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EXTRACTS AND PURIFIED SUBSTANCES OF *Cabralea canjerana* INHIBIT HATCHING AND EXTRACTS OF *Schinus terebinthifolius* KILL JUVENILES OF MELOIDOGYNE INCOGNITA

Vanessa Alves GOMES[1], Carolina Alves GOMES[2], Fabíola de Jesus SILVA[3], Márcio Santos SOARES[4], Everaldo Antonio LOPES[5]

[1] Plant Protection/PhD in Agronomy, São Paulo State University, Botucatu, São Paulo, Brazil.
[2] Crop Production/MSc in Agronomy, Federal University of Viçosa, Rio Paranaíba, Minas Gerais, Brazil.
[3] Plant Pathology/PhD in Agronomy, Federal University of Lavras, Lavras, Minas Gerais, Brazil.
[4] Department of Chemestry, Federal University of Viçosa, Rio Paranaíba, Minas Gerais, Brazil.
[5] Department of Crop Production, Federal University of Viçosa, Rio Paranaíba, Minas Gerais, Brazil.

Corresponding author:
Vanessa Alves Gomes
Email: vavvgomes@gmail.com

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Abstract

Nematicidal substances have been identified from plants and are potentially useful for the management of plant-parasitic nematodes. *Cabralea canjerana*, (Meliaceae) and *Schinus terebinthifolius* (Anacardiaceae) produce bioactive compounds during their secondary metabolism and little is known about the effect of such substances on plant-parasitic nematodes. In the present study, we assessed the effect of aqueous and ethanolic extracts of *C. canjerana* and *S. terebinthifolius* at 1% (m:v) and purified substances from *C. canjerana* (gedunin, ocotillone, cabraleadiol, a mixture of ocotillone + cabraleadiol and a mixture of shoreic acid + eichlerianic acid) on hatching and mortality of *Meloidogyne incognita* juveniles. Aqueous extracts of *C. canjerana* fruits and seeds reduced hatching by 70.3 to 95.7%. Aqueous extracts of *S. terebinthifolius* fruits killed 42.8 to 77.1% of juveniles. The purified substances of *C. canjerana* inhibited the hatching of *M. incognita* from 57 to 90% and did not increase the mortality of juveniles. Therefore, *C. canjerana* extracts and its purified substances reduce *M. incognita* hatching and aqueous extracts of *S. terebinthifolius* kill J_2 of this nematode.

Keywords: Botanical nematicides. Brazilian pepper tree. Plant extracts. Mortality. Root-knot nematode.

1. Introduction

Root-knot nematodes are one of the main plant-pathogens worldwide, causing losses ranging from 20% to 100%, depending on population density, cultivar susceptibility, nematode species, soil texture, and environmental conditions (Nicol et al. 2011). In Brazil, *Meloidogyne incognita* (Kofoid and White) Chitwood and *M. javanica* (Treub) Chitwood are widespread over the country, damaging cash, and staple crops (Ferraz et al. 2010). The efficient management of RKN depends on the combination of different control strategies, including crop rotation, genetic resistance, physical methods, biological control and nematicides (Charchar et al. 2007; Neves et al. 2007; Silva and Pereira 2008; Jones et al. 2017; Gomes et al. 2020). Nematicidal substances can be produced by plants during their secondary metabolism and such substances may be useful for managing plant-parasitic nematodes (Ferraz et al. 2010).

Essential oils, crude extracts, isolated compounds, and volatile organic compounds of several plant species have been reported to suppress or kill root-knot nematodes under greenhouse and field conditions.

Plant-origin compounds may kill active forms of nematodes, inhibit hatching, impair nematode movement, interfere with root location, or even activate plant defense mechanisms (Ferraz et al. 2010). A plethora of chemicals from plants may have nematicidal properties, including phenolic compounds, alkaloids, tannins, fatty acids, terpenes, among others (Neves et al. 2008; Gardiano et al. 2011; Mateus et al. 2014; Barros et al. 2019; Gomes et al. 2020; Silva et al. 2020a; Silva et al. 2020b).

Cabralea canjerana belongs to the Meliaceae, the same family of the nematicidal plants: Neem (*Azadirachta indica*) and Chinaberry (*Melia azedarach*). A number of bioactive triterpenes and limonoids have been isolated from *C. canjerana* (Soares et al. 2004; Braga et al. 2006; Soares et al. 2006; Cazal et al. 2009). *Schinus terebinthifolius* belongs to the Anacardiaceae family and is grown as an ornamental plant. This species is rich in polyphenols, which are unevenly distributed in leaves, fruits, seeds, and bark (Feuereisen et al. 2017). Despite the richness of bioactive compounds found in *C. canjerana* and *S. terebinthifolius*, little is known about the potential of these plants as sources of nematicidal substances. Thus, this work aimed to evaluate the nematicidal potential of extracts of *C. canjerana* and *S. terebinthifolius* and purified substances of *C. canjerana* against *M. incognita*.

2. Material and Methods

Nematode inoculum

The nematode *M. incognita* race 3 was multiplied and kept in roots of potted tomato (cv. Santa Clara) in the greenhouse. Nematode eggs were extracted by the technique of Hussey and Barker (1973), modified by Boneti and Ferraz (1981). Hatching chambers were prepared to obtain second-stage juveniles (J_2) (Southey 1970). The concentration of eggs and juveniles was adjusted using Peters slide under an inverted microscope.

Plant extracts preparation

The aqueous and ethanolic extracts of *C. canjerana* and *S. terebinthifolius* were evaluated for their ability to inhibit hatching and kill *M. incognita* J_2 in laboratory experiments. Leaves, branches, fruits, and seeds of *C. canjerana* and leaves and fruits of *S. terebinthifolius* were dried in a forced-air oven for 72 h at 45 °C (Table 1). The dried material was ground in a Willey mill to pass through a 1.27-mm mesh. The resulting crushed plant material (hereafter “plant powder”) was stored in hermetically sealed jars until use. Aqueous and ethanolic extracts were prepared for the extraction of secondary metabolites from plants. To obtain the aqueous extract, 1 g of plant powder was added in a beaker containing 100 mL of deionized water and kept in a water bath at 65 °C for 90 min. Then, the material was filtered through Whatman number 1 filter and a 0.45-µm nitrocellulose membrane. The filtrates were kept in amber glasses and stored in a refrigerator. To prepare ethanolic extracts, 1 g of plant powder was added to 100 mL of ethanol (96° GL), followed by grinding with a domestic blender for 1 minute at low speed. The grinding process was performed three times. The extracts were filtered through Whatman number 1 filter and a 0.45-µm nitrocellulose membrane and stored in a refrigerator. The final concentration of the extracts was 1% (m:v).

Table 1. Plant used for preparing aqueous and ethanol extracts.

Aqueous extracts		Ethanolic extracts	
Cabralea canjerana	*Schinus terebinthifolius*	*Cabralea canjerana*	*Schinus terebinthifolius*
Leaves	Leaves	Leaves	Leaves
Branches	Fruits	Branches	Fruits
Fruits		Fruits	
Seeds		Seeds	

Nematicidal effect of aqueous and ethanolic extracts of *Cabralea canjerana* and *Schinus terebinthifolius*

To investigate the anti-hatching activity of the extracts, 100 µL of egg suspension containing 30 eggs were placed in a well of ELISA plate. The cells were then filled with 100 µL of aqueous or ethanolic extract

and 100 µL of phosphate saline buffer (16.0 g NaCl; 4.2 g Na2HPO4.7H2O; 0.4 g KH2PO4 and 0.4 g KCl pH 7.0). In the controls, the extracts were replaced by 100 µL of distilled water or ethanol (96° GL). The number of eggs and eventual J_2 per cell was evaluated soon after the beginning of the experiment, with the aid of a light microscope with inverted objectives. The plates were then sealed with film paper and kept in an incubator at 27 ºC. Every 24 h, over a period of five days, the number of hatched juveniles was evaluated. In the experiments to evaluate the nematicidal effect of extracts on J_2, the procedures were similar to hatching experiments. However, the inoculum was composed of 30 J_2 and the numbers of mobile and immobile J_2 were evaluated shortly after 5 days. After this period, immobile J_2 were considered dead. The experiments with aqueous and ethanolic extracts were carried out twice, in a completely randomized design (CRD) in a 5 x 2 (control; leaf, branches, seeds or fruit extracts of *C. canjerana* x water or ethanol) or 3 x 2 (control; leaf or fruit extracts of *S. terebinthifolius* x water or ethanol) factorial scheme. Each treatment was repeated 10 times.

Nematicidal effect of purified substances of *Cabralea canjerana*

Cabraleadiol, eichlerianic acid, gedunin, ocotillone, and shoreic acid were extracted from *C. canjerana* according to Soares et al. (2006). Seed extracts were initially fractionated using the microcrystalline Cellulose-D stationary phase column chromatography technique and solvents in increasing order of polarity as eluents (hexane, CH2Cl2, AcOEt, and MeOH). This stationary phase was chosen because the extract had high polarity and a silica column could retain some secondary metabolites of interest in the study. 59 fractions were collected, which were grouped according to their similarities by comparison by CDDA, yielding 11 subfractions. Fruit extracts with greater mass than seed extracts were analyzed by the 1H NMR spectrum, which revealed the presence of triterpenes. Assuming that triterpenes were masking the signals of other more polar metabolites, 88.3 g of the extract was fractionated through a liquid-liquid partition. At the end of the partition, four extracts were obtained. These extracts were submitted to a liquid-liquid extraction with 120 mL of solution. The ethanolic fruit extract was fractionated in hexane, dichloromethane, ethyl acetate, or methanol and the substances were extracted according to Soares et al. (2006). The ethanolic seed extract was subjected to column chromatography using microcrystalline cellulose, where the mobile phase was composed of hexane/dichloromethane/ethyl acetate in increasing polarity order, resulting in eleven fractions. The chemical identification of the substances was performed according to Sarria et al. (2014).

Two milligrams of each substance were diluted in a 3-µL solution containing 50% of water, 50% of ethanol, and one drop of Tween 20. The potential of gedunin, ocotillone, cabraleadiol, the mixtures of ocotillone + cabraleadiol, and a mixture of shoreic and eichlerianic acids in inhibiting hatching and killing second-stage juveniles of *M. incognita* was investigated following the same procedures as described for aqueous and ethanolic extracts. A solution containing water 50%, ethanol 50%, Tween 20, and without *C. canjerana* substances was used as control. The experiments with purified compounds of *C. canjerana* were carried out twice in a CRD with 10 replicates.

Statistical Analysis

The experiments were carried out in a completely randomized design (CRD) in a 5x2 and 3x2 factorial scheme. Data of all experiments were subjected to analysis of variance and means were compared by Tukey test ($p = 0.05$) using the statistical package R version 3.1.1 (R Core Team 2016).

3. Results and Discussion

The effect of *C. canjerana* and *S. terebinthifolius* extracts on *M. incognita* hatching varied due to the interaction between the extracts and the solvents in both experiments (Tables 2 and 3). Aqueous extracts of leaves, branches, fruits, and seeds of *C. canjerana* reduced hatching in both experiments (Table 1). No ethanol extract of *C. canjerana* reduced hatching (Table 2). Aqueous extracts of *C. canjerana* fruits and seeds reduced hatching by 70.3 to 95.7% (Table 2).

Aqueous extracts of leaves and fruit of *S. terebinthifolius* reduced hatching by more than 97% in the experiment 1 but had no effect in the experiment 2 (Table 3). No ethanolic extract of *S. terebinthifolius* reduced nematode hatching in both experiments. For both the plants, nematode hatching was lower when ethanol was used as solvent.

Table 2. Effect of aqueous and ethanolic extracts of *Cabralea canjerana* on hatching of second-stage juveniles (J_2) of *Meloidogyne incognita* race 3.

	Hatching (%)			
Extract	Experiment 1		Experiment 2	
	Aqueous	Ethanolic	Aqueous	Ethanolic
Leaves	13.6^{Ab}	13.8^{Aa}	2.4^{Ac}	0.6^{Aa}
Branches	15.5^{Ab}	3.5^{Bb}	15.0^{Ab}	2.3^{Ba}
Fruits	6.5^{Ac}	0.4^{Bb}	5.9^{Ac}	0.5^{Ba}
Seeds	4.2^{Ac}	0.0^{Bb}	3.6^{Ac}	0.7^{Aa}
Control	21.9^{Aa}	0.8^{Bb}	83.7^{Aa}	2.16^{Ba}

Means followed by the same uppercase letter in the row and lowercase in the column do not differ from each other by the Tukey test (p = 0.05).

Table 3. Effect of aqueous and ethanolic extracts of *Schinus terebinthifolius* on hatching of second stage juveniles (J_2) of *Meloidogyne incognita* race 3.

	Hatching (%)			
Extract	Experiment 1		Experiment 2	
	Aqueous	Ethanolic	Aqueous	Ethanolic
Leaves	2.0^{Ab}	1.2^{Aa}	96.1^{Aa}	1.2^{Ba}
Fruits	1.1^{Ab}	1.1^{Aa}	97.6^{Aa}	2.0^{Ba}
Control	84.5^{Aa}	2.16^{Ba}	29.2^{Ab}	0.8^{Ba}

Means followed by the same uppercase letter in the row and lowercase in the column do not differ from each other by the Tukey test (p = 0.05).

The effect of *C. canjerana* and *S. terebinthifolius* extracts on mortality of J_2 varied due to the interaction between types of extracts and solvents in both experiments (Tables 4 and 5). No aqueous extract of *C. canjerana* increased mortality of J_2 (Table 4). In general, ethanolic extracts of *C. canjerana* and ethanolic control caused high mortality of juveniles. Aqueous extracts of *S. terebinthifolius* fruits killed 42.8 to 77.1% of juveniles (Table 5). Ethanolic extracts of *S. terebinthifolius* did not increase the mortality of J_2 in comparison to ethanolic control (Table 5).

Table 4. Effect of aqueous and ethanolic extracts of *Cabralea canjerana* on mortality of the second stage juveniles (J_2) of *Meloidogyne incognita* race 3.

	Mortality (%)			
Extract	Experiment 1		Experiment 2	
	Aqueous	Ethanolic	Aqueous	Ethanolic
Leaves	22.4^{Ab}	33.8^{Ac}	12.5^{Bc}	54.5^{Aa}
Branches	54.2^{Aa}	53.4^{Abc}	34.5^{Bb}	68.4^{Aa}
Fruits	17.0^{Bb}	75.5^{Aa}	13.3^{Bc}	69.9^{Aa}
Seeds	49.0^{Aa}	60.6^{Bab}	29.2^{Bbc}	66.9^{Aa}
Control	45.5^{Ba}	72.4^{Aab}	58.3^{Ba}	72.5^{Aa}

Means followed by the same uppercase letter in the row and lowercase in the column do not differ from each other by the Tukey test (p = 0.05).

Table 5. Effect of aqueous and ethanolic extracts of *Schinus terebinthifolius* on mortality of second-stage juveniles (J_2) of *Meloidogyne incognita* race 3.

	Mortality (%)			
Extract	Experiment 1		Experiment 2	
	Aqueous	Ethanolic	Aqueous	Ethanolic
Leaves	26.2^{Bb}	71.5^{Aa}	31.1^{Bb}	72.2^{Ab}
Fruits	42.8^{Ba}	85.9^{Aa}	77.1^{Aa}	79.8^{Aab}
Control	11.7^{Bb}	84.7^{Aa}	7.3^{Bc}	92.6^{Aa}

Means followed by the same uppercase letter in the row and lowercase in the column do not differ from each other by the Tukey test (p = 0.05).

All the purified substances of *C. canjerana* and mixtures inhibited hatching of *M. incognita* race 3 in both experiments, with no difference between them (Table 6). The inhibition ranged from 57 to 90%. However, no substance increased mortality of *M. incognita* J_2 (Table 6).

Table 6. Effect of purified substances of *Cabralea canjerana* on hatching and mortality of second-stage juveniles (J_2) of *Meloidogyne incognita* race 3.

Substance	Hatching (%)		Mortality (%)	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Gedunin	12.4[b]	19.1[b]	17.9[b]	18.5[b]
Ocotillone	9.2[b]	6.1[b]	71.3[a]	76.0[a]
Cabraleadiol	7.1[b]	9.8[b]	39.1[b]	39.9[b]
Ocotillone + Cabraleadiol	12.5[b]	8.5[b]	31.3[b]	27.4[b]
Shoreic acid + Eichlerianic Acid	4.1[b]	5.0[b]	35.1[b]	26.5[b]
Control	42.5[a]	44.9[a]	70.5[a]	77.3[a]

Means followed by the same uppercase letter in the row and lowercase in the column do not differ from each other by the Tukey test (p = 0.05).

Plants from the Meliaceae family produce several biocidal compounds. The nematicidal effect of extracts, essential oil, seed cake, and purified substances of neem (*Azadirachta indica*) has been widely reported (Silva and Pereira 2008; Ferraz et al. 2010; Oka 2010). *Cabralea canjerana*, a neglected species from the Meliaceae family, is rich in limonoids and triterpenes (Braga et al. 2006). Extracts of this plant have antifungal and insecticidal properties (Lopes et al. 2008; Smaniotto et al. 2010; Magrini 2011; Mata and Lomonaco 2013). Here, we observed that aqueous extracts of fruit and seeds and purified substances of *C. canjerana* reduce hatching and did not cause significant mortality of *M. incognita* J_2. It is likely that the extracts and the purified substances have nematostatic rather than nematicidal activity on *M. incognita*. The lower mortality of nematodes exposed to aqueous extracts and purified substances of *C. canjerana* in comparison to the control can be a result of this nematostatic activity. After five days, the nematodes immobilized by the extracts and purified substances had higher body energy than those in water (control). As a result, the nematodes in the water remained moving, wasting energy, and, because of this, died sooner (Silva et al. 2018; Silva et al. 2019).

The extracts of *S. terebinthifolius* had no activity on inhibiting hatching and aqueous extracts of this plant cause mortality of second-stage juveniles. Little is known about the properties of this plant in reducing plant-parasitic nematodes. In one of the few studies, Schwengber et al. (2017) reported the effect of the essential oil of this plant against *Pratylenchus zeae* in the greenhouse. Borges et al. (2018) also related to the suppression of root-knot nematode by S. *terebinthifolius* essential oil. It is likely that nematicidal compounds of *S. terebinthifolius* are concentrated in the essential oils. Further studies are required to elucidate which compounds found in aqueous extracts and essential oils and if the concentration of the aqueous extracts higher than 1% may provide a nematicidal effect against *M. incognita* (Silva et al. 2020a; Silva et al. 2020b).

The ethanolic extracts were highly toxic against J_2 but similar to control with ethanol only. However, ethanolic extracts did not reduce hatching in comparison to aqueous extracts. Such results support previous works that demonstrated the strong effect of ethanol solutions against plant-parasitic nematodes, especially on second-stage juveniles (Silva et al. 2017; Pedroso et al. 2019; Fujita et al. 2020). Ethanol may kill J_2 (Silva et al. 2017). To better understanding the effect of *C. canjerana* substances on J_2 and eggs of root-knot nematodes, researchers must consider diluting the compounds just in water and Tween 20.

Botanical nematicides may be additional control tools in integrated management of plant-parasitic nematodes (Jardim et al. 2018; Silva et al. 2018; Gomes et al. 2020; Silva et al. 2020a; Silva et al. 2020b). The nematicidal potential of extracts and essential oils of a plethora of plant species have not been studied yet. Some plants can be sources of substances that may ultimately be active ingredients of plant-based nematicides. Here, we found that *C. canjerana* extracts and purified substances inhibit hatching of *M. incognita* J_2, while *S. terebinthifolius* extracts kill the active forms of the nematode. These results should stimulate further research to evaluate whether formulations containing these extracts and compounds can be useful for the management of root-knot nematode under field conditions (Borges et al. 2018).

4. Conclusions

Cabralea canjerana aqueous extracts of fruits and seeds reduce *Meloidogyne incognita* race 3 hatching and aqueous extracts of seeds cause mortality of second-stage juveniles (J_2) this nematode. All *P*urified substances of *C. canjerana* reduce *M. incognita race 3* hatching and all purified, except ocotillone, kill J_2 of this nematode. *Schinus terebinthifolius* aqueous extracts of fruits cause mortality of J_2 of *M. incognita* race 3.

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Ethics Approval: This study has been reviewed by all authors and all are in agreement. The study has not been tested in animals and humans. The study has not been published elsewhere.

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