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Original article

Chemical composition and efficacy in the egg-hatching inhibition of essential oil of *Piper aduncum* against *Haemonchus contortus* from sheep

Gisele Lopes Oliveira[a], Thallyta Maria Vieira[b], Vanessa Francisca Nunes[c], Munic de Oliveria Ruas[c], Eduardo Robson Duarte[d], Davyson de Lima Moreira[e,], Maria Auxiliadora Coelho Kaplan[f], Ernane Ronie Martins[d]*

[a]*Programa de Pós-graduação em Biotecnologia Vegetal, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*
[b]*Programa de Pós-graduação em Ciências Agrárias, Universidade Federal de Minas Gerais, Montes Claros, MG, Brazil*
[c]*Ciências Biológicas, Faculdade de Saúde Ibituruna, Montes Claros, MG, Brazil*
[d]*Instituto de Ciências Agrárias, Universidade Federal de Minas Gerais, Montes Claros, MG, Brazil*
[e]*Departamento de Produtos Naturais, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil*
[f]*Núcleo de Pesquisa de Produtos Naturais, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*

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A B S T R A C T

Piper aduncum L., Piperaceae, has been used to treat mainly inflammatory diseases and has shown several biological activities such as insecticidal and larvicidal. The aim of this study was to analyze the chemical composition of essential oil of *P. aduncum* and its efficacy to egg-hatching inhibition of *Haemonchus contortus* from sheep. The essential oil was obtained from leaves and analysed by gas chromatography coupled to flame ionization detector and gas chromatography coupled to mass spectrometry. It was possible to characterize 22 different substances, among them *monoterpenes* (80.6%) and *sesquiterpenes* (13.9%). The major compound was identified as 1,8-cineole (55.8%). Eggs of the nematode were exposed to four concentrations of the essential oil. Levamisole phosphate was used as positive control. The essential oil showed to be effective in inhibiting *H. contortus* hatchability and the LC_{90} was calculated as 8.9 $mg.ml^{-1}$. These results can point out the *P. aduncum* essential oil and its chemical components as potential alternative to control of *H. contortus*.

Introduction

Essential oils have shown interesting biological activity such as antimicrobial, cytostatic, insecticidal, larvicidal and anthelmintic (Franzios et al., 1997; Bakkali et al., 2008; Nerio et al., 2010; Lara Junior et al., 2012; Oliveira et al., 2013a).

Considering the different plant families showing essential oils, special interest in Piperaceae, which has the one of its great features the presence of structures that produce essential oils especially in inflorescences and leaves (Pessini et al., 2003). The family Piperaceae is distributed throughout tropical regions around the world, with the *Piper* being the most

* *Corresponding author.*
E-mail: dmoreira@far.fiocruz.br (D.L. Moreira).

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representative genus (Yuncker, 1972; Souza and Lorenzi, 2005). *Piper aduncum* L., Piperaceae, stands especially by showing biological activity associated with its extracts and essential oil such as insecticide, larvicide, antimicrobial, molluscicidal and leishmanicidal (Orjala et al., 1994; Bernard et al., 1995; Moreira et al., 1998; Torres-Santos et al., 1999; Souto, 2006; Sousa et al., 2008; Lara Junior et al., 2012). This specie is popularly known as pepper jack or false jaborandi and it is considered an opportunistic plant that invades deforested areas, and features rusticity with high resilience to climate change (Lorenzi and Matos, 2002; Sousa et al., 2008). The biological activities attributed to *P. aduncum* and the great chemical diversity of its essential oil lead us to investigate this plant for veterinary applications.

Several helminths infect herds of any age and gender and are a major obstacle for its development (Silva, 2004; Mali and Mehta, 2008). The gastrointestinal nematode *Haemonchus contortus* is the most pathogenic and is responsible for over 80% of the parasite load in small ruminants, causing loss of weight and appetite, significant decrease in the production of milk and meat, submandibular edema, ascites, hypoproteinemia, hipoalbunemia, severe gastritis bleeding, severe anemia and death (Urquhart et al., 1990; Arosemena et al., 1999; Melo, 2005). The treatment of nematode parasites in sheep is most often carried out exclusively with synthetic antihelminthics. This practice has promoted the rapid selection of multiresistant populations of parasites to commercial antihelminthics (Melo and Bevilaqua, 2002; Furtado, 2006).

Thus, the purpose of this study was to the evaluate chemical composition of *P. aduncum* essential oil and its efficacy to egg-hatching inhibition of *H. contortus* from sheep.

Materials and methods

Plant materials, essential oil extraction and analysis

Leaves of *Piper aduncum* L., Piperaceae, were collected in May 2012 in the Gallery Forest of the Angico River, in the rural region of Bocaiúva site, Minas Gerais State, Brazil (S 16° 57,582', W 43° 51,912'). A voucher specimen was deposited at the Herbarium of the Botanical Garden of Rio de Janeiro (RB 501.330). The essential oil was extracted from fresh leaves (150 g) by hydrodistillation in a modified Clevenger-type apparatus, yielding 0.9% (p/v).

The samples were subjected to analysis by gas chromatography coupled to flame ionization detector (HP-Agilent 6890 GC-FID) and by gas chromatography coupled to mass spectrometry (HP Agilent GC 6890 – MS 5973), in the Analytical Platform of Farmanguinhos, Fiocruz, Rio de Janeiro. Initially, the essential oils were diluted in dichloromethane (1 mg.ml^{-1}) and analyzed by GC-MS to obtain the mass spectra and to performer chemical characterization. Concomitantly, the diluted samples of essential oils (0.5 mg.ml^{-1}) were analyzed by GC-FID for quantification of chemical constituents and to determine the retention indices (RI). Each essential oil component was quantified based only in the individual component's relative peak area in the chromatogram. The substances in the essential oil were identified by comparing their mass spectra with database registration (WILEY7n) and by comparison of RI calculated those records from literature (Adams, 2007). RI were calculated using GC data of a homologous series of saturated aliphatic hydrocarbons within C8 to C20 (Sigma-Aldrich), performed at the same column and the conditions used in the GC analysis for the essential oils, and using the equation proposed by van Den Dool and Kratz (1963).

GC-FID parameters: HP-5ms column (30 m × 0.32 mm × 0.25 µm), temperature programming from 60 to 240°C, with increase of 3°C.min^{-1}, using the hydrogen and synthetic air to the carrier gas, with a flow rate of 1 ml.min^{-1} and injection volume of 1 µl.

GC-MS parameters: HP-5ms column (30 m × 0.32 mm × 0.25 µm), temperature programming from 60 to 240°C, with increase of 3°C.min^{-1}, using helium as the carrier gas to the, with a flow rate of 1 ml.min^{-1} and injection volume of 1 µl.

Egg-hatching inhibition

The nematode eggs were obtained from the feces of three castrated male sheep Santa Inés infected with *Haemonchus contortus* only that were raised in pens at the Experimental Farm of the Institute of Agricultural Sciences of the Federal University of Minas Gerais. The average fecal egg count of > 3,000 g^{-1} and the infection was determined using the modified McMaster technique (Gordon and Whitlock, 1939). For the recovery of infective larvae it was used the technique of coproculture in accordance with Ueno and Gonçalves (1998) and larvae of *H. contortus* were confirmed according to the key of Keith (1953). Experimental procedures were carried out in accordance with Experience the Animal Ethical Committee of Minas Gerais Federal University and approved by this committee, under protocol number 042/2008. About 100 g of feces were macerated, homogenized, filtered and washed in sieves with meshes of 106, 53 and 20 mm. The eggs were suspended in supersaturated aqueous sodium chloride solution and subsequent centrifugation according to Bizimenyera et al. (2006). It was standardized at the final concentration of 100 eggs in 240 µl of sterile distilled water. Assess to the activity of the essential oil of *P. aducum* in egg-hatching inhibition (EHI) tests were done with five replicates (Coles et al., 1992). The essential oil was diluted in 1% of tween 80 in sterile distilled water solution. In test tubes it was added 240 µl of the solution with approximately 100 eggs and added equal volume of essential oil solution at the end concentrations of 50.0, 12.0, 6.0 and 3.6 mg.ml^{-1}, respectively. Solution of levamisole phosphate (15 mg.ml^{-1}) was used as control positive and pure distilled water and 1% tween 80 solutions were used to the negative controls. The cultures were kept at 28°C in biological oxygen demand chamber for 48 h. Subsequently, unembryonated eggs, embryonated eggs, and first-stage larvae (L1) were counted according Coles et al. (1992).

The counting of eggs and L1 was transformed into relative values for the initial number of eggs for replicate. The results were submitted to variance analysis and compared in media tests ($p \leq 0.05$) of the statistical package SAEG 9.1 (2007). Probit regression was employed to analyze the data using this statistical package to determine the concentrations sufficient to inhibit 50% (LC_{50}) and 90% (LC_{90}) of egg hatching. The following formula was used to determine the EHI effectiveness: % Efficacy = 100 × (1 - L1 larvae / initial number of eggs).

L1 = 1st stage larvae

Ethics committee

The procedures used in this experiment are in accordance with the ethical principles of animal experimentation, approved in the protocol 42/2008 by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais, Minas Gerais State, Brazil.

Results and discussion

Chemical composition

It was possible to characterize 22 substances in the essential oil from leaves of *P. aduncum*, distributed among monoterpenes (80.6%) and sesquiterpenes (13.9%). The major compound was the identified the oxygenated monoterpene 1,8-cineole (eucalyptol), comprising 55.8% of the sample. Previous analysis essential oil from leaves of *P. aduncum* from the same site of collection showed 1,8-cineole content of 53.9% evaluated by GC-FID and 48.0% by with external standard calibration curve (Oliveira et al., 2013b). The two concentrations achieved are quite similar and by this mean it can be assumed the 1,8-cineole relative percentage achieved by GC-FID. The following monoterpenes was incredible identified in great amounts: α-terpineol (5.9%), *trans*-ocimene (4.8%), β-pinene (4.7%) and α-pinene (4.5%). These five monoterpenes comprises 75.7% of the mixture. The more representative sesquiterpene was assigned the bicyclogermacrene (4.4%). Arylpropanoids, usually identified in *P. aduncum* essential oil from North of Brazil, were not registered in the sample (Maia et al., 1998) (Table 1).

Egg-hatching inhibition

The essential oil of *P. aduncum* at different concentrations was effective in EHI of *H. contortus* (Table 2). It was the most effective that observed concentrations (6.0 and 12.0 $mg.ml^{-1}$) was incredible more effective in the inhibition of initial embryo development, since it was in these concentrations obtained greater unembryonated eggs and larvae averages lesser than in negative controls (Table 2).

It was observed increase effectiveness of the EHI with the increase in the concentration of the essential oil, indicating dose-dependent activity. The LC_{50} and LC_{90} for the essential oil of *P. aduncum* were calculated at 2.4 and 8.9 $mg.ml^{-1}$, respectively (Fig. 1).

Regarding the chemical constitution and nematicide activity, despite of 1,8-cineole (eucalyptol) being the major compound identified (55.8%) of the studied *P. aduncum* essential oil, the action of EHI may be due to the synergic action between the different substances that constitute the essential oil, including those in lower concentrations. Literature records have shown anthelmintic activity is essential oils rich in

Table 1
Substances identified in essential oil from leaves of *Piper aduncum* L.

Substances	RI	RIlit	% relative
α-pinene	921	939	4.5
β-pinene	964	980	4.7
β-mircene	974	991	1.9
limonene	1022	1031	0.3
1,8-cineole (eucalyptol)	1027	1033	55.8
trans-ocimene	1032	1050	4.8
γ-terpinene	1044	1062	0.5
δ-terpineol	1164	1166	1.2
4-terpineol	1176	1177	1.0
α-terpineol	1181	1189	5.9
E-cariophyllene	1405	1418	1.0
α-humulene	1434	1454	0.5
germacrene D	1469	1480	1.5
bicyclogermacrene	1482	1500	4.4
cubebol	1498	1514	0.5
nerolidol	1551	1564	1.0
not identified sesquiterpene	1553	-	1.3
caryophyllene oxide	1558	1581	0.5
guaiol	1594	1600	0.6
tau-cadinol	1624	1638	0.3
tau-muurolol	1628	1640	0.4
α-muurolol	1632	1645	0.3
Monoterpenes			80.6
Sesquiterpenes			13.9
Total			94.5

RI, retention index; RI_{lit}, retention index from Adams (2007).

Table 2
Effects of different concentration of essential oil of *Piper aduncum* L. on egg-hatching of *Haemonchus contortus* from sheep.

Treatments ($mg.ml^{-1}$)	Unembryonated eggs mean	Embryonated eggs mean	Larvae L1 mean	Efficacy (%)
1.5	3.0 C	10.5 b	23.5 b	37.2
3.6	7.3 C	26.5 ab	32.3 b	52.1
6.0	26.3 B	31.0 ab	7.8 a	87.9
12.0	24.3 B	40.8 a	4.3 a	95.0
levamisole *	61.3 A	23.8 b	0.0 a	100.0
Sterile water (SW)	4.3 C	20.5 b	51.5 b	-
SW and Tween 80	3.3 C	31.0 b	49.3 b	-
C.V.	35.9	25.3	24.4	

Note: Means followed by the same lowercase letters in the column are statistically similar to the Duncan test ($p \leq 0.05$). Means followed by capital letters in the same column are statistically similar to the Tukey test ($p \leq 0.05$). C.V., Coefficient of variation (%).
* Levamisole (15.0 $mg.ml^{-1}$).

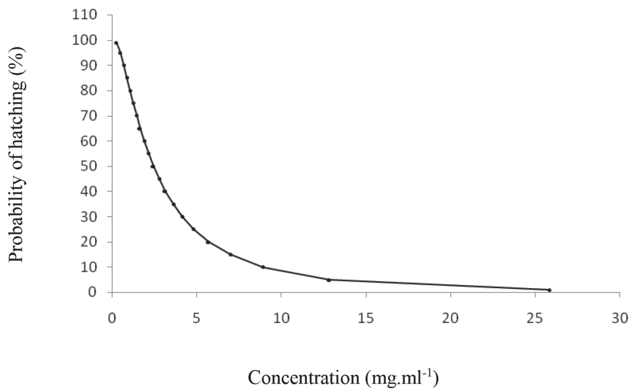

Figure 1 – Probability of hatching of *Haemonchus contortus* in different concentrations of the essential oil from leaves of *Piper aduncum* L.

1,8-cineole. Macedo et al. (2009) demonstrated an anthelmintic activity for the essential oil *Eucalyptus globules* Labill on EHI and larval development of *H. contortus*. The major compound was identified as 1,8-cineole (83.9%). The essential oil at 17.4 $mg.ml^{-1}$ showed 87.3% efficacy in EHI. Our results showed that the essential oil from leaves of *P. aduncum* was twice more effective than the *E. globulus* Labill essential oil as half of the concentration of the essential oil from *P. aduncum* has similar anthelmintic activity.

Conclusion

The essential oil from leaves of *P. aduncum*, rich in 1,8-cineole, antihelmintic showed *in vitro* efficacy. This essential oil showed 95% egg-hatching inhibition to *H. contortus* at 12.0 $mg.ml^{-1}$ and was more effective in the inhibition of initial embryo development. This Piperaceae species has great potential for alternate control of nematodes, since this plant can easily be cultivated with the great changes in the essential oil chemical composition (Oliveira et al., 2013b). Future studies should the evaluate the efficacy antihelmintic of 1,8-cineole in combination with other compounds of the essential oil *P. aduncum* such as α-terpineol, *trans*-ocimene, β-pinene and α-pinene. Its effect may be analyzed also to different cycle stages of the nematode as well as the possible toxic effects to animals, to represent a safe and effective alternative in the control of ovine helminthiasis.

Authors' contributions

GLO (PhD student) contributed in collecting plant, confection of herbarium sample to taxonomic identification, running the laboratory work (essential oil extraction, analysis of the chromatograms and mass spectra), and helped in the draft of the paper. TMV (Msc student), VFN (College student) and MOR (College student) contributed to biological studies, regarding to anthelmintic activity. ERD supervised the biological tests. DLM supervised the GC-MS and GC-FID analysis and contributed to critical reading of the manuscript. MACK contributed to critical reading of the manuscript. ERM supervised the essential oil extraction and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the late submission.

Conflicts of interest

The authors declare no conflicts of interest.

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