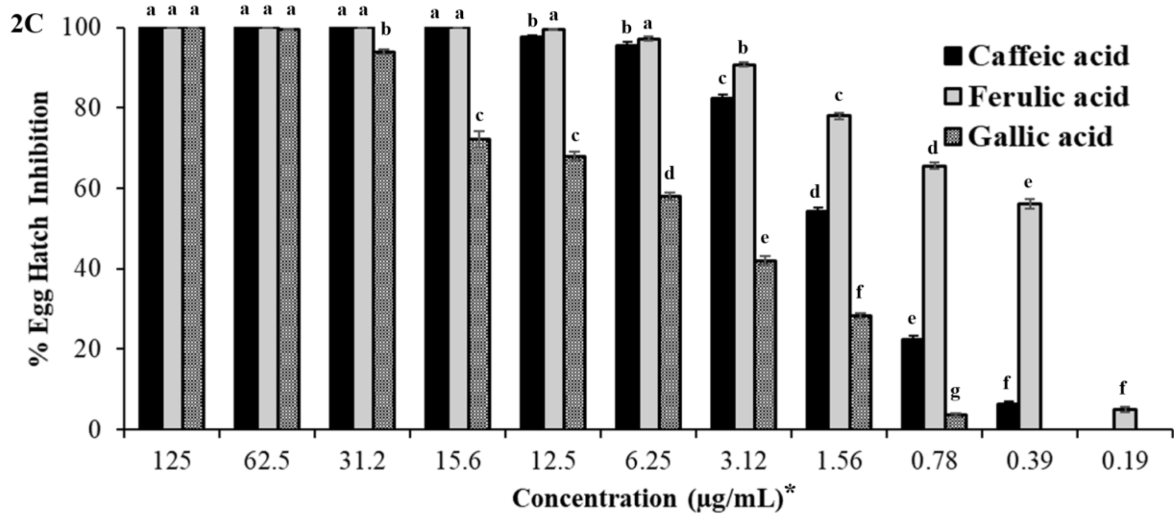

2C
% Egg Hatch Inhibition
Caffeic acid
Ferulic acid
Gallic acid
Concentration (µg/mL)*
125
62.5
31.2
15.6
12.5
6.25
3.12
1.56
0.78
0.39
0.19

Figure 2. *In vitro* effect of (2A) ethanolic extracts of leaves (EEL) and fruits (EEFR), (2B) flavonols (quercetin, rutin, and morin), (2C) phenolic acids (caffeic acid, ferulic acid, and gallic acid) on the egg hatching of *Haemonchus contortus*. *Different lower-case letters in the same column correspond to significant differences between treatments of the same solvent concentration by the Tukey test ($p \leq 0.05$).

In contrast, the flavonols (quercetin, rutin, and morin) inhibited the egg hatch more than 50%. Morin presented the lowest EC_{50} value (663 µg/mL), followed by quercetin with EC_{50} of 880 µg/mL. Rutin had EC_{50} values twice less effective than morin ones. Figure 2B shows the inhibition percentage at different concentrations calculated for the flavonols. At 500 µg/mL concentration the percentage inhibition values were statistically similar (33%, 27% and 32% for quercetin, rutin and morin, respectively).

Based on EC_{50} values and high inhibition percentages at low concentrations, the phenolic acids (caffeic, ferulic, and gallic acid) were identified as the most potent bioactive compounds. Ferulic acid had an EC_{50} (0.56 µg/mL) three times more potent than caffeic acid (EC_{50} = 1.48 µg/mL) against egg hatching. Gallic acid was less effective among the phenolic acids tested. Figure 2C shows the inhibition of egg hatching of phenolic acids. At concentration 15.6 µg/mL the caffeic and ferulic acid showed 100% of egg hatching inhibition, however, gallic acid had 30% inhibition decrease (Figure 2C).

Moreover, our results show that the phenolic acids were able to inhibit the egg hatching (Figure 3) at two different concentrations (500 and 6.25 µg/mL). The eggs that did not hatch even had the larvae developed inside after incubation for 24 h with phenolic acids at two different concentrations. Hatched larvae appeared sluggish and were often dead at high concentrations of compounds (Figure 3), indicating that they had died during or after incubation but before subsequent observation by microscopy.

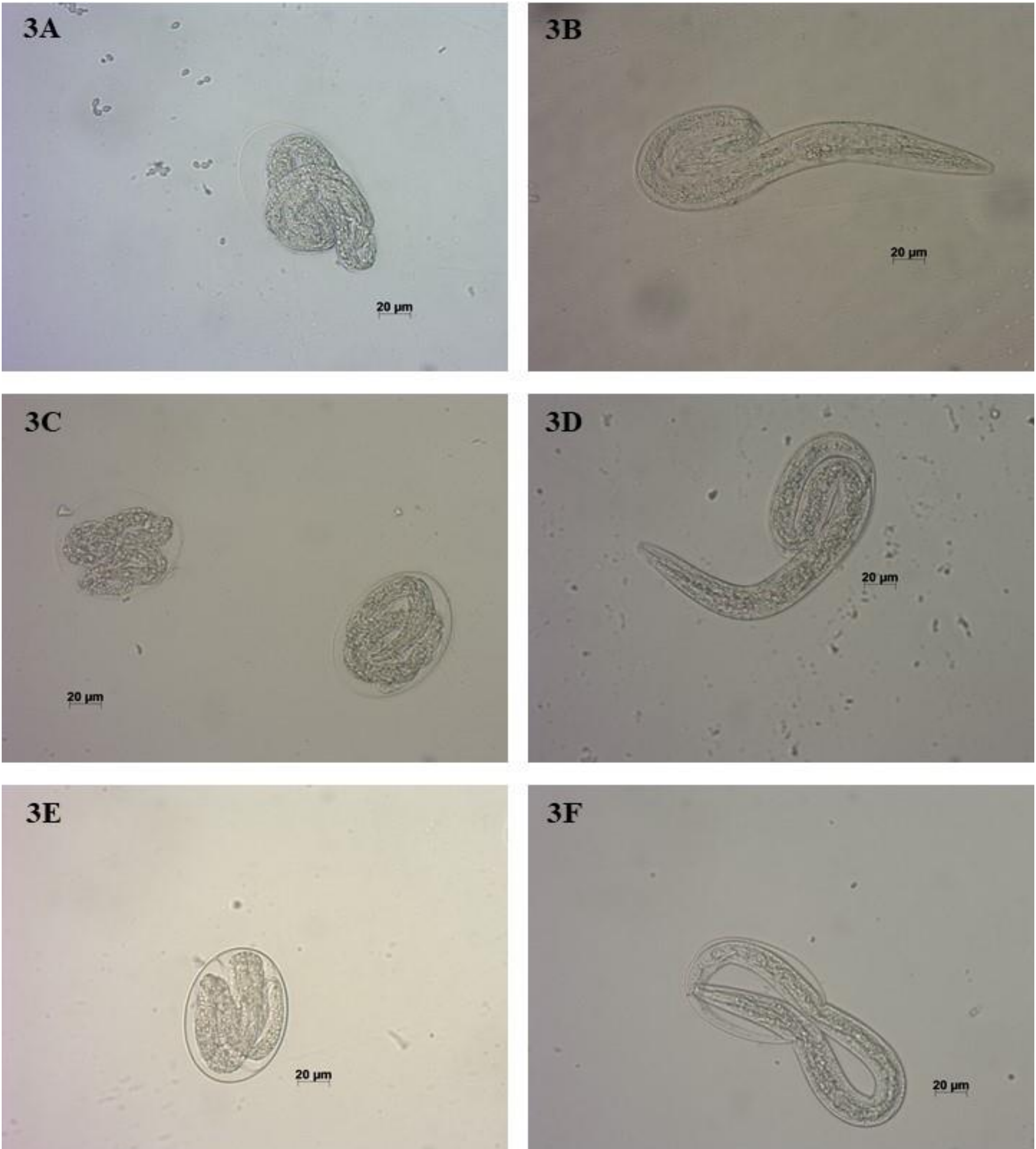


Figure 3. Images of *Haemonchus contortus* eggs and hatched larvae under optic microscopy (x400) after incubation (27 °C , RU ≥ 80%) for 24 h in (3A) 500 µg/mL, (3B) in 6.25 µg/mL of caffeic acid; (3C) in 500 µg/mL, (3D) in 6.25 µg/mL of ferulic acid and (3E) in 500 µg/mL and (3F) in 6.25 µg/mL of gallic acid. The hatched larvae appeared sluggish and were often dead at the concentrations of phenolic acids, indicating that they had died during or after incubation but before subsequent observation by microscopy.

3.2 Larval Development Assay (LDA)

In relation to the LDA results, the EC_{50} values were calculated (Table 1). The ethanolic extracts showed EC_{50} values indicating a potent effect to inhibit the larval development process. The EEFR (EC_{50} = 35 µg/mL) were slightly more active than EEL (EC_{50} = 47 µg/mL), however both extracts presented the concentration at which 50% larval development inhibition statistically similar ($p \geq 0.05$).

The ethanolic extracts presented a concentration-dependent response in the LDA. There was a non-significant difference between the anthelmintic activity of EEL and EEFR ($p \leq 0.05$) until the concentration of 156 µg/mL. In the lower concentrations, the proportion of larvae developed from L_1 to L_3 decreased with the increase of concentrations of ethanolic extracts (Figure 4A).

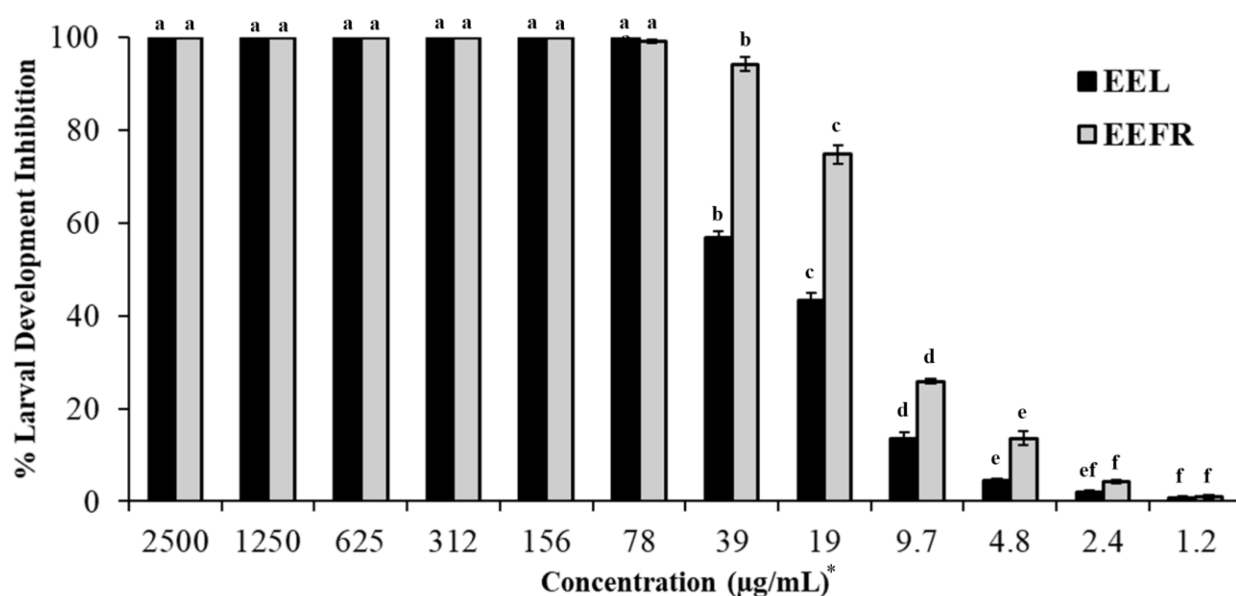


Figure 4. *In vitro* effect of ethanolic extracts from leaves (EEL) and fruits (EEFR) from *Pterogyne nitens* on the larval development of *Haemonchus contortus*. *Different lower-case letters in the same column correspond to significant differences between treatments of the same solvent concentration by the Tukey test ($p \leq 0.05$).

Comparing the microscopy images of *H. contortus* larvae after incubation for 7 days from the negative control (PBS, Figure 5A) and from EEL (Figure 5B) and EEFR treatment (Figure 5C), both extracts were able to impact the larvae structure at 2500

µg/mL. The undeveloped larvae appeared sluggish and were often dead at the extract's incubation plate at the tested concentration, indicating that they could die during or after seven days of incubation but before subsequent observation by microscopy.

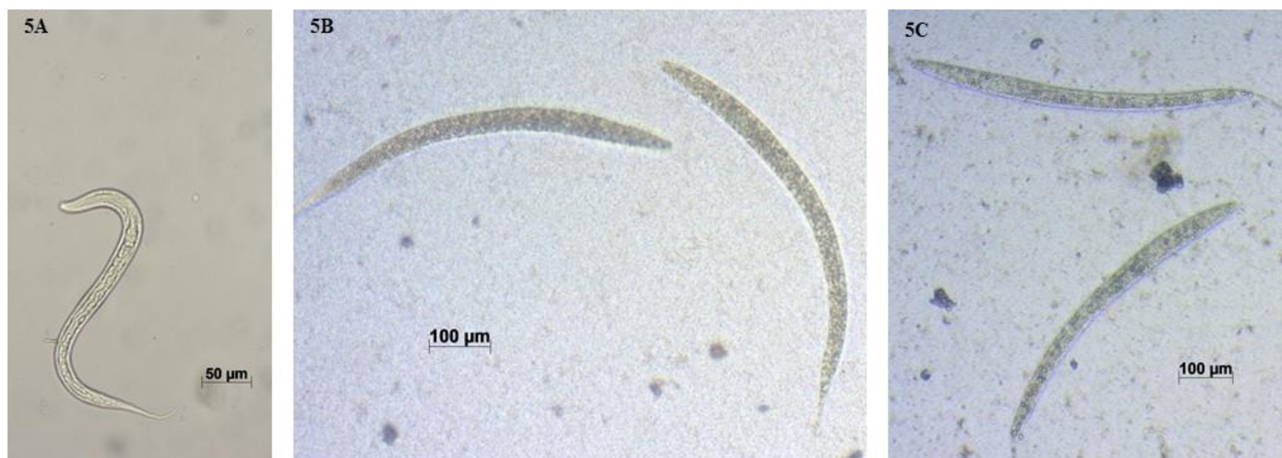


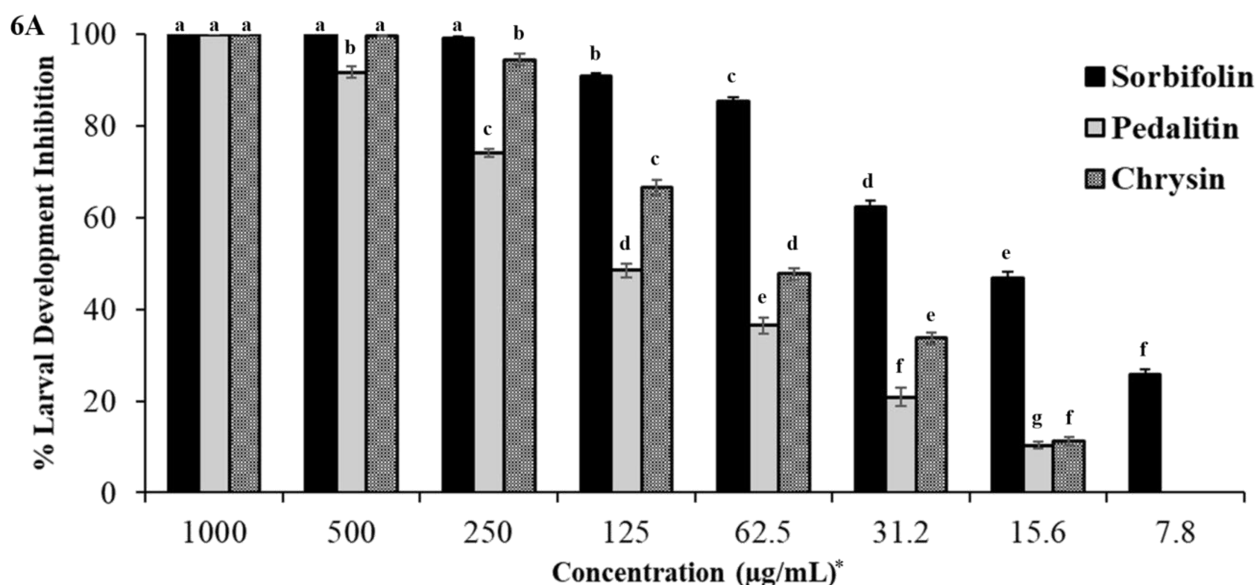
Figure 5. Images of *Haemonchus contortus* larvae under optic microscopy (x400) after incubation (27 °C, RU ≥ 80%) for 7 days in (5A) negative control (PBS), (5B) in 2500 µg/mL of EEL and (5C) in 2500 µg/mL of EEFR and with magnification x100. The undeveloped larvae appeared sluggish and were often dead at the extract's incubation plate indicating that they could die during or after seven days of incubation but before subsequent observation by microscopy.

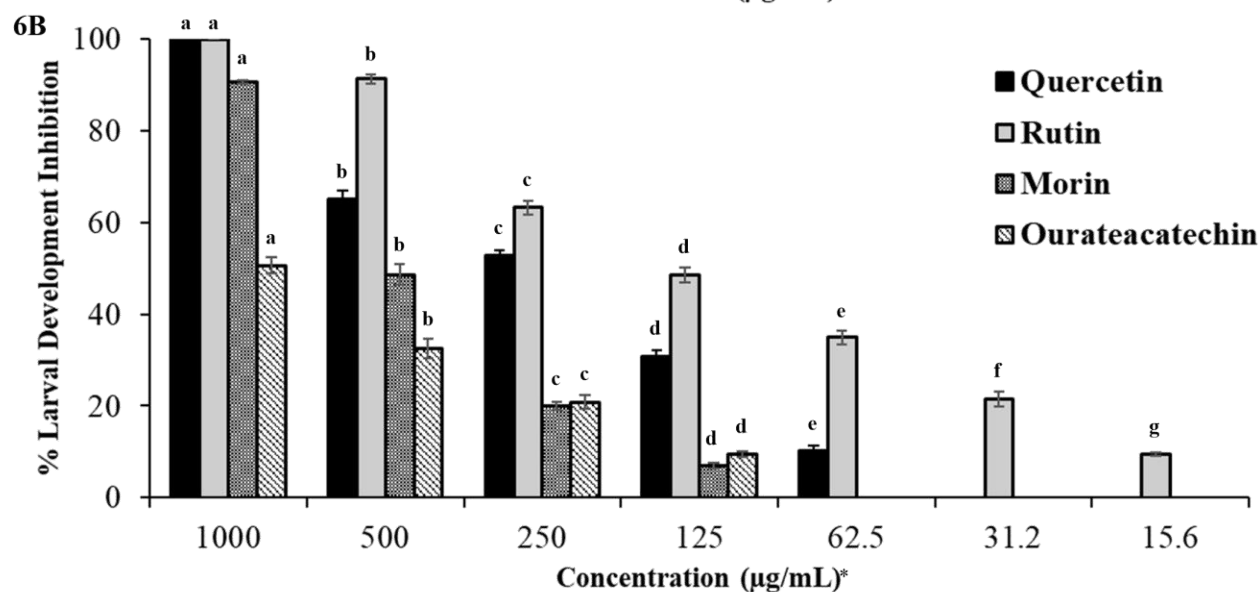
Among the flavones, the most effective compound was sorbifolin (EC_{50} = 18 µg/mL). The other two flavones tested were less effective (EC_{50} = 58 µg/mL for chrysin and EC_{50} = 83 µg/mL for pedalitin). While sorbifolin was significantly more effective than the other flavones, with 90% inhibition of larval development at 125 µg/mL ($p \leq 0.05$). Pedalitin and chrysin had the same response at 1000 µg/mL (Figure 6A).

Overall, the flavonols showed lower EC_{50} values comparing with the flavones. Rutin (EC_{50} = 104 µg/mL) presented a larvicidal activity twice more effective than quercetin (EC_{50} = 231 µg/mL) and four times more than morin (EC_{50} = 448 µg/mL). Morin showed 90% inhibition of larval development at 1000 µg/mL (Figure 6B) while quercetin and rutin caused 100% inhibition at the same concentration. Ouratecatechin appeared the less effective flavonoid with high EC_{50} (989 µg/mL). In the highest concentrations

(1000 µg/mL) tested this compound was able to inhibit 50% larval development (Figure 6B).

The phenolic acids showed similar larvicidal activity, which no statistical differences in EC_{50} values ($p \geq 0.05$). Ferulic acid presented EC_{50} value (22 µg/mL) below the caffeic acid (31 µg/mL) and gallic acid (33 µg/mL). When the concentration was 250 µg/mL, the proportion of larval developed was similar for all phenolic acids (99%, 100% and 95% for caffeic, ferulic and gallic, respectively). Figure 6C shows that the percentage of larval development inhibition decreased with the decreasing of the concentrations of phenolic acids. In the negative control wells, 95% of the larvae developed in L_3.





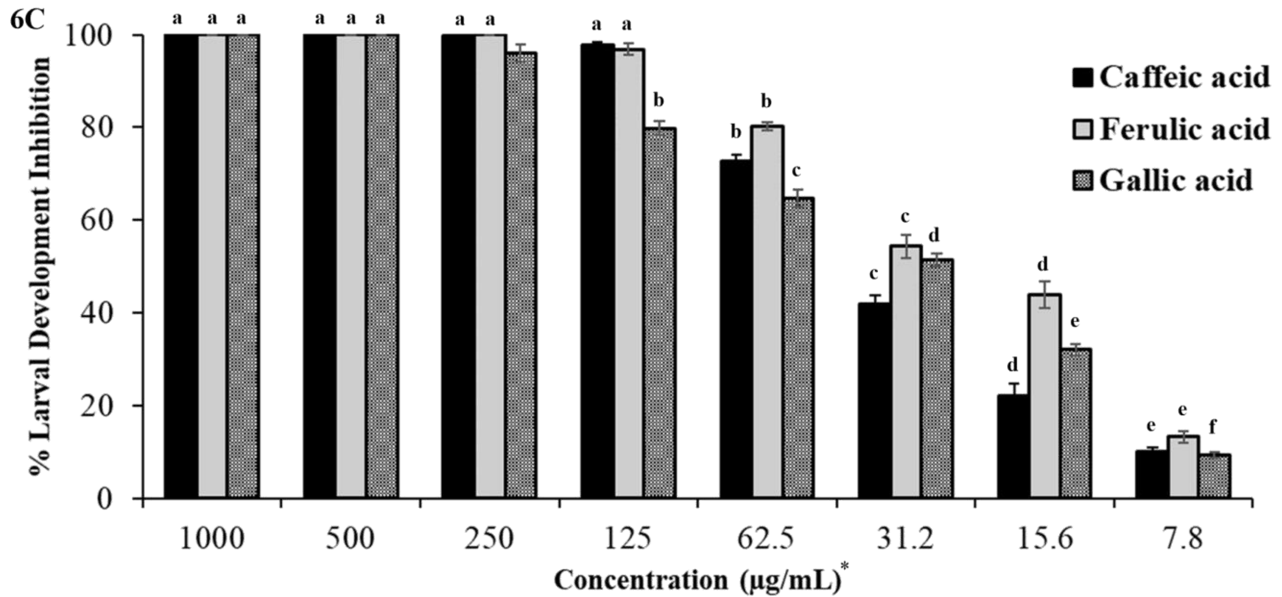


Figure 6. *In vitro* effect of (6A) flavones from *Pterogyne nitens* (sorbifolin and pedalitin) and commercial flavone (chrysin), (6B) flavonols (quercetin and rutin) and flavan-3-ol (ourateacatechin) from *Pterogyne nitens,* and commercial flavonol (morin), and (6C) phenolic acids from *Pterogyne nitens* (caffeic acid, ferulic acid, and gallic acid) on the larval development of *Haemonchus contortus*. *Different lower-case letters in the same column correspond to significant differences between treatments of the same solvent concentration by the Tukey test ($p \leq 0.05$).

4. Discussion

The results of this study show that extracts and phenolic compounds from *P. nitens* can disrupt the life cycle of *H. contortus* by preventing their eggs from hatching and by preventing L_1 larvae from developing to the infective L_3 stage. Although some legumes containing phenolic compounds, as condensed tannins have been shown potent inhibitory effects on some NGI species (Mueller-Harvey et al., 2019). This is the first demonstration that the *P. nitens* extracts and compounds are able to inhibit free-living stages of this nematode species.

Egg hatching is not so disturbed by the phenolic compounds as is larval development. This may be due to the duration of the assay. Eggs are exposed for only 24 h. In contrast, in the LDA the L_1 and L_2 larvae are exposed to test compounds for 7 days. Also, in the LDA the larvae are in the feeding stage of their life cycle so they may

ingest the compounds. Either extracts or phenolic compounds are able to achieve the complete inhibition of egg hatching and larval development (Molan et al., 2002).

It is important to note that only the benzimidazoles class can act in low concentrations as an ovicidal, inhibiting the embryogenesis of GIN eggs (Egerton, 1969). However, the nematode resistance problem to this anthelmintic group has already been reported (Coles and Simpkin, 1977). Therefore, it became necessary to identify anthelmintic alternative methods, such as the use of medicinal plants and their extracts and compounds.

In the EHA, the phenolic acids showed potent anthelmintic activity against the egg hatching. Probably their low molecular weight could be an important factor for the passive diffusion that the compounds are able to penetrate the nematode eggshell, which is one of the most resistant biological structures. It is impermeable to most substances, with the exception of gases and lipid solvents (Arthur and Sanborn, 1969). The penetration of compounds and the effect of temperature on the permeability of the eggshell suggests that the lipid layer provides the main permeability barrier (Wharton, 1980).

Escareño-Díaz et al. (2019) evaluated the *in vitro* anthelmintic activities of caffeic acid, coumarin, quercetin, rutin and their combination against egg hatching and L_3 larvae exsheathment of *Cooperia punctata*. They observed that quercetin and rutin did not demonstrate bioactivities on the eggs and larvae. However, when evaluated in combination with caffeic acid and coumarin they observed an effective synergic interaction against the egg hatching, which could suggest a linear correlation between a lower molecular weight and a higher ovicidal activity since the EC_{50} values were measurement higher to the smaller molecules (caffeic acid and coumarin).

The addition of a hydroxyl group on flavonol structures significantly increased the activity when compared with the flavones. There is a correlation between the number of hydroxy groups in the C-ring and inhibitory activity on egg hatching: sorbifolin, pedalitin, and chrysin, flavones, were less active than the quercetin, rutin and morin, flavonols. A crude ethanolic extract from the aerial parts of *Artemisia campestris* inhibited eggs hatching and caused death and paralysis in adults of *H. contortus*. The extracts contained predominantly derivatives of quercetin and apigenin (Makkar et al., 2007).

The same trend was observed to ourateacatechin, which has a hydroxyl group in its C-ring, however, it was statistically equal from PBS in the EHA at a concentration of 1000 µg/mL. Other investigations demonstrated that the tri-hydroxylation on B-ring of condensed tannins monomers, as catechin and flavan-3-ols, showed a higher effect comparing with non-substituted. Molan et al. (2003) observed this situation in egg hatching, larval development and larval migration of *T. colubriformis*. Brunet and Hoste (2006) in the infective larva (L_3) exsheathment process of *H. contortus* and *T. colubriformis.*

In the LDA, the ethanolic extracts were able to inhibit efficiently the larval development in different concentrations. Moreover, at 2500 µg/mL there are larval structure differences between the extracts and PBS, which presented small bubbles in their body under microscopy observation previously demonstrated in Figure 6.

Among the phenolic compounds, there are appears to be no structure-activity correlation, only concentration-dependence. Phenolic acids and sorbifolin exhibited complete inhibition of larval development at 500–1000 µg/mL and ourateacatechin inhibited less than 50% at the same concentrations. The flavonols and their glucose derivatives were less active than flavones in the LDA. In addition, Ayers et al. (2008) isolated methoxylated flavones from a *Struthiola argentea* methanolic extract, which were effective against larval development of *H. contortus*.

The larvicidal activity of flavonols was considered weak, comparing quercetin with its isomer morin, which on the other hand was less active against the larval development of *H. contortus*. This suggest that, the displacement of the hydroxyl group from position 3’ to 2’ (ring B) modified the anthelmintic activity. The replacement by a sugar subunit (rutinose) into the quercetin structure can improve twice the larvicidal activity of rutin.

Some authors had proposed that the interactions of the phenolic compounds with the surface proteins of larvae by hydrogen bonds and/or hydrophobic interactions (Hagerman et al., 1998; Poncet-Legrand et al., 2006) can explain its effects on the development process (Kahn and Diaz-Hernandez, 1999; Molan et al., 2003). Once they are able to complex with collagen of the nematode cuticle (Bravo, 1998; Jerónimo et al., 2016).

According to Echevarria et al. (1996) 95% of *H. contortus* populations are found in the pasture, and approximately 5% are found in the host. Thus, focus on building a blend of natural substances that are able to inhibit free-living stages is an interesting approach for an integrate control. However, effective concentrations, synergistic effects, enhanced bioavailability, cumulative effects, or simply the addictive properties of the constituents must be defined previously (Williamson, 2001). As well as consider absorption by the digestive tract and elimination in the feces (% in the feces in relation to the supplied orally). Also binding with other compounds, for example, condensate tannins bind to proteins and heavy metals, but are pH dependent bonds.

5. Conclusion

In conclusion, our results showed that ethanolic extracts and phenolic compounds from *P. nitens* present potent anthelmintic effects against egg hatching and larval development of *H. contortus*. The ovicidal and larvicidal activity of flavonoids can increase with substitutions into the chemical structure, as the addition of the hydroxyl group and the rutinose group, respectively. In addition, the phenolic acids showed potent anthelmintic activity, which suggests a promising alternative to control the free-living stages. Further studies will be needed to explore these interactions at the molecular level, as well as *in vivo* experiments should also be carried out to explore the therapeutic efficacy of *P. nitens* to control gastrointestinal nematodes in small ruminants.

Acknowledgments

This work was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES), Brazilian Council for Scientific and Technological Development (CNPq) (Grants 471129/2013-5, 306251/2016-7 and 429322/2018-6), and São Paulo Research Foundation (FAPESP) (Grants 2014/18330-0 and 2018/15083-2). C. S. L. thanks *CAPES (finance code 001)* for the scholarships and the Doctoral Program Sandwich Abroad at the Institut National de la Recherche Agronomique of Toulouse, France (Grant 88881.187940/2018-01).

Conflict of interest

The authors declare that there are no conflicts of interest.

Ethical approval

All procedures were approved by the Embrapa Pecuária Sudeste Ethics Committee on Animal Experimentation (process no. 04/2017), and are in accordance with national and international principles and guidelines for animal experimentation adopted by the Brazilian College of Experimentation (CONCEA).

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Capítulo Final

CONCLUSÕES GERAIS

A) Três extratos etanólicos de folhas, frutos e flores de *P. nitens*, foram preparados com rendimento variando de 0,25 a 12% em relação a matéria seca. Oito substâncias foram isoladas, duas flavonas (sorbifolina e pedalitina) foram isoladas do extrato etanólico de folhas (EEL), dois flavonóis (quercetina e rutina) purificados do extrato etanólico de frutos (EEFR); e um flavan-3-ol (ourateacatequina) e três ácidos fenólicos (ácido caféico, ácido ferúlico e ácido gálico) isolados do extrato etanólicos de flores (EEFL).

B) No teste *in vitro* de inibição de eclosão de ovos os valores de CI_{50} dos extratos e substâncias foram mais elevados, demonstrando que o ensaio pode ser menos sensível, ou a estrutura dos ovos de NGIs ser mais difícil de ser afetada. Os flavonóis foram os mais ativos contra ovos de *T. colubriformis* e ácidos fenólicos demonstraram potente atividade inibitória de eclosão de ovos de *H. contortus* originário do Brasil.

C) De modo geral, os extratos foram mais ativos contra o desenvolvimento larval de L_1 para L_3 de *T. colubriformis* quando comparado com as substâncias isoladas. Podendo sugerir um efeito sinérgico destes na composição dos diferentes extratos testados. Em contraste, para *H. contortus* os compostos foram mais ativos, em que a sorbifolina e os ácidos fenólicos demonstraram efeito larvicida potente.

D) Dentre os ensaios realizados, o ensaio de desembainhamento larval se mostrou o mais sensível para os extratos e substâncias. De modo geral, os extratos etanólicos demonstraram potente atividade AH *in vitro* contra isolados susceptíveis *T. colubriformis* e *H. contortus*. Em relação as substâncias, os flavonoides foram mais ativos contra ambas espécies de nematoides, sendo a quercetina o mais potente na inibição do desembainhamento de larvas infectantes L_3.

E) A comparação dos valores de CI_{50} dos flavonoides demonstrou que o número e a posição de grupos hidroxila na estrutura química altera a atividade AH contra ambas espécies de nematoides nos três ensaios biológicos.

F) Nosso estudo estabeleceu dados preliminares de relação estrutura-atividade, além de identificar possíveis substâncias naturais como promissoras candidatas a fármacos contra nematoides gastrintestinais de pequenos ruminantes.