Piper aduncum against Haemonchus contortus isolates: cross resistance and the research of natural bioactive compounds

Piper aduncum contra isolados de Haemonchus contortus: resistência cruzada e a pesquisa de bioativos naturais

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Abstract

The anthelminthic activity of the essential oil (EO) of Piper aduncum L. was tested in vitro on eggs and larvae of resistant (Embrapa2010) and susceptible (McMaster) isolates of Haemonchus contortus. The EO was obtained by steam distillation and its components identified by chromatography. EO concentrations of 12.5 to 0.02 mg/mL were used in the egg hatch test (EHT) and concentrations of 3.12 to 0.01 mg/mL in the larval development test (LDT). Inhibition concentrations (IC) were determined by the SAS Probit procedure, and significant differences assessed by ANOVA followed by Tukey's test. In the EHT, the IC₅₀ for the susceptible isolate was 5.72 mg/mL. In the LDT, the IC₅₀ and IC₉₀ were, respectively, 0.10 mg/mL and 0.34 mg/mL for the susceptible isolate, and 0.22 mg/mL and 0.51 mg/mL for the resistant isolate. The EO (dillapiole 76.2%) was highly efficacious on phase L₁. Due to the higher ICs obtained for the resistant isolate, it was raised the hypothesis that dillapiole may have a mechanism of action that resembles those of other anthelmintic compounds. We further review and discuss studies, especially those conducted in Brazil, that quantified the major constituents of P. aduncum-derived EO.

Keywords: Isolates, Haemonchus contortus, cross resistance, dillapiole, synergy.

Resumo

Este estudo avaliou a atividade anti-helmíntica in vitro do óleo essencial (OE) de Piper aduncum L. sobre ovos e larvas de Haemonchus contortus, verificando se um isolado resistente (Embrapa2010), apresentaria o mesmo comportamento que um sensível (McMaster). O OE foi obtido por arraste a vapor e analisado por cromatografia para identificação dos constituintes. O óleo foi avaliado nas concentrações de 12,5 a 0,02 mg/mL no Teste de eclosão dos ovos (TEO) e nas concentrações de 3,12 a 0,01 mg/mL no Teste de desenvolvimento larvar (TDL). As concentrações inibitórias (CI) foram determinadas pelo procedimento Probit do SAS e as diferenças estatísticas geradas pela ANOVA seguida pelo teste de Tukey. Para o isolado sensível obteve-se CI₅₀ de 5,72 mg/mL no TEO. No TDL o óleo apresentou CI₅₀ e CI₉₀ de 0,10 mg/mL e 0,34 mg/mL para o isolado sensível e 0,22 mg/mL e 0,51 mg/mL para o resistente, respectivamente. Demonstrou-se que o OE (dilapiol 76,2%) teve alta eficácia sobre a fase L₁. Devido às elevadas CIs obtidas para o isolado resistente, levantou-se a hipótese de que o dilapiol talvez possua um mecanismo de ação que ressemble aos outros antihelminticos. O artigo faz uma revisão e discute estudos de quantificação dos constituintes majoritários do OE de P. aduncum, destacando os realizados no Brasil.

Palavras-chave: Isolados, Haemonchus contortus, resistência cruzada, dilapiol, sinergismo.

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Introduction

The nematode *Haemonchus contortus* parasitizes the gastrointestinal tract of ruminants, causing anemia, weight loss, and, in severe infections, death (URQUHART *et al.*, 1998). *Haemonchus* infections affect hundreds of millions of small ruminants worldwide causing estimated losses of US$ 10 billion per year (WALLER & CHANDRAWATHANI, 2005; ROEBER *et al.*, 2013). The use of synthetic anthelmintic substances constitutes the main course of action against gastrointestinal parasitism (FREEMAN *et al.*, 2003). However, resistance to currently available anthelmintic drugs drastically reduces the effects of parasite control programs (JAMES *et al.*, 2009; SUTHERLAND & LEATHWICK, 2011). Therefore, phytotherapeutic strategies have become the target of researchers aiming at developing environmentally-friendly tools for the control of nematodes in small ruminants.

Secondary metabolites probably mediate many of the biological activities of medicinal plants. These metabolites have become an alternative treatment, especially in organic farming where regulation forbids the routine use of synthetic anthelmintic drugs (CHAGAS, 2004; LISONBEE *et al.*, 2009). Among such compounds, essential oils (EOs) represent an important category. They can be obtained from hydro- or dry distillation of plant material (ISO, 2013), and consist of a mixture of volatile, lipophilic, often odoriferous and liquid substances. Interaction among these substances may interfere in nematode metabolism, inhibiting and disorganizing vital functions since the early stages of development (OKA *et al.*, 2000).

Some plant species, when processed, yield large amounts of EOs, especially those in the Apiaceae, Asteraceae, Cupressaceae, Hypericaceae, Clusiaceae, Lamiaceae, Lauraceae, Fabaceae, Liliaceae, Myrtaceae, Pinaceae, Piperaceae, Rosaceae, Rutaceae, Santalaceae, Zingiberaceae and Zygophyllaceae families (HUSNU *et al.*, 2007; FIGUEIREDO *et al.*, 2008). Specifically, Piperaceae family EOs have been tested in many different contexts worldwide (GUPTA, 1995; OVIEDO-RONDON *et al.*, 2006; SRINIVASAN, 2007; SCOTT *et al.*, 2008; GUERRINI *et al.*, 2009; SILVA *et al.*, 2009; SINGH *et al.*, 2009; PINO *et al.*, 2011; ARAUJO *et al.*, 2012). The biological activity of the *Piper* genus, native to tropical and subtropical regions (JARAMILLO & MANOS, 2012), has been more extensively studied. Within this genus, the spiked pepper *Piper aduncum* L. produces a biodegradable EO with high commercial value due to its fungicidal, bactericidal, molluscicidal, acaricidal, insecticidal and larvicidal activities (MAIA *et al.*, 1998). The species has a high tolerance to different weather conditions and can be cultivated in a wide range of areas (LORENZI & MATOS, 2002; SOUSA *et al.*, 2008). Finally, spiked pepper cultures may represent a crucial stepping stone for regional economic development based on a renewable source of chemical raw materials, which can be processed in large or small scales (FAZOLIN *et al.*, 2006).

The distinct biological activities attributed to the varying chemical composition of *P. aduncum* EO fuel studies on the potential veterinary applications of this species. Thus, the present work aims at assessing the *in vitro* anthelmintic activity of *P. aduncum* EO on eggs and larvae of resistant and susceptible isolates of *H. contortus*. Because these parasites had never been exposed to the plant extract, we assessed if the resistant isolate would present similar response that of susceptible isolate.

Materials and Methods

*P. aduncum* cultivation and EO extraction

*P. aduncum* plants were cultivated at Embrapa Amazônia Ocidental, located in the city of Manaus, state of Amazonas, Brazil, in 2014. The cultivation took place in field plots with soil classified as Dystrophic Yellow Latosol, which was fertilized with chicken manure (1.0 kg m$^{-2}$) added before cultivation. The field plots were located at the geographic coordinates 03º 06’ 23.04” S and 60º 01’ 35.14” W, at an average altitude of 50 m, average temperature of 25.6 °C, and average annual rainfall of 2,200 mm. The equatorial climate is characterized as Af, according to the world map of the Köppen-Geiger climate classification. Samples of *P. aduncum* were deposited in the EAFM herbarium of the Amazon Federal Institute under the number 10.480.

After harvest, in July 2014, leaves were separated from branches and shade-dried, in a barn. After drying for one week, leaves were taken to the laboratory where extraction was conducted by hydro-distillation in a modified Clevenger-type apparatus.

EO chromatographic analyses

Chemical composition analysis of the EO was conducted at Embrapa Agroindústria de Alimentos, Rio de Janeiro, Brazil, with gas chromatography coupled to mass spectrometry (GC-MS) in an Agilent 5973 N system (Agilent Technologies, DE, USA) equipped with a HP-5MS capillary column (5%-diphenyl, 95%-dimethyl-silicone, 30 m × 0.25 mm; film thickness 0.25 μm). Helium was used as the carrier gas (1.0 mL min$^{-1}$). A total volume of 1.0 μL of a 1% solution of the oil in dichloromethane was injected into an injector heated to 250 °C, operating in split mode (split ratio 1:100). Oven temperature was raised from 60 °C to 240 °C, at a rate of 3 °C min$^{-1}$. The mass detector was operated in electronic ionization mode (70 eV) with the mass analyzer maintained at 150 °C, the ionization source at 220 °C, and the transfer line at 260 °C. Linear retention indices were calculated by injection of a series of n-alkanes (C$_{10}$-C$_{20}$) in the same column and conditions as above. Identification of the essential oil components was done by comparison of both mass spectra and retention indices with the Wiley sixth edition spectral database and other data from the literature (ADAMS, 2007).

For quantification, the essential oil was analyzed in an Agilent 7890A chromatograph equipped with a flame ionization detector (FID) kept at 280 °C and fitted with an HP-5MS capillary column (5% diphenyl-95%-dimethyl-silicone; 30 m × 0.25 mm; film thickness 0.25 μm). The same injection and chromatographic conditions described above were applied, but hydrogen was used as the carrier gas at 1.5 mL min$^{-1}$. Results were expressed as relative area (percentage area).
**Donor animals and isolates of H. contortus**

Four Santa Inês animals were obtained from the ovine herd of Embrapa Pecuária Sudeste (CPPSE), São Carlos/SP. These animals had between three and four months of age, and weighed an average of 30 kg. They were initially treated with Zoalix® (monepantel, 2.5 mg/kg body weight) for the removal of natural parasitic infections. Seven and 14 days after deworming, we performed fecal counts of eggs per gram (EPG) to be sure the animals were clean of nematodes. Fecal material was collected directly from the rectum and eggs were enumerated using the McMaster x 50 technique (ROBERTS & O'SULLIVAN, 1950). Then, two animals were mono-specifically infected with 4000 L₁ of the isolate H. contortus McMaster and the other two with 4000 L₁, the isolate H. contortus Embrapa2010. Each pair of animals was placed in separate stalls, supplemented ad libitum with corn silage, water and mineral salt. All procedures involving donor animals were approved by the Committee on Animal Research and Ethics of the CPPSE (Protocol no. 06/2012).

**H. contortus** McMaster displays susceptibility to numerous chemical groups and has no history of exposure to anthelmintic agents (GILL et al., 1995). H. contortus Embrapa2010 was originally isolated from the CPPSE herd, and cryopreserved since 2006. The isolate was registered in the anthelmintic resistance consortium SNPs-parasite isolate database (CARS). It has displayed resistance to benzimidazole (20% efficacy), ivermectin (52%), moxidectin (85%), levamisole (93%) and high susceptibility to closantel (100%) and monepantel (100%) (CHAGAS et al., 2013).

**In vitro tests**

The P. aduncum EO was assessed in the egg hatch test (EHT) in 12 concentrations (0.02, 0.04, 0.09, 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 mg/mL) and in the larval development test (LDT) in seven concentrations (0.04, 0.09, 0.19, 0.39, 0.78, 1.56 and 3.12 mg/mL). To improve EO emulsification in water, Tween 80 at 2% was added in the EHT and DMSO at 0.5% in the LDT and the solutions were homogenized in a vortex mixer until oil, solvent and water formed a stable emulsion.

**Recovery of H. contortus eggs**

Fresh feces from the donor animals described above were obtained for the isolation of nematode eggs, according to the method described by COLES et al. (1992), and adapted by CHAGAS et al. (2011). In summary, approximately 5 g of feces, directly collected from the rectum, were mixed with tepid water (37 °C) and filtered through sieves with mesh sizes of 1 mm, 106 μm, 53 μm and 25 μm. The last and finer mesh retained the eggs. Recovered eggs were added to a saturated NaCl solution, centrifuged at 3000 rpm for 5 min, then floating eggs were collected using a 25 μm sieve and washed with distilled water. Eggs were separated and quantified.

**EHT**

One hundred eggs in 10 μL of distilled water were added to each well of a 24-well microplate. Then the EO, water (negative control 1), 2% Tween 80 (negative control 2) or thiabendazole (Sigma, T8904) at 60 μg/mL (positive control) was added to the wells. Each EO concentration and each control had six repetitions performed in three independent experiments, making 18 wells for each treatment or approximately 1800 eggs evaluated. Each line/treatment of 24-well plates was identified. Then the plate was sealed with PVC film and incubated at 27 °C and ≥80% relative humidity for 24 h. After this time, eggs and L₁ larvae were counted with an inverted microscope to calculate the egg hatching inhibition (CHAGAS et al., 2011).

**LDT**

As described in CHAGAS et al. (2011), 100 eggs were added to each well of a 24-well microplate with distilled water for a total volume of 250 μL in each well. The plates were incubated for 24 h at 27 °C and ≥80% relative humidity to obtain L₁ larvae. After this time, nutritive medium was added to each well according to Hubert & Kerboeuf (1992). All concentrations of essential oils, water (negative control 1), DMSO at 0.5% (negative control 2), and ivermectin (Sigma, I8898) at 10 μg/mL (positive control) were tested in six repetitions performed in three independent experiments (approximately 1,800 L₁ evaluated/treatment). The plates were incubated under the same conditions for six days, when each well was analyzed with an inverted microscope and all L₁ and undeveloped larvae were counted, to estimate larval development inhibition.

**Statistical analysis**

In the EHT and LDT, the percentage of inhibition or efficacy of each treatment was determined based on the arithmetic mean (X) of the egg hatching or larval development, according to the following equation (COLES et al., 1992):

\[
\text{Inhibition} \% = \left( 1 - \frac{X_{\text{Obs}}}{X_{\text{Exp}}} \right) \times 100
\]

where, \(X_{\text{Obs}}\) represents the number of unhatched eggs in the EHT, or the number of larvae that did not develop until L₁ in the LDT, and \(X_{\text{Exp}}\) corresponds to the total number of eggs + L₁ in the EHT, or the number of L₁ + L₂ + L₃ in the LDT.

The efficacy data were subjected to a variance analysis by the GLM procedure of SAS following a randomized experimental design with two isolates (resistant and susceptible) and 13 levels of concentration, describing a factorial 2 x 13, with 6 repetitions. We studied the effect of isolate, concentration and their interaction “ISOxCONC”. Then it was performed a Tukey’s multiple comparison test (SAS 9.0 for Windows) in order to detect significant statistical differences (p ≤ 0.01). The calculation of the inhibition concentrations (ICs) was performed by the SAS Probit procedure to estimate the IC₅₀ and IC₉₀.

**Results**

We obtained an average yield of P. aduncum EO of 3.6% (v/w). The EO chemical composition and retention times are indicated in Table 1. We identified a total of 36 substances, representing
99.1% of the oil extracted from the leaves. Dillapiole represented the major compound (76.2%), followed by \((E)\)-caryophyllene (3.8%) and myristicin (3.6%).

Results from the EHT indicated that the EO had better effect on the susceptible isolate. At the two lowest concentrations, the EO was not efficacious and results were statically similar to negative controls (2% tween 80 and distilled water). On the other hand, better effects were seen in the highest concentrations; at 0.39, 0.78, 1.56 and, 3.12 mg/mL results of egg inhibition were statistically similar. In the case of the resistant isolate Embrapa2010, the highest EO effect was seen in the concentration 12.5 mg/mL, which was statistically similar to the concentrations 6.25 and 3.12 mg/mL. Again, the EO results were statistically similar to negative controls at the two lowest concentrations. When the isolates were compared in the same concentration, all results were significantly different \((p \leq 0.01)\) (Table 2). The highest EO concentration (12.5 mg/mL) inhibited hatching by 55.80% of the susceptible eggs, and by 14.68% of the resistant eggs. The dose-response was linear for susceptible eggs, and the IC\(_{50}\) was calculated at 5.72 mg/mL (Table 3). Because of the relatively low effect of the EO on egg hatching, we could not define the ICs for the resistant isolate, or the IC\(_{50}\) for the susceptible one.

The percentage inhibitions in larval development from L\(_1\) to L\(_3\), mediated by the EO are displayed on Table 4. At the three highest concentrations, the EO was highly efficacious, inhibiting larval development of the two \(H. contortus\) isolates by more than 99%. There was no significant difference among concentrations from 0.37 to 3.12 mg/mL in the McMaster isolate. The same occurred among concentrations from 0.78 to 3.12 mg/mL to the Embrapa2010 isolate. LDT results were also significantly different.

| Compound              | LRI | % relative |
|-----------------------|-----|------------|
| α-pinene              | 931 | 0.5        |
| β-pinene              | 974 | 1.7        |
| myrcene               | 989 | 0.1        |
| α-phellandrene        | 1003| 0.2        |
| limonene              | 1025| 0.6        |
| (Z)-β-ocimene         | 1034| 1.0        |
| (E)-β-ocimene         | 1045| 2.2        |
| γ-terpinene           | 1054| 0.1        |
| linalool              | 1100| 0.2        |
| α-terpineol           | 1189| 0.1        |
| n.i.                  | 1210| 0.1        |
| ciclostricene         | 1359| 0.1        |
| α-copaene             | 1368| 0.2        |
| β-elemene             | 1385| 0.2        |
| α-gurjaneene          | 1401| 0.1        |
| (E)-caryophyllene     | 1412| 3.8        |
| β-copaene             | 1420| 0.1        |
| α-\(\alpha\)-trans-bergamotene | 1429| 0.1  |
| α-humulene            | 1445| 0.6        |
| allo-aromadendrene    | 1452| 0.2        |
| germacrene D          | 1472| 0.9        |
| ar-curcumene          | 1477| 0.1        |
| \(trans\)-4(14),5-muroladiene | 1482| 0.1  |
| \(\alpha\)-cubebol     | 1487| 0.6        |
| \(\alpha\)-murolene    | 1493| 0.4        |
| cubebol               | 1508| 0.4        |
| myristicin            | 1521| 3.6        |
| n.i.                  | 1535| 0.1        |
| germacrene B          | 1546| 0.2        |
| isoelemicene          | 1557| 0.3        |
| \((E)\)-nerolidol      | 1561| 0.9        |
| spathulenol           | 1571| 1.7        |
| viridiflorol          | 1584| 1.0        |
| ledol                 | 1593| 0.2        |
| humulene epoxide II   | 1599| 0.1        |
| n.i.                  | 1602| 0.2        |
| n.i.                  | 1614| 0.1        |
| dillapiole            | 1640| 76.2       |
| α-cadinol             | 1654| 0.4        |
| n.i.                  | 1667| 0.3        |
| apiole                | 1680| 0.3        |

Table 1. Composition of the essential oil of \(Piper aduncum\) L. assessed by GC/MS, retention time \((t_{k \text{ subst}})\) and linear retention index available in the literature (LRI).

| Concentrations | McMaster | Embrapa2010 |
|----------------|----------|-------------|
| 0.02           | 2.68 ± 0.31 | 1.10 ± 0.15 |
| 0.04           | 7.03 ± 0.35 | 2.53 ± 0.21 |
| 0.09           | 12.10 ± 0.44 | 4.63 ± 0.19 |
| 0.19           | 23.04 ± 0.85 | 6.58 ± 0.41 |
| 0.39           | 35.13 ± 1.03 | 8.34 ± 0.62 |
| 0.78           | 37.04 ± 0.80 | 9.57 ± 0.29 |
| 1.56           | 37.55 ± 1.05 | 11.32 ± 0.54 |
| 3.12           | 38.73 ± 0.66 | 12.85 ± 0.72 |
| 6.25           | 44.80 ± 2.25 | 13.88 ± 0.65 |
| 12.5           | 55.80 ± 2.63 | 14.68 ± 0.67 |
| 2% Tween 80    | 2.35 ± 1.08 | 2.47 ± 0.19 |
| Distilled water| 1.92 ± 1.08 | 1.67 ± 0.23 |

Table 2. Inhibition efficacy (mean percentage ± S.E.) of the essential oil extracted from \(Piper aduncum\) L. (mg/mL) on hatching of eggs from susceptible McMaster and resistant Embrapa2010 isolates of \(Haemonchus contortus\) in the egg hatch test (EHT). Also shown are controls containing water, Tween 80 and thiabendazole.

Different upper-case letters in the column (concentrations) and different lower-case letters in the line (isolates) indicate significant differences according to Tukey’s test \((p \leq 0.01)\).

Table 3. IC\(_{50}\), IC\(_{90}\) (mg/mL) and confidence intervals obtained in the egg hatch test (EHT) and larval development test (LDT) using essential oil extracted from \(Piper aduncum\) L. against susceptible McMaster and resistant Embrapa2010 isolates of \(Haemonchus contortus\).

Different upper-case letters in the column (concentrations) and different lower-case letters in the line (isolates) indicate significant differences according to Tukey’s test \((p \leq 0.01)\).
Table 4. Inhibition efficacy (mean percentage ± S.E.) of the essential oil extracted from *Piper aduncum* L. (mg/mL) on larval development of susceptible McMaster and resistant Embrapa2010 isolates of *Haemonchus contortus* in the larval development test (LDT). Also shown are controls containing water, DMSO and ivermectin.

| Concentrations      | McMaster | Embrapa2010 |
|---------------------|----------|-------------|
| 0.01                | 3.12 ± 0.33<sup>a,b</sup> | 0.47 ± 0.32<sup>a</sup> |
| 0.02                | 12.16 ± 0.32<sup>b</sup> | 1.91 ± 0.35<sup>a</sup> |
| 0.04                | 20.59 ± 0.69<sup>Ch</sup> | 6.00 ± 0.45<sup>Ba</sup> |
| 0.09                | 41.41 ± 0.52<sup>Ch</sup> | 13.11 ± 0.46<sup>Ca</sup> |
| 0.19                | 60.26 ± 0.97<sup>Ch</sup> | 24.37 ± 0.78<sup>Da</sup> |
| 0.37                | 99.37 ± 0.40<sup>Ch</sup> | 85.58 ± 1.09<sup>Es</sup> |
| 0.78                | 100 ± 0.00<sup>Fa</sup>  | 99.52 ± 0.32<sup>Es</sup> |
| 1.56                | 100 ± 0.00<sup>Fa</sup>  | 99.68 ± 0.20<sup>Es</sup> |
| 3.12                | 100 ± 0.00<sup>Fa</sup>  | 100 ± 0.00<sup>Es</sup> |
| 0.5% DMSO           | 2.33 ± 0.05<sup>a</sup>  | 2.90 ± 0.27<sup>Ab</sup> |
| Distilled water     | 1.86 ± 0.02<sup>a</sup>  | 2.28 ± 0.21<sup>Ab</sup> |
| Ivermectin          | 1.00 ± 0.00<sup>G</sup>  | 100 ± 0.00<sup>G</sup>  |

Different upper-case letters in the column (concentrations) and different lower-case letters in the line (isolates) indicate significant differences according to Tukey’s test (<i>p</i> ≤ 0.01).

between the two isolates (<i>p</i> ≤ 0.01) at every concentration except for the three highest ones. A dose-response relation was observed for both isolates (Table 4).

The IC<sub>50</sub> for both isolates calculated from the LDT are summarized in Table 3. The IC<sub>50</sub> for the susceptible isolate was 0.10 mg/mL, and it reached an approximately twofold higher value (0.22 mg/mL) for the resistant isolate.

### Discussion

An estimated 500 thousand plant species exist in the world, and 16% of them are in the Amazon forest in Brazil. The diversity of this country’s flora represents an enormous reservoir of mostly unexplored plant secondary compounds. Despite increasing research efforts to unveil new species and understand their roles over the past two decades, our knowledge remains scant (FAZOLIN et al., 2007). Nevertheless, different industrial sectors have increasingly sought Amazon plant compounds, including the agro-business sector.

Few reports discuss the potential of EOs derived from *P. aduncum* L. against nematodes that parasitize ruminants. In the present study, EO anti-hatching activity was only evident against susceptible eggs at the highest concentration (55.8% inhibition with 12.5 mg/mL), which was statistically different of the other concentrations on McMaster isolate, as well as at the same concentration (12.5 mg/mL) on the Embrapa2010 isolate. Other work using the EHT showed that the EO from *P. aduncum* L. was 95% efficacious at 12 mg/mL against *H. contortus* eggs (OLIVEIRA et al., 2014). We believe this divergent results stem from the chemical composition of EOs, which in the previous study primarily contained 1,8-cineole (55.8% against 0% dillapiole), whereas in the present study 76.2% of the oil was composed of dillapiole with no 1,8-cineole. Qualitative and quantitative differences in plant composition result from several factors including genetic background and plant variety (BARBOSA et al., 2007; HABER, 2008), site of cultivation (HABER, 2008; FURLAN et al., 2010), climatic and agricultural conditions (MARCO et al., 2007), harvest season and timing (BLANK et al., 2007; FURLAN et al., 2010), as well as plant developmental stage (LAHLOU & BERRADA, 2003; LEAL et al., 2003). The extraction method (e.g., super-citric fluid, steam distillation, solvent) also affects oil composition (QUISPE-CONDORI et al., 2008), much like drying (BLANK et al., 2007; BARBOSA et al., 2008) and storage conditions (GUIMARÃES et al., 2008).

In contrast to the mild effects on egg hatching, the EO significantly inhibited larval development of both isolates in a dose-dependent manner. Eggs in their hard and resistant shell usually resist chemicals to a larger extent than L. larvae (KATIKI et al., 2011). At the four highest concentrations the EO was more than 99% efficacious in inhibiting larval development of the susceptible isolate and, at three highest concentrations of the resistant isolate. For both isolates the results were statistically similar at those concentrations. When isolates were compared at the same concentration, the larval development inhibition was statistically similar at 0.78, 1.56 and 3.12 mg/mL. The IC<sub>50</sub> represented low EO doses for both isolates, but the dose required to kill 50% of the resistant larvae was twofold greater than required for the susceptible ones. This in vitro effect observed with the LDT may be related to the arylpropanoid dillapiole, to which many of the pharmacological activities of the EO extracted from *P. aduncum* L. have been attributed (MAIA et al., 1998; POHLIT et al., 2006). In fact, dillapiole has been shown to have acaricidal (SILVA et al., 2009; PINO et al., 2011; ARAÚJO et al., 2012), anti-bacterial (LARA-JÚNIOR et al., 2012; BRAZÃO et al., 2014), anti-fungal (LARA-JÚNIOR et al., 2012; GUERRINI et al., 2009), leishmanicidal (PARISE-FILHO et al., 2012), anti-inflammatory (PARISE-FILHO et al., 2011) and insecticidal activities (BELZILE et al., 2000; SOUTO, 2006; LING et al., 2009). When 99% pure, this compound has been evaluated and identified as the mediator of many of the biological activities above (ALMEIDA, 2004). Dillapiole may also associate with other minor bioactive compounds in the EO potentiating their action. For example, myristicin, present at 3.6% in the tested EO, sarisan (BIZZO et al., 2001) or safrole (HUANG et al., 1999), all of which have a methylenedioxyphenyl group in their structure are natural insecticides that could be the target of such association. Brazão et al. (2014), when assessing the EO extracted from *P. aduncum* against susceptible and resistant isolates of *Staphylococcus* spp., also showed the combined bactericidal action of dillapiole and myristicin (1 to 2%), lending further support to our conclusions.

Previous work revealed dillapiole as the major component of the EO of different varieties of *P. aduncum* collected in the Amazon, such as *P. aduncum* L. (MAIA et al., 1998; ALMEIDA et al., 2009), *P. aduncum* var. aduncum and *P. aduncum* var. cordulatum (GOTTTLIEB et al., 1981). However, individuals from this same species collected in other regions have other major constituents, including 1,8-cineole (LARA-JÚNIOR et al., 2012; OLIVEIRA et al., 2013), (E)-nerolidol (MESQUITA et al., 2005; OLIVEIRA et al., 2006) and nerolidol (NAVKIENNE et al., 2006), asaricine (FERREIRA, 2011; POTZERNHEIM et al., 2012) and piperitone
Table 5. Major chemical compounds making up the essential oils from *Piper aduncum* L. leaves, as reported by different authors.

| Chemical compound | Relative abundance (%) | Origin | Reference |
|-------------------|-----------------------|--------|-----------|
| β-caryophyllene    | 17.4                  | Panama City/Panama | Vila et al. (2005) |
|                   | 86.9                  | Amazonas/Brazil | Almeida et al. (2009) |
|                   | 45.9                  | Morona/Ecuador | Guerrini et al. (2009) |
|                   | 43.3                  | Morobe/Papua New Guinea | Rali et al. (2007) |
|                   | 82.2                  | Pinar do Río/Cuba | Pino et al. (2004) |
|                   | 31.5                  | Amapa/Brazil | Maia et al. (1998) |
|                   | 50.8-86.9             | Para/Brazil |                     |
| Dillapiole         | 91.1-97.3             | Amazonas/Brazil |                     |
|                   | 88.1                  | Acre/Brazil |                     |
|                   | 64.5                  | Kuala Lumpur/Malaysia | Jantan et al. (1994) |
|                   | 90.0                  | Panama | Gupta et al. (1983) |
|                   | 58.0                  | Fiji | Smith & Kassim (1979) |
|                   | 61.8                  | Cartago y San José/ Costa rica | Ciccio & Ballestero (1997) |
|                   | 74.5-88.4             | Amazonas/Brazil | Gottlieb et al. (1981) |
|                   | 49.5                  | Brasilia/Brazil | Potzernheim et al. (2012) |
|                   | 40.5                  | Cochabamba/Bolivia | Vila et al. (2005) |
|                   | 42.0-42.5             | Cochabamba/Bolivia | Arze et al. (2008) |
| 1,8-cineole        | 48.0-53.9             | Minas Gerais/Brazil | Lara-Júnior et al. (2012) |
|                   | 23.3-37.2             | Minas Gerais/Brazil | Oliveira et al. (2013) |
| (E)-nerolidol      | 79.2-82.5             | Pernambuco/Brazil | Oliveira et al. (2006) |
|                   | 14.2                  | Minas Gerais/Brazil | Mesquita et al. (2005) |
| Nerolidol          | 10.4                  | São Paulo/Brazil | Navickiene et al. (2006) |
|                   | 10.5                  | Cochabamba/Bolivia | Arze et al. (2008) |
| Asaricin           | 49.0                  | São Paulo/Brazil | Ferreira (2011) |
|                   | 15.8                  | Brasilia/Brazil | Potzernheim et al. (2012) |
| Piperitone         | 22.7-24.9             | Brasilia/Brazil | Potzernheim et al. (2012) |

(POTZERNHEIM et al., 2012). Table 5 summarizes the major components found in leaf EOs from *P. aduncum* L., highlighting the results of studies conducted in Brazil. The Piperaceae taxonomy is very complex (KATO & FURLAN, 2007). For different reasons, *P. aduncum* L. was botanically described in the Neotropical region by several authors in distinct times and places. In Brazil, there also were diverging points of view as to its taxonomic identity, and two varieties or subspecies were described by Gottlieb et al. (1981): *P. aduncum var. aduncum* and *P. aduncum var. cordulatum*. Recently, a single nomenclature – *P. aduncum var. aduncum* – was adopted (GUIMARÃES et al., 2015).

The chemical variation observed in the EO of *P. aduncum* L. may result from the predominance of different biosynthetic pathways in two chemotypes (ALMEIDA et al. 2009). In the Amazon forest the shikimate pathway took precedence, producing the phenylpropanoid dillapiole, whereas in the Atlantic forest chemotype, the mevalonate/acetate pathway was favored, generating the terpenoids (E)-nerolidol and linalool. These authors showed the occurrence of both chemotypes in both ecosystems, with their individual predominance depending on environmental factors. In addition, Andrade et al. (2009) conducted a hierarchical analysis based on EO chemical composition and clustered 21 *P. aduncum* specimens into four groups, from A to D. The EO examined in the present study fits the A chemical profile of the Amazon *P. aduncum* variety. This complex taxonomy and classification system indicates that differences in results may well stem from misclassifications at the subspecies level. Moreover, in face of such complexity, the phytochemical study of each oil’s efficacy against parasites becomes of paramount importance. Every pharmaceutic and veterinary product must be chemically qualified, i.e., the EOs must have known compositions. Bioactive markers must be established and, for any given use, the EOs must have these markers within the appropriate range to elicit the expected effects. Thus, we propose that the stages of taxonomic identification, extraction and phytochemistry should be clearly developed for *P. aduncum* to further define its anthelminthic activity, as shown in Figure 1.

The action of *P. aduncum* extracts on *H. contortus* may be explained at the molecular level as an effect of the methylenedioxyphenyl group present in dillapiole (Figure 2) and in other substances of the Piperaceae-derived EOs (FAZOLIN et al., 2007). This group...
Figure 1. Required steps in the research of essential oils derived from *Piper aduncum* L. as anthelmintic agent against *Haemonchus contortus*.

Figure 2. Major aryl-propanoids present in the EO of *Piper aduncum*, highlighting the presence of a methylenedioxyphenyl group and its structural analogy to piperonyl butoxide.
represents an important inhibitor of cytochrome P450-dependent monoxygenases (MUKERJEE et al., 1979; BERNARD et al., 1990; FAZOLIN et al., 2014). Monoxygenase inhibition reduces the detoxification capacity of insects, which are then poisoned by plant compounds that would otherwise be gradually eliminated (BERNARD et al., 1995). According to Kotze et al. (2006), piperonyl butoxide, which is related to dillapiole, acts synergistically with insecticides by inhibiting cytochrome P450-mediated metabolism of the insecticide. In the same study, the anti-helminthic properties of rotenone and its activity in combination with piperonyl butoxide, were examined in vitro. Rotenone was toxic to larvae of *H. contortus* and *Trichastrengus calabriformis*, with IC₅₀ values in the LDT of 0.54 and 0.64 μg/mL, respectively. The compound also caused complete cessation of movement in adult *H. contortus* at 20 μg/mL. Rotenone toxicity towards larvae and adults increased in the presence of piperonyl butoxide. Thus, the significant synergism observed suggests that these two nematode species use a cytochrome P450 pathway to detoxify rotenone, and indicates that a role may exist for cytochrome P450 inhibitors, such as piperonyl butoxide, to potentiate the action of anthelmintic substances that undergo oxidative metabolism within the nematode (KOTZE et al., 2006). The role of oxidative pathways in the metabolism of benzimidazoles in susceptible and resistant nematodes is unknown. Thus, the ability of a synergist to increase the toxicity of this chemical group towards both susceptible and resistant strains remains to be determined (KOTZE et al., 2006). Resistance to macrocyclic lactones does not involve enhanced oxidative metabolism and will likely require other types of assessments to have its mechanism determined (KOTZE, 2000).

In conclusion, the EO was highly efficacious against *L.*, but not against egg hatching. The action of this plant species against *H. contortus* is likely associated with dillapiole, the major EO component. Thus, the results obtained in the present study suggest that the EO derived from *P. aduncum* causes nematode toxicity via dillapiole-mediated cytochrome P450 inhibition in both isolates of *H. contortus*. We started from the premise that this EO, specifically dillapiole, could provide a natural source of anthelmintic agents. However, the differences observed in IC₅₀ indicate that the development of anti-parasitic drugs based on dillapiole, or other chemicals containing the methylenedioxyphenyl group, may be of little value. While the results indicate there may be a relationship between resistance status to commercially available chemicals and the plant-derived compound described in the present study, further research should expand the number of resistant and susceptible populations assessed in the bioassay.

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