



## Research paper

## *In vitro* anthelmintic activity of the crude hydroalcoholic extract of *Piper cubeba* fruits and isolated natural products against gastrointestinal nematodes in sheep



Matheus Souza de Paula Carlis<sup>b</sup>, Aline Féboli<sup>a</sup>, Antonio Carlos de Laurentiz<sup>b</sup>, Rosemeire da Silva Filardi<sup>b</sup>, Anna Helena Prizantelli de Oliveira<sup>c</sup>, Márcio Luis Andrade e Silva<sup>c</sup>, Luciano Alves dos Anjos<sup>b</sup>, Lizandra Guidi Magalhães<sup>c</sup>, Rosângela da Silva de Laurentiz<sup>a,\*</sup>

<sup>a</sup> Departamento de Física e Química, Faculdade de Engenharia de Ilha Solteira, Universidade Estadual Paulista, Av Brasil, 56, Centro, Ilha Solteira, CEP 15385-000, São Paulo, Brazil

<sup>b</sup> Departamento de Biologia e Zootecnia, Faculdade de Engenharia de Ilha Solteira, Universidade Estadual Paulista, Rua Monção, 226 - Zona Norte, Ilha Solteira, CEP 15385-000, São Paulo, Brazil

<sup>c</sup> Núcleo de Pesquisa em Ciências Exatas e Tecnológicas, Universidade de Franca, Av Sales de Oliveira 201, Parque Universitário, Franca, CEP 14404-600, São Paulo, Brazil

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## ABSTRACT

This study describes the *in vitro* anthelmintic activity of a hydroalcoholic extract from the fruit of *Piper cubeba* and its major isolated components against the eggs and larvae of gastrointestinal nematodes obtained from naturally-infected ovines. *In vitro* anthelmintic activity was evaluated using the egg hatch test (EHT), larval development test (LDT) and L3 migration inhibition test (LMT). The extract showed ovicidal and larvicidal activity, with an EC<sub>50</sub> of 200 µg/mL and 83.00 µg/mL in the EHT and LDT, respectively. The extract inhibited 100% of larval migration at the lowest tested concentration (95 µg/mL). The crude extract was purified using successive silica gel chromatographic columns, which revealed the lignans hinokinin, cubebin and dihydrocubebin as the major compounds that were present, which were then used in *in vitro* tests. Cubebin, dihydrocubebin and hinokinin showed higher activity than the crude extract, with an EC<sub>50</sub> for ovicidal activity of 150.00 µg/mL, 186.70 µg/mL and 68.38 µg/mL, respectively. In the LDT, cubebin presented an EC<sub>50</sub> of 14.89 µg/mL and dihydrocubebin of 30.75 µg/mL. Hinokinin inhibited 100% the larval development at all concentrations evaluated. In the LMT, dihydrocubebin inhibited 100% the larval migration in all concentrations evaluated while cubebin and hinokinin showed EC<sub>50</sub> values of 0.89 µg/mL and 0.34 µg/mL, respectively. *P. cubeba* extract is rich in several classes of active compounds, but here we demonstrate that the described anthelmintic activity may be related to the presence of these lignans, which are present in larger concentrations than other components of the extract. Our results demonstrate for first time the anthelmintic activity against gastrointestinal nematodes in sheep for this class of special metabolites that are present in *P. cubeba* fruit. However, future detailed studies are needed to evaluate the effectiveness of *P. cubeba* fruits extract and active lignans in *in vivo* tests.

## 1. Introduction

The administration of synthetic anthelmintics has for decades helped to control parasitic infections of gastrointestinal nematodes (GINs) in small ruminants. However, the indiscriminate use of synthetic anthelmintics without epidemiological criteria has selected resistant parasites, further aggravating the problem of parasitosis (Jabbar et al., 2006; Demessie et al., 2016). *Haemonchus contortus* is the most

prevalent and pathogenic nematode found in small ruminants in the tropics (Besier et al., 2016); it is the main cause of loss of sheep due to its hematophagous behaviour, causing severe anaemia in animals, which at high levels of infection causes death.

In recent decades, plants have been used as a promising source of target molecules for development of new drugs to treat cancer, pain and parasitic infections. Several research groups around the world have carried out studies on the use of plants and extracts for the treatment of

\* Corresponding author.

E-mail address: [rosangela@dfq.feis.unesp.br](mailto:rosangela@dfq.feis.unesp.br) (R. da Silva de Laurentiz).

gastrointestinal parasites in sheep. These studies have demonstrated the importance of this research area as an alternative in the control of the infection (Qi et al., 2015; Féboli et al., 2016; Katiki et al., 2017; Oliveira et al., 2017; Abidia et al., 2018). Studies examining plant extracts, plant essential oils and condensed tannins have been carried out to evaluate their *in vitro* anthelmintic activity against *H. contortus* (Katiki et al., 2013; Qi et al., 2015; Féboli et al., 2016; Katiki et al., 2017; Mengistu et al., 2015; Oliveira et al., 2017). The anthelmintic activity of these plants is generally associated with the presence of tannins (Katiki et al., 2017; Mengistu et al., 2015; Oliveira et al., 2017). Among these tannins, the main emphasis has been on condensed tannins due to their occurrence in forages used to feed sheep and their effect on the various life stages of nematodes (Alonso-Díaz et al., 2008; Chan-Pérez et al., 2016). However, some studies have also been conducted using non-tanniferous plants, such as *Cymbopogon citratus*, whose anthelmintic properties are related to essential oil components (Macedo et al., 2015), or plants used in ethnoveterinary practices such as *Cissus quadrangularis*, used in livestock against helminthiasis in Ethiopia, with flavonoids as the active compounds (Zenebe et al., 2017).

In addition to the plants described above, *Piper cubeba* is non-tanniferous plant of Asian origin whose fruit is used in Asian cuisine and Indian traditional medicine to relieve gastric pain, enteritis, diarrhoea and inflammation, and to treat acute jaundice (Chopra et al., 1956). *P. cubeba* fruit contains an oil that is rich in terpenes, with sabinene and eucalyptol being of the highest proportion (Magalhães et al., 2012). Moreover, *P. cubeba* fruit hydroalcoholic extract presents about 24 lignans with highly varied chemical structures (Elfahmi Ruslan et al., 2007). Despite the structural diversity of the components present in the *P. cubeba* extract, the biological activities of this extract are generally attributed to the lignans cubebin and hinokinin, which are the compounds at the highest concentrations (Lima et al., 2018).

The biological properties attributed to *P. cubeba* fruit extracts, both in terms of its essential oil and isolated compounds, are analgesic, anti-inflammatory (Silva et al., 2005), antimicrobial (Silva et al., 2007; Laurentiz et al., 2015), antioxidant (AlSaid et al., 2015) and antiparasitic (Magalhães et al., 2012; Esperandim et al., 2013a). Due to these properties and its low toxicity (Graidist et al., 2015), *P. cubeba* fruit appears to be a promising candidate for evaluation against gastrointestinal nematodes in sheep. Therefore, the aim of this study was to evaluate the *in vitro* anthelmintic activity of the hydroalcoholic extract and isolated compounds from *P. cubeba* fruit against gastrointestinal nematodes eggs and larvae obtained from the faeces of naturally infected sheep using the egg hatch test (EHT), larval development test (LDT) and L3 migration inhibition test (LMT).

## 2. Materials and methods

### 2.1. Plant materials and isolation of major compounds

The dried fruits of the *P. cubeba* were purchased from Floral Seed Company, Dehradun, India. The powdered *P. cubeba* fruits were exhaustively extracted by maceration for five days with 70% aqueous ethanol (500 g seed for 2 L ethanol). The extract was filtered and concentrated under vacuum to furnish the crude hydroalcoholic extract (PCE), which was fractionated by the partition between the phases of hexane and methanol/water (9:1) (Laurentiz et al., 2015). The crude methanol/water fraction (50 g) was submitted to silica gel column chromatography Bajpai et al. (2016). Elution with increasing proportions of hexane, hexane/ethyl acetate and ethyl acetate yielded 16 fractions (200 mL), of which six, with similar chromatographic profile, were pooled, concentrated under vacuum and again submitted to silica gel column chromatography using a gradient crescent of the mixture of hexane/ethyl acetate. This latter chromatography sequentially presented three compounds as major products. Compound 1 was eluted with hexane-EtOAc (9:1), compound 2 was eluted with hexane-EtOAc (7:3) and compound 3 was eluted with hexane-EtOAc (1:1). These

compounds were compared with known lignan standards by thin layer chromatography (TLC) and submitted for analysis by  $^1\text{H}$  and  $^{13}\text{C}$  Nuclear Magnetic Resonance (NMR). The PCE and the isolated compounds were kept frozen into use and after diluted in 0.5% DMSO to obtain the concentrations used in the *in vitro* tests.

### 2.2. NMR data

$^1\text{H}$  and  $^{13}\text{C}$  NMR analyses for structural determination of isolated compounds were performed using a Bruker ARX 500 spectrometer (Bruker-Germany). Samples for the analyses were prepared by dissolving 10 mg of each isolated compound in 0.5 mL of  $\text{CDCl}_3$ . Proton chemical shifts in  $\text{CDCl}_3$  are associated with the middle of the residual singlet ( $\delta = 7.28$  ppm). Carbon chemical shifts were reported in parts per million ( $\delta$ ) relative to  $\text{CDCl}_3$  (77 ppm), and J (coupling constant) values were reported in hertz. The splitting patterns of protons are described as s (singlet), brs (broad singlet), d (doublet), dd (doublet of doublets), t (triplet) and m (multiplet) (Silverstein et al., 2005).

### 2.3. Recovery and preparation of eggs

The faeces used in *in vitro* tests were obtained from sheep naturally infected with gastrointestinal nematodes, being 95% *H. contortus* and 5% *Trichostrongylus* sp, the nematode species were determined according to Wyk & Mayhew (2013). The faeces samples were obtained directly from the rectum of a donor animal. The eggs were recovered with the sequential use of sieves, according to the method described by Coles et al. (1992), with some modifications. The eggs retained in the last sieve were washed with distilled water, transferred to Falcon tubes (50 mL) and centrifuged at 2054 x g for 5 min. Then, the supernatant was removed and a saturated NaCl solution was added to resuspend the sedimented eggs. After centrifugation under the same conditions, the supernatant was transferred to 25  $\mu\text{m}$  sieves; the eggs were again washed with distilled water and transferred to another tube. The concentration of eggs in this tube was estimated by counting the number of eggs in 50  $\mu\text{L}$  aliquots (5 counts) using the McMaster slide technique and the suspension was diluted to achieve a concentration of 100 eggs/100  $\mu\text{L}$ .

### 2.4. Egg hatch test

The eggs suspension (100 eggs/100  $\mu\text{L}$ ), and 100  $\mu\text{L}$  of PCE at different concentrations (190–12000 g/mL) or lignans (75–1250  $\mu\text{g}/\text{mL}$ ) were incubated for 48 h at 28 °C in 24-well plate. The eggs and L1 were counted under an inverted microscope. Albendazole (12.5  $\mu\text{g}/\text{mL}$ ) was used as a positive control, while 0.5% DMSO was used as a negative control (Coles et al. (1992). Five replicates for the controls and for each concentration of the PCE and lignans were performed.

### 2.5. Larval development test

This test was carried out according to the method described by Lacey et al. (1995), with some modifications. The egg suspension (100  $\mu\text{L}/100$  eggs), 90  $\mu\text{L}$  of nutritive media (1 g of yeast in 90 mL of normal saline and 10 mL of Earle's balanced salt) and 10  $\mu\text{L}$  of amphotericin (Sigma-Aldrich) (25  $\mu\text{g}/\text{mL}$ ) were added to each well of a 24-well plate. The plates were incubated for 48 h under humidified conditions at 28 °C, after which 200  $\mu\text{L}$  of PCE or lignans at same concentrations described for the EHT were then added to each well. Ivermectin (10  $\mu\text{g}/\text{mL}$ ) and 0.5% DMSO were used as a positive and negative control, respectively. Five replicates were carried out for each concentration and for the controls. The plates were incubated for 5 days. The number of L1/L2 and L3 in each well were counted under an inverted microscope at 40x magnification.

## 2.6. Larval migration test

Infective L3 larvae were obtained by faeces coproculture and collected by sedimentation using Baermann's devices (Van Wyk and Mayhew, 2013). This material was washed three times with PBS and transferred to a Falcon tube (Eppendorf®) reservoir. The number of L3 in the reservoir was counted under an inverted microscope at 40x magnification through 10% aliquots (6-well plate, 6 counts) and then the suspension in the tube was diluted to reach a concentration of 500 L3 /mL. LMT was performed with live L3 stage larvae (1000/tube) which were added to Falcon (Eppendorf®) tubes containing either 2 mL of the negative control (PBS; pH 7.2), an anthelmintic control (levamisole at 1.25 mg/mL), PCE or lignans at concentrations as described for the EHT. After incubation for 3 h at 28 °C, the L3 in each tube were washed with PBS and centrifuged (2054 x g) three times. The tubes were capped with 25 µm steel mesh and placed on a Petri dish. After 3 h of incubation, the number of larvae that migrated through the mesh was counted under an inverted microscope at 40x magnification, based on aliquots of 10% (5 counts) (Rabel et al., 1994).

## 2.7. Scanning electron microscopy (SEM) processing of L3 stage larvae

The larvae (L3 stage) for SEM analysis were recovered from the negative control and lignans (150 µg/mL) treatments in the larval migration test (five L3 for treatment). The L3 were individually preserved in 2% glutaraldehyde solution in phosphate buffer (0.1 M, pH = 7.4) and refrigerated at 4 °C until analysis. The fixed larvae were washed in phosphate buffer (0.1 M, pH = 7.4) and dehydrated in a graded acetone series (15%, 30%, 50%, 70%, 95% and 100%). The dehydrated larvae were dried by critical point drying with Leica EM CPD300 (Leica-Germany) and coated with gold for 2 min at 10 Å min<sup>-1</sup>. Parasites were then observed with an EVO LS15 scanning electron microscope (ZEISS-Germany) at an accelerating voltage of 15 kV.

## 2.8. Statistical analysis

The PCE and lignans concentrations used in the statistical analysis were the initial concentrations divided in half due to the final dilution performed during the *in vitro* tests. Comparisons of mean percentages of hatching inhibition, larval development inhibition and larval migration inhibition at different concentrations with the controls were performed by one-way ANOVA followed by Tukey's test ( $p < 0.05$ ) using Sisvar version 5.6 program (Universidade Federal de Lavras, MG, Brazil). The results were expressed as means  $\pm$  S.E. EC<sub>50</sub> (the effective concentrations to inhibit 50% of larvae hatching, larval development and larval migration of L3), R<sup>2</sup>, hill slope (HS) and EC<sub>95</sub> (the effective concentrations to inhibit 95% of larvae hatching, larval development and larval migration of L3) were calculated using a nonlinear regression analysis with 95% confidence (log(agonist) vs. response-variable slope), using the Graphpad Prism version 8.0 (Graphpad software).

## 3. Results

### 3.1. Plant materials, compound isolation and NMR identification

After extraction with ethanol, 500 g of the powdered *P. cubeba* fruit furnished 75 g of PCE (15% yield). Sequential silica gel column chromatography of the crude methanol/water fraction from PCE (50 g) with a mixture of solvent in a crescent polarity (hexane /ethyl acetate, dichloromethane) produced three major compounds with mass of 1.04 g (compound 1), 1.15 g (compound 2) and 1.00 g (compound 3). Results of the NMR analysis (Table 1) confirmed the structures of compounds 1, 2 and 3 as those of hinokinin (HNK), cubebin (CB) and dihydrocubebin (DHC), respectively, (Fig. 1). The NMR data obtained for these compounds are in agreement with literature data (Silva et al., 2005; Laurentiz et al., 2015).

### 3.2. Egg hatch test

The percentages of egg hatch inhibition are presented in Table S1 and S2 (Supplementary material). PCE, in the highest evaluated concentrations, inhibited egg hatching by 100% (Table S1), showing ovicidal activity similar to the albendazole control (6.25 µg/mL); it had an EC<sub>50</sub> of 200.0 µg/mL with 95% confidence interval (95% CI) range from 190.0 to 210.0, R<sup>2</sup> = 0.99 and HS = 1.2. HNK was the most efficient lignan to inhibit egg hatchability, with EC<sub>50</sub> of 68.38 µg/mL (95% CI 58.00–85.70, R<sup>2</sup> = 0.98 and HS = 0.62). CB showed lower activity than HNK, with EC<sub>50</sub> = 150.0 µg/mL (95% CI 143.9–172.0, R<sup>2</sup> = 0.98, HS = 0.87), however the lignan less active was the DHC with EC<sub>50</sub> = 186.7 µg/mL (95% CI 173.2–201.2, R<sup>2</sup> = 0.98, HS = 0.90) (Table 2). The values of HS and R<sup>2</sup> (Table 2) indicate that the PCE had an effect dose-dependent higher than the lignans. EC<sub>95</sub> value for PCE was 2300 µg/mL (95% CI 2059–2570), for CB, DHC and HNK could not be determined because the evaluated concentrations did not reach inhibitions percentage (Table S2) values necessary for the accurate EC<sub>95</sub> calculations.

### 3.3. Larval development test

PCE inhibited the development of L1 to L3, even at the lowest evaluated concentration (Table S1), with an EC<sub>50</sub> of 83.00 µg/mL (95% CI 60.50–114.10), R<sup>2</sup> = 0.89 and HS = 0.54. CB showed EC<sub>50</sub> value of 14.89 µg/mL (95% CI 10.87–20.38), R<sup>2</sup> = 0.93 and HS = 3.03, while DHC with EC<sub>50</sub> of 30.75 µg/mL (95% CI 26.86–35.2), R<sup>2</sup> = 0.95 and HS = 1.25 (Table 2) was the lignan less active. EC<sub>50</sub> value for HNK could not be determined, because it exhibited 100% of activity at all evaluated concentration. DHC showing higher dose-dependent effect when compared to PCE (Table 2). Although HS for CB have been 3.03 this values cannot be used to evaluate dose-dependent effect, because only in the concentration of 35.00 µg/mL there was percentage of inhibition different of 100% (Table S2). Among the EC<sub>95</sub> values determined, CB showed EC<sub>95</sub> = 39.48 µg/mL (95% CI 37.48–41.59) while for DHC and PCE the EC<sub>95</sub> were 320.00 µg/mL (95% CI 233.2–437.3) and 20,300 µg/mL (95% CI 9,610–42,880), respectively, (Table 2). PCE was the least active with the highest confidence interval (95% CI). Overall, larvae were more sensitive to PCE and lignans than eggs and HNK was the most active (Table S2).

### 3.4. Larval migration test

PCE and lignans significantly inhibited larval migration ( $P < 0.05$ ). PCE showed 100% efficacy, in all concentrations, with activity similar to the positive control (Table S1). The EC<sub>50</sub> values for CB and HNK were respectively, 0.89 µg/mL (95% CI 0.55.60–1.43) and 0.34 µg/mL (95% CI 0.09–1.02). The R<sup>2</sup> values of 0.9 for CB and 0.84 for HNK together with HS values (0.19 and 0.20, respectively for CB and HNK) indicate a lower dose-dependent effect of these compounds on LMT than on EHT and LDT. The percentage of inhibition for PCE and DHC at the lowest concentration (35 µg/mL) did not reach values below 50% for obtaining of accurate EC<sub>50</sub> calculations, so their EC<sub>50</sub> could not be determined (Table 2). DHC showed EC<sub>95</sub> = 240 µg/mL (95% CI 145.0–384.4), for other lignans and PCE the EC<sub>95</sub> could not be determined, because the evaluated concentrations did not reach inhibitions percentage values necessary for the accurate EC<sub>95</sub> calculations (Table S1 and S2).

### 3.5. Larval migration test and scanning electron microscopy (SEM)

Structural changes induced in the L3 isolated from LMT performed with the lignans were assessed using SEM (Fig. 2A–D). The main changes observed between control (Fig. 2A) and treated L3 involved mainly the surface of the body (cuticle) (Fig. 2B–D). L3 cuticle was injured, with internal content exposure and loss of cylindrical shape mainly after treatment with HNK and DHC (Fig. 2C and D).

**Table 1**<sup>1</sup>H and <sup>13</sup>C NMR chemical shifts  $\delta$  (ppm), multiplicities and coupling constants  $J$  for hinokinin (1), cubebin (2) and dihydrocubebin (3) in CDCl<sub>3</sub>.

$\delta_H$ (multiplicity, H number and J)	$\delta_C$
Hinokinin (1) 6.8-6.4 (m, H arom), 5.9 (br.s, 4H), 4.15 (dd, 1H, J = 7.1 Hz and J = 9.3 Hz), 3.85 (dd, 1H, J = 7.1 Hz and J = 9.1 Hz), 3.0 (dd, 1H, J = 5.1 Hz and J = 14.2 Hz), 2.85 (dd, H, J = 7.3 Hz and J = 14.2 Hz), 2.6 (d, 2H, J = 7.1 Hz), 2.55 (m, 1H), 2.45 (d, 1H, J = 8.6 Hz), 2.4 (m, 1H).	178.4, 147.9, 147.8, 146.5, 146.4, 131.6, 131.3, 122.2, 121.55, 109.4, 108.8, 108.4, 108.3, 101.0, 71.2, 46.4, 41.3, 38.4, 34.8.
Cubebin (2) 6.8-6.5 m (6H arom), 6.6 (s, 4H), 5.2 (s, 1H), 4.1 (dd, 1H, J = 7.1 Hz and J = 14.4 Hz), 4.0 (dd, 1H, J = 7.1 Hz and J = 8.6 Hz), 3.8 (dd, 1H, J = 7.8 Hz and J = 8.4 Hz), 3.6 (dd, 1H, J = 7.1 Hz and J = 8.4 Hz), 2.7-2.2 (m, 4H), 2.0 (m, 1H).	148.0, 147.9, 143.6, 146.1, 134.9, 134.5, 122.1, 121.8, 109.7, 109.5, 108.6, 108.5, 101.3, 101.2, 99.2, 72.6, 53.5, 46.2, 39.6, 34.0.
Dihydrocubebin (3) 6.6 (m, 6H), 5.8 (s, 4H), 3.7 (dd, 2H, J = 1.3 Hz and J = 11.3 Hz), 3.4 (dd, 2H, J = 4.1 Hz and J = 11.3 Hz), 3.2 (s, 2H), 2.7 (d, 1H, J = 8.7 Hz), 2.65 (d, 1H, J = 8.7 Hz), 2.56 (d, 1H, J = 5.7 Hz), 2.51 (d, 1H, J = 5.7 Hz), 1.75 (m, 2H).	147.6, 145.7, 134.3, 121.9, 109.3, 108.1, 100.8, 60.2, 44.3, 35.9.

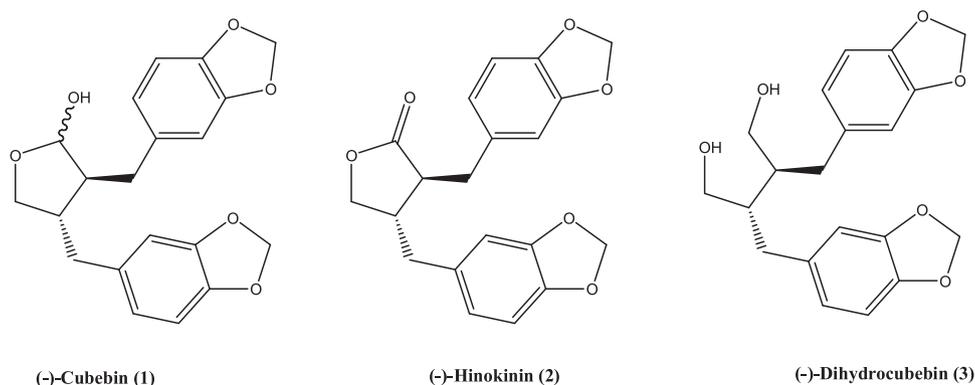
#### 4. Discussion

In the present study, we investigated the *in vitro* anthelmintic effects of PCE and its isolated compounds against gastrointestinal nematodes from sheep. The lignans CB, HNK and DHC were isolated from PCE as the major constituents. The *in vitro* evaluation showed that PCE presented activity against eggs and larvae of the parasite, at all evaluated concentrations. In addition, the lignans CB, HNK and DHC when evaluated separately showed significant *in vitro* anthelmintic activity, superior to that presented by the crude extract, mainly in relation to their ovicidal action.

Studies on anthelmintic activity of other plant of the *Piper* genus were described by Gaínza et al. (2016) which evaluated the effects of the essential oil of *P. aduncum*, against eggs and larvae of *H. contortus*. The authors obtained results lower than those described in our study, with ED<sub>50</sub> values of 5.72 mg/mL and 0.1 mg/mL, respectively, for the EHT and LDT. *P. aduncum* essential oil contains no lignans and anthelmintic activity has been attributed to its main constituent, dillapiole, which is a phenylpropanoic compound. In another study carried out by Carvalho et al. (2012), the methanol extract of *P. tuberculum* was evaluated against GINs and presented excellent ovicidal activity with an ED<sub>50</sub> of 0.031 mg/mL. However, this activity was attributed to a set of piperamide compounds and not the lignans. Other plants of the genus *Piper* have been reported to have anthelmintic properties against several types of parasites (Adate et al., 2012; Koorse et al., 2018; Paul et al., 2018). However, our study is the first to report the activity of *P. cubeba* on the eggs and larvae of gastrointestinal nematodes of sheep and to relate this activity to the presence of individual lignans in the compounds found in *P. cubeba*. There are no previous reports on the *in vitro* evaluation of lignans against this class of parasite. The promising activity we have presented for these lignans raises an interest in studying the mechanism by which they act in the different stages of the life cycle of parasite. It is clear that they are able to cross and damage the cuticle of eggs and larvae, preventing hatching, larval development and motility.

Although effective against all stages of the life cycle of the parasite that were evaluated, both PCE and lignans were most active against larvae. Differences in the structure of the egg membrane and the cuticle of larvae may interfere with the anthelmintic activity of the compounds evaluated and alter their mechanisms of action. The nematode cuticle is an extracellular protein complex with trace amounts of lipid and carbohydrate that can vary between developmental stages (Mansfield et al., 1992; Riou et al., 2005). These differences may have contributed to higher PCE and lignan efficiency in larvae than in eggs, however is not the only factor for be considerate. Just like in our result, Oliveira et al. (2017) also found results with higher values of EC<sub>50</sub> for EHT than for LDT in the *in vitro* anthelmintic evaluation of eight plant extracts from Brazilian savanna. However Araújo Filho et al. (2018) found higher values of EC<sub>50</sub> for LDT than EHT in the *in vitro* anthelmintic evaluation of the *Eucalyptus citriodora* essential oil. The results of these two studies demonstrate that the greater or lesser ovicidal or larvicidal activity of a compound (plant extract or essential oil) depends not only on the morphological differences between eggs or larvae, but also on their chemical nature.

Lignans caused serious lesions in the integument of larvae that led to death, as observed in SEM studies, especially in relation to treatment with HNK (Fig. 2C) and dihydrocubebin (Fig. 2D). SEM assesses the interaction of compounds with the helminth cuticle and has been used to demonstrate direct effects of compounds with potential anthelmintic effects (Martínez-Ortiz-de Montelhanó et al., 2013; Andre et al., 2016). The effect of the lignans on the cuticle of the larvae was different from that obtained by Engstrom et al. (2019) when evaluating the effect of tannins obtained from various plant sources. According to the authors, the damage to the cuticle of the larvae caused by tannins was small and isolated, while with lignans we observed that the lesions were over the entire body of the parasite. The loss of motility of the L3 after treatment with lignans occurred due to cuticle damage and cylindrical form alterations, and not due to paralysis as occurs with ivermectin (Laing et al., 2017). The cuticle provides the worms their shape. It is also involved in their motility and in the exchanges with the parasite



**Fig. 1.** Chemical Structures of cubebin (1), hinokinine (2) and dihydrocubebin (3).

**Table 2**

EC<sub>50</sub> (R<sup>2</sup>, Hill slope) and EC<sub>95</sub> with their respective 95% confidence intervals (95% CI) for inhibition of egg hatching (EHT), inhibition of larval development (LDT) and inhibition of larval migration (MLT) of sheep gastrointestinal nematodes of the lignans CB, DHC, and HNK extracted from *Piper cubeba* fruits and PCE.

	EHT				LDT				MLT			
	PCE	CB	DHC	HNK	PCE	CB	DHC	HNK	PCE	CB	DHC	HNK
EC <sub>50</sub> (µg/mL)	200.0	150.0	186.7	68.38	83.00	14.89	30.75	ND	ND	0.89	ND	0.34
95% CI	190.0–210.0	143.9–172.0	173.2–201.2	58.00–85.70	60.50–114.10	10.87–20.38	26.86–35.20	ND	ND	0.55.60–1.43	ND	0.09–1.02
R <sup>2</sup>	0.99	0.98	0.98	0.98	0.89	0.93	0.95	ND	ND	0.90	ND	0.84
Hill slope	1.20	0.87	0.90	0.62	0.54	3.03	1.25	ND	ND	0.19	ND	0.20
EC <sub>95</sub> (µg/mL)	2300	ND	ND	ND	20300	39.48	320.0	ND	ND	ND	240.0	ND
95% CI	2059–2570	ND	ND	ND	9610–42880	37.48–41.59	233.2–437.3	ND	ND	ND	145.0–384.4	ND

ND -not determined because the evaluated concentrations did not reach percentage of inhibitions values  $\leq 50\%$  for the EC<sub>50</sub> calculations and /or the did not reach inhibitions percentage values necessary for the accurate EC<sub>95</sub> calculations; PCE-hydroalcoholic extract of *P. cubeba* fruits; CB-Cubebin; DHC- Dihydrocubebin; HNK-Hinokinin.

environment, including metabolic exchanges with the local environment in the digestive tract of the host. The structural cuticle changes described in the current study might lead to possible impairments in the free movement of the nematodes. Preventing the penetration of mucosal abomasum needed to develop to the L4 stage (Lucius et al., 2017).

Our findings contribute to the search for molecules whose chemical structures can be used as targets for the development of new anthelmintics. Recent studies have indicated an increased resistance of GINs to the most commonly used anthelmintics (Kotze and Prichard, 2016), as well as to monepantel, which belongs to the class of amino-acetonitrile derivatives and has recently been commercialized (Lecová et al., 2014; Ramos et al., 2018). This aggravating factor increases the need for research into new alternatives for parasite control, such as the inclusion of active extracts like PCE in sheep feed, and the identification of new bioactive molecules for the development of novel anthelmintics. In addition, PCE and its lignans also have numerous biological properties that may be beneficial to animals, namely their anti-inflammatory, analgesic and antioxidant activity (Silva et al., 2005; Nahak and Sahu, 2011) and low toxicity. Several authors have evaluated the toxicity of PCE and lignans by different protocols. Perazzo et al. (2013) evaluated the toxicity of *P. cubeba* extract using male albino Wistar rats and found that it had an LD<sub>50</sub> = 2000 mg/kg (body weight), and this result was later confirmed by Mouid et al. (2016). Esperandim et al. (2013b) evaluated the trypanocidal potential of CB and HNK in *T. cruzi*-infected mice at a dose of 50 mg/kg (bw) and did not observe any toxicity, with the lignans-treated animals having a higher survival in relation to negative control. Rezende et al. (2016) evaluated the cytotoxicity of CB and HNK in LLCMK2 fibroblast cells using the MTT method and found that these lignans do not present

significant cytotoxicity. Therefore, these studies indicate that these lignans and PCE are safe for use at the concentrations evaluated here.

Therefore, research evaluating the anthelmintic activity PCE and its lignans should be pursued, particularly with the objectives of verifying whether the effectiveness of these compounds *in vitro* also occurs *in vivo* in GINs-infected animals, and evaluating the possible forms of administration and safe doses of these compounds for use in infected animals.

## 5. Conclusion

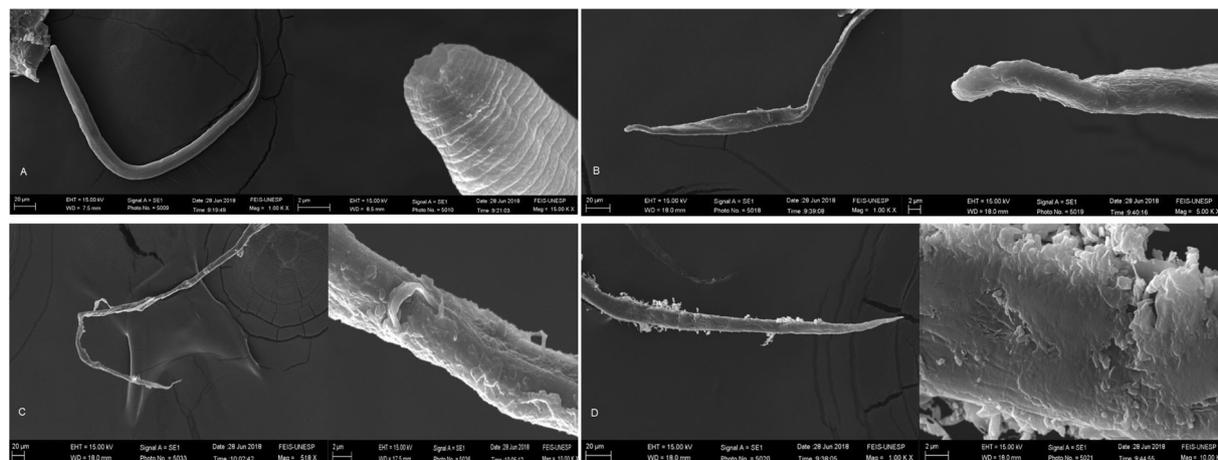
PCE showed promising anthelmintic activities against the eggs and larvae of GINs. The activity of *P. cubeba* against GINs can be supported by the anthelmintic activities of the lignans HNK, CB and DHC. Therefore, *P. cubeba* may be an alternative source of new anthelmintic agents to control gastrointestinal nematodes in sheep. However, future studies determining the toxicity and adequate doses of these compounds are necessary prior to *in vivo* evaluation with infected animals, as well molecular modelling studies using the structure of the active lignans as potential targets for development of new anthelmintic drugs.

## Declaration of Competing Interest

The authors declared that they have no conflicts of interest to this work.

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**Fig. 2.** SEM micrographs of L3 stage recuperated from the migration larval assays after 3 h of incubation at room temperature. A: with negative control (0.5% DMSO), B: cubebin at concentration of 150 µg/mL, C: hinokinin at concentration of 150 µg/mL, D: DHC at concentration of 150 µg/mL.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetpar.2019.108932>.

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