1-1-2019

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Recommended Citation

Machado, C. D., Raman, V., Rehman, J. U., Maia, B. H. L. N. S., Meneghetti, E. K., Almeida, V. P., Silva, R. Z., Farago, P. V., Khan, I. A., & Budel, J. M. (2019). Schinus molle: anatomy of leaves and stems, chemical composition and insecticidal activities of volatile oil against bed bug (Cimex lectularius). Revista Brasileira de Farmacognosia, 29(1), 1–10. https://doi.org/10.1016/j.bjp.2018.10.005

Revista Brasileira de Farmacognosia 29 (2019) 1–10

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Schinus molle: anatomy of leaves and stems, chemical composition and insecticidal activities of volatile oil against bed bug (*Cimex lectularius*)

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ARTICLE INFO

Article history:
Received 17 July 2018
Accepted 29 October 2018
Available online 11 December 2018

Keywords:
Aroeira-salsa
Bed bug
Calcium oxalate crystals
Light and scanning electron microscopy
Phytoinsecticide
Quality control

ABSTRACT

The present work investigates the leaf and stem anatomy, chemical composition and insecticidal activities (against *Cimex lectularius* Linnaeus, 1758) of the volatile oils of *Schinus molle* L., Anacardiaceae, a Brazilian native traditional medicinal plant. Noteworthy micro-morphological features that can help in the identification and quality control of the species include the presence of isobilateral and amphistomatic leaves, anomocytic and cyclocytic stomata, capitate glandular and conical non-glandular trichomes, large secretory ducts in the midrib, presence of druses and prismatic crystals, and the petiole vascular system comprising of five vascular bundles arranged in U-shape and an additional dorsal bundle. The major components of the volatile oil include β-pinene (14.7%), α-pinene (14.1%), limonene (9.4%) and muurolol (11.8%). Insecticidal activities of the volatile oil against bed bugs were investigated for the first time; strong toxicity by fumigation with the volatile oil of *S. molle* was observed and reported herein.

Introduction

Anacardiaceae R.Br. is pantropical and includes 72 genera and approximately 550 species used as ornamentals, food and medicines. *Schinus* L. is an important genus of this family, comprising 29 species which inhabit areas from Central America to Argentina (Lorenzi and Matos, 2002; The Plant List, 2018). In Brazil, the genus is represented by nine species (Flora do Brasil 2020, 2018). The genus includes several medicinally important species, such as *Schinus areira* L., *S. longifolia* (Lindl.) Speg., *S. molle* L., and *S. terebinthifolia* Raddi.

Schinus molle is popularly known in Brazil as aroeira, aroeira-salsa, periquita, anacauíta, and molho (Flora Digital, 2010). In other regions, it is called aguaribay, peruvian-pepper, american-pepper, peruvian-peppertree, escobilla, false-pepper, molle-del-peru and pepper-tree (USDA, 2017). It is an evergreen tree that reaches 3–15 m in height with dark brown, latescent and intensely fissured bark. The leaves are imparipinnate with a winged rachis and 20–40 leaflets. The leaflets are linear-lanceolate, 2–5 × 0.4–0.8 cm, and entire or dentate along margins (Martínez-Millán and Cevallos-Ferriz, 2005).

In general, the common names of medicinal plants are problematic for classification because a single species often has numerous folk names, and a single popular name can occasionally be used for a range of plants (Upton et al., 2011). *S. molle* is frequently confused with other species of *Schinus* as well as species of other genera in the family. The common name "aroeira" is applied to different species of *Lithraea* and *Astronium* thus creating further confusion in species identification (Queiroz et al., 2002).

Schinus molle is used in folk medicine as an analgesic, antifungal, antitumoral, antispasmodic, diuretic, topical antiseptic, and to treat hypertension, wounds, bacterial infections and asthma (Goldstein and Coleman, 2004; Martins et al., 2014; Maema et al., 2016). Pharmacological studies have reported several properties such as sedative (Taylor et al., 2016), anti-inflammatory (Yueqin et al., 2003), antimicrobial activity against *Staphylococcus aureus* and *Streptococcus pyogenes* (Guerra-Boone et al., 2013), trypanocidal (Molina-Garza et al., 2014), repellent and insecticidal properties against *Triatoma infestans*, the vector of Chagas' disease (Ferrero et al., 2006). Biological activities have also been described for the

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https://doi.org/10.1016/j.bjp.2018.10.005

volatile oil, such as antibacterial (Pellegrini et al., 2017), antifungal (Martins et al., 2014), cytotoxic (Díaz et al., 2008), and insecticidal against *Haematobia irritans* L. (López et al., 2014). Moreover, the volatile oil is used as an adjuvant in various applications in food products because of its antimicrobial and antioxidant properties or as antiparasitary in cattle and beekeeping (Guala et al., 2016).

The literature shows high production of volatile oils in *S. molle*; however, differences in the chemical composition of the volatile oils have been reported, especially in relation to the major compounds Gomes et al., 2013). Variations in the edaphic, climatic and geographic conditions, post-harvest drying and storage methods, extraction method and the type of plant material used can interfere in the yield and chemical composition of volatile oils (Raut and Karuppayil, 2014; Saulle et al., 2018), and consequently their biological activities.

Cimex lectularius Linnaeus, 1758, popularly known bed bug, is a hematophagous pest that feeds on human blood. It is a difficult pest to control as it typically feeds at night, whereas, during the day, it hides in the folds of furniture, inside cracks and crevices, wooden or non-wood structures (Ghauri, 1973). Over the past decades it has resurged and became a major concern in the USA and other countries (Koganemaru and Miller, 2013).

Considering the morphological similarities among different species of *Schinus* and the variability in the chemical composition of volatile oils as well as their biological activities, the present study aims to (1) investigate the anatomical features of the leaf and stem of *S. molle* to provide anatomical characters in order to support the correct identification of the species, (2) characterize the volatile oil composition of the species collected in Campos Gerais, Paraná, South Brazil, and (3) test the insecticidal activities of the volatile oil against bed bugs (*Cimex lectularius*).

Materials and methods

Plant material

Vegetative aerial parts of *Schinus molle* L., Anacardiaceae, were collected from plants growing in open and sunny areas in the Botanical Garden of Pharmacy School of State University of Ponta Grossa (latitude 25°5′23″ S and longitude 50°6′23″ W), Brazil in March 2017. Mature leaves and stems (at least three samples) obtained from the sixth node and below (median, intercostal and margin regions), as well as stem fragments from 5 to 15 cm from the shoot were prepared for the anatomy and histochemical analyses. For the extraction of volatile oil, only dried leaves were used. The plant material containing inflorescences was used to prepare voucher specimens, that were identified by a Taxonomist and stored at the Herbarium of University of Ponta Grossa under the numbers 20048, 22240 and 22239 HUPG. The access to the botanical material was authorized by Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN) and was registered under number A8C5B94, as required for Brazilian legislation.

Anatomical assays

Freshly collected samples of the leaves and stems of *S. molle* were fixed in FAA solution (formalin–acetic acid–alcohol) (Johansen, 1940), and stored in 70% ethanol (Berlyn and Miksche, 1976). Transverse sections of leaves (lamina and petiole) and stems were prepared by free-hand sectioning using razors. These sections were stained with toluidine blue (O'Brien et al., 1964) or double stained with Astra blue and basic fuchsin (Roeser, 1972), mounted in a drop of glycerin (50%) on a glass slide, covered with a cover slip, and sealed with transparent nail polish (Kraus and Arduin, 1997).

The diaphanization of the leaves was performed by following the technique of Kraus and Arduin (1997). For the crystals, descriptions by He et al. (2012) were used. The length and width of stomata, secretory ducts and starch grains were measured from twenty structures at different locations on the leaf blade, midrib and stem to determine the average size. Stomatal index (SI) was performed by taking twenty measurements from multiple leaves and calculated using the following formula wherein S = number of stomata per unit area, and E = number of epidermal cells (including trichomes) in the same unit area.

$$SI = \frac{S}{E+S} \times 100$$

Histochemical tests

For the histochemical tests, sudan III was used to detect lipophilic compounds (Foster, 1949); hydrochloric phloroglucin to reveal traces of lignin (Sass, 1951); potassium dichromate (10%) (Gabe, 1968) and ferric chloride (1%) for phenolic substances (Johansen, 1940); and iodine-iodide solution (2%) was used to identify starch grains (Berlyn and Miksche, 1976). Appropriate controls were performed in parallel with the tests.

Photomicrographs were prepared using an Olympus CX 31 optical microscope equipped with Olympus C-7070 digital camera. The semi-permanent and histochemical test slides were then analyzed in the Laboratory of Pharmacognosy at the State University of Ponta Grossa (UEPG) for a detailed description of the leaf and stem tissues. Calcium oxalate crystals were observed using a Nikon E600 POL polarized microscope equipped with Nikon DSFiv camera systems and Nikon Elements imaging software (Nikon Inc., Tokyo, Japan) at the University of Mississippi.

Field emission scanning electron microscopy (FESEM) and energy-dispersive X-ray spectroscopy (EDS)

A Tescan Mira 3 FESEM was used for micro-morphological analysis and imaging. The samples were dehydrated in a series of ethanol solutions of increasing concentrations and then dried in a critical point dryer. Afterwards, the dried samples were mounted on aluminum stubs using glued carbon ribbon and then coated with gold using a sputter coater (Quorum, SC7620). The samples were observed and imaged in the FESEM in high vacuum mode with an accelerating voltage 15 kV.

EDS analysis was performed on selected crystals as well as the cells devoid of crystals (control) using an EDS detector attached to the FESEM. This procedure was carried out at the multi-user laboratory (C-Labmu) of UEPG.

Extraction of the volatile oil (VO)

Leaves of *S. molle* were air-dried and then distilled using a Clevenger type apparatus for 4 h. The oil obtained from 100 g of dried leaves was separated and dried over anhydrous Na_2SO_4. The values for VO yield of the three replications were averaged. The VO was stored in amber glass vials with Teflon-sealed caps at 4 ± 0.5 °C in the absence of light until used. This procedure was performed at UEPG.

GC–MS analysis

The analysis of the VO was performed on a Shimadzu 2010 Plus gas chromatograph, coupled with a Shimadzu TQ8040 mass selective detector and equipped with a Rtx-5MS capillary column (30 m × 0.25 mm × 0.25 μm). GC–MS was carried out in split mode (ratio 1:40), with an injection volume of 1 μl of the sample (1%

Table 1
Chemical compounds in the volatile oil of *Schinus molle*.

Compound	Peak area (%)	RI cal.	RI lit.
α-Pinene	14.1	934	932
Camphene	0.6	948	954
β-Pinene	14.7	977	979
Limonene	9.4	1029	1024
trans-Pinocarveol	4.7	1140	1135
trans-Verbenol	0.5	1146	1140
Pinocarvone	0.8	1165	1160
trans-Linalool oxide	1.3	1169	1173
Myrtenol	4.6	1199	1194
trans-Carveol	1.1	1221	1215
Carvone	0.9	1247	1239
(*E*)-Caryophyllene	2.1	1423	1417
9-*epi*(*E*)-Caryophyllene	1.2	1465	1464
Bicyclogermacrene	1.4	1500	1500
α-Muurolene	0.5	1504	1500
γ-Cadinene	3.1	1518	1513
Zonarene	1.9	1527	1528
Spathulenol	5.5	1581	1577
Caryphyllene oxide	7.4	1587	1582
Globulol	0.8	1596	1590
Ledol	0.8	1608	1602
Humulene epoxide II	0.6	1614	1608
1,10, di-*epi*-Cubenol	2.5	1620	1618
Muurolol	11.8	1646	1644
α-Cadinol	0.6	1660	1652
Compounds identified	93.1		
Monoterpenes hydrocarbons	38.8		
Oxygenated monoterpenes	14.0		
Sesquiterpenes hydrocarbons	10.3		
Oxygenated sesquiterpenes	30.0		

RI lit., retention index from Adams (2007); RI calc, calculated retention index. The identification of the compounds was performed using RI lit, RI calc and mass spectra from NIST02 library and Adams (2007). The compounds in bold represent the major compounds.

(w/v) in hexane), and an injector temperature of 250 °C, the column set at 60 °C, with heating ramp of 3 °C/min (final temperature: 240 °C), and the interface ion source was at 300 °C, mass range of *m/z* 40–400. Helium was used as the carrier gas with a flow of 1.0 ml/min, with the ionization mode: electron impact 70 eV. Quantitative analysis was performed using a Hewlett-Packard 5890 gas Chromatograph equipped with a flame ionization detector under the same conditions previously described. The relative areas were the average of triplicate analysis.

Identification of the individual compounds was based on comparison of their GC retention indices (RI) on apolar column, and mass spectra of components with spectra from NIST02 library and with literature data (Adams, 2007) (Table 1). This analysis was carried out at UFPR.

Insecticidal studies

Rearing of bed bugs

The bed bug strains (*Cimex lectularius*) (Bayonne 'Insecticide resistant' and Ft. Dix 'Susceptible') were provided by Dr. Changlu Wang, Department of Entomology, Rutgers University, New Brunswick, NJ and are mass reared at NCNPR since Dec. 2017. The bed bug colony was raised and fed in 473 ml clear glass jars containing paper strip (standard 92 multipurpose paper) as harborages. The jars were covered with fine mesh cloth for ventilation, which were held in place with a rubber band (Campbell and Miller, 2015). Jars were kept in a growth chamber at 26 °C and 60% RH with a photoperiod of 13:11 h light/dark. The bed bugs were fed with defibrinated rabbit blood (Hemostat, CA) weekly. The bed bugs were fed according to method explained by Montes et al. (2002). Blood feeders (CG-1836-75 ChemGlass, NJ) were attached to a water bath using latex tubing and a piece of parafilm 'M' membrane was stretched across the bottom of the feeder. A quantity of 5 ml of defibrinated rabbit blood (Hemostat) was placed in the hollow center of the glassware and pooled on the parafilm. The parafilm provided a barrier for the bed bug to feed through. The water bath was set to circulate and warm the blood to 37 °C. Bed bugs drop down after feeding and jars were removed.

Topical bioassay

Insecticidal studies with VO of *S. molle* were conducted according to the procedure mentioned by Romero et al. (2009). Adult bed bugs were separated using feather weight forceps in the Petri dishes. Bed bugs were anesthetized with CO_2 and 1 μl of a treatment solution in acetone was delivered onto the dorsal surface of the abdomen with a hand-held repeating dispenser (Hamilton Co., Reno, NV) equipped with a 50 μl glass syringe (Hamilton Co.). Control insects received 1 μl of acetone alone. After treatment, bed bugs were transferred into the 20 ml clear glass vials containing paper strip (standard 92 multipurpose paper) and kept in the growth chamber. Data for the mortality of the bed bugs was recorded at 1, 2, 3, 5 and 7 days after treatment. Bed bugs were considered dead if no body part moved after touching the body with a needle. There were 3 replicates with 10 bed bugs (mixed sex)/replicate. Four doses *viz.* 125, 50, 25 and 12.5 μg VO/bed bug were tested. Deltamethrin was used as the standard insecticide.

Residual bioassay

Residual studies were done using the filter paper method in a Petri dish as described by Campbell and Miller (2015) after slight modifications. A filter paper disk of 20 cm^2 (Whatman# 1) was treated with an aliquot 100 μl of each treatment concentration (25, 50 and 100 μg VO/cm^2) using pipette and allowed to dry for 10 min. The 100 μl aliquot of treatment fully covered the filter paper. The treated filter papers were then placed in the Petri dish (50 mm × 9 mm, Falcon). Control treatments received only acetone. Ten adult bugs were released on the filter paper and mortality was recorded for 7 days as mentioned in previously. There were three replicates with ten bed bug/replicate. Deltamethrin was used as the standard.

Fumigation bioassay

Bed bugs were subjected to vapor toxicity in 125 ml of clear glass jars. A small piece of paper was placed in the jar's bottom to provide a substrate for the bed bugs to rest during the volatile oil volatile tests. Bed bugs were introduced in the jars 2–4 h before treatment to acclimatize. A treatment solution or acetone aliquot of 2 μl was deposited directly onto the internal surface of the bottle side wall ~4 cm from bottle bottom using a 50 μl gas-tight syringe (Hamilton Company, Reno, NV) attached to a PB600 (Hamilton Company, Reno, NV) repeating dispense. Five concentrations *viz.*, 15.6, 31.25, 62.5, 125 and 250 μg VO/125 sq. cm were tested against the bugs. The jars were placed in the growth chamber and data for mortality were recorded 24 h after the treatment. The 2,2-diclorovinil-dimetylphosphate (DDVP) was used as the standard.

Treatment means of vapor toxicity were compared using Two-factor analysis of variance (ANOVA) and separated by Tukey's honestly significant difference (HSD). The LC_{50} and LC_{90} values were calculated using Probit analysis. Analysis were performed in SAS® 9.3 and JMP® 10.0.

Results and discussion

Anatomical studies

The leaflet of *S. molle* (Fig. 1a, b), in frontal view, showed epidermal cells with straight and thickened anticlinal walls (Fig. 1c, e), prominently raised above the leaf surface. The external periclinal walls have striate cuticle and epicuticular waxes in crusts shape

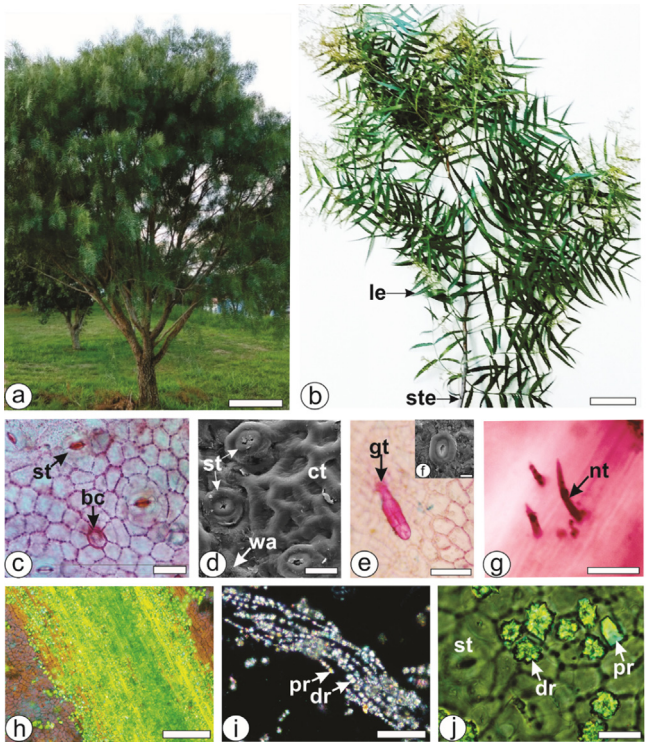

Fig. 1. Morpho-anatomy of leaves of *Schinus molle* [c, e, g: light microscopy; stained in safranin; h, i, j: polarized microscopy; d, f: FESEM]. a: Plant habit. b: A twig with leaves. c–j: Leaf epidermis in surface view (e, f, g, j: adaxial side; c, d, h, i: abaxial) [bc: basal cell of trichome; ct: cuticle; dr: druse; gt: glandular trichome; nt: non-glandular trichome; pr: prismatic crystal; st: stomata: wa: waxes in crusts]. Scale bars: a = 10 cm, b = 5 cm, h = 200 μm, i = 100 μm, c, d, e and g = 50 μm, j = 25 μm, f = 10 μm.

(Fig. 1d). The stomata are anomocytic and cyclocytic and are present on both sides (Fig. 1c–f). They measured 28 × 23 μm on average on both sides and the stomatal index was 7.27 on the abaxial and 4.1 on the adaxial sides.

Different types of stomata were observed in the genus, such as cyclocytic type in *S. polygama* (Cav.) Cabrera, *S. latifolius* (Gillies ex Lindl.) Engl., *S. meyeri* F.A. Barkley and *S. molle*, actinocytic in *S. polygama* (Cav.) Cabrera, *S. lentiscifolius* Marchand and *S. venturii* F.A. Barkley, and anomocytic in *S. weinmannifolius* Engl. (Martínez-Millán and Cevallos-Ferriz, 2005).

Amphistomatic leaves have been reported for several species of *Schinus* (Perrotta and Arambarri, 2004; Martínez-Millán and Cevallos-Ferriz, 2005; Duarte et al., 2006; Delbón et al., 2010; Dias et al., 2013; Pires et al., 2015). However, hypostomatic leaves were found in *S. latifolius*, *S. meyeri*, *S. venturii*, *S. weinmannifolius* (Martínez-Millán and Cevallos-Ferriz, 2005) and *S. terebinthifolius* (Azevedo et al., 2015). *Schinus longifolia* var. *longifolia* shows stomata measuring 25 × 22 μm and 1.96% of stomatal index on adaxial, whereas the stomata have 24.1 × 20.4 μm and 9.46 for stomatal index on abaxial side (Perrotta and Arambarri, 2004).

Several druses and prismatic crystals in different shapes are found in the leaves, especially in the veins and they can be better evidenced by a polarized microscope (Fig. 1h, i, j). Prismatic crystals and druses were frequently reported in species of *Schinus* and were found in the veins and in the mesophyll (Perrotta and Arambarri, 2004; Martínez-Millán and Cevallos-Ferriz, 2005; Duarte et al., 2006; Delbón et al., 2010; Dias et al., 2013; Azevedo et al., 2015; Pires et al., 2015).

Glandular and non-glandular trichomes are rarely present in *S. molle*. Glandular trichomes are capitate with short pedicel and pluricellular biseriate head (Fig. 1e). The non-glandular are conic, unicellular or pluricellular and the walls are thick (Fig. 1g). Martínez-Millán and Cevallos-Ferriz (2005) have stated that trichomes in *Schinus* are scarce, but reported non-glandular trichomes in *S. polygama*, *S. latifolius*, *S. lentiscifolius*, *S. meyeri* and *S. weinmannifolius* and glandular trichomes in *S. meyeri*, *S. venturii* and *S. weinmannifolius*.

In cross-section, the epidermis is uniseriate formed by cells in rectangular shape. Beneath the epidermis, a subepidermal layer can be seen on both sides (Fig. 2a, b). Druses can be seen in the subepidermal layer (Fig. 2b). The mesophyll is isobilateral and is formed

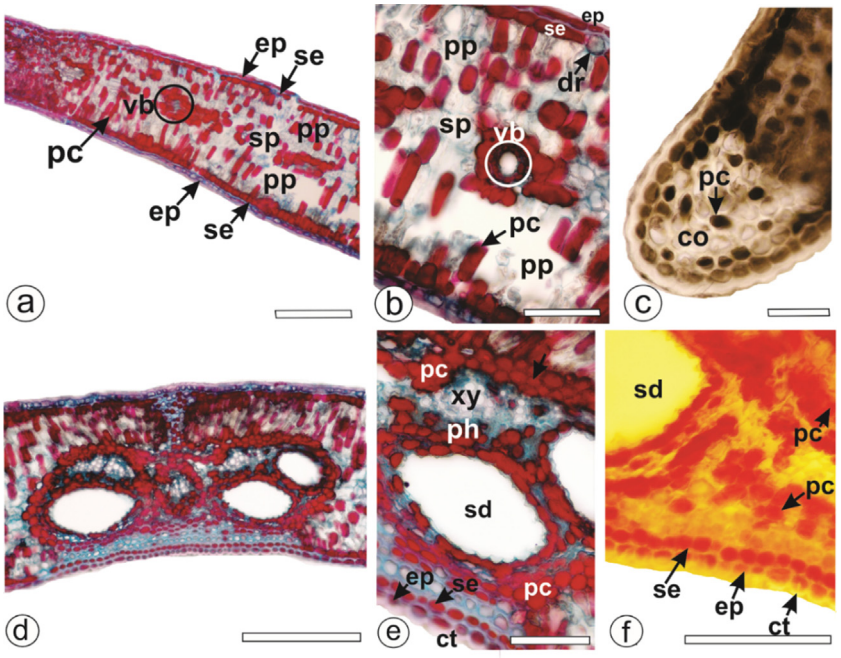

Fig. 2. Leaf anatomy of *Schinus molle* – transverse sections of leaf blade (a–c) and midrib (d–f). [a, b, d, e: Stained in Astra blue/basic fuchsin; c: ferric chloride solution, and f: potassium dichromate solution] [co: collenchyma; ct: cuticle; dr: druse; ep: epidermis; pc: phenolic compounds; ph: phloem; pp: palisade parenchyma; sd: secretory duct; se: subepidermal layer; sp: spongy parenchyma; vb: vascular bundle; xy: xylem]. Scale bars: a, d = 100 μm, b, c, e, f = 50 μm.

by 2–3 layers of palisade parenchyma on both sides and about three layers of spongy parenchyma. Small collateral vascular bundles are immersed in the mesophyll and they are surrounded by a parenchymatous sheath (Fig. 2a, b). Prismatic crystals and druses are also observed in the mesophyll (Fig. 2b). In the edges of the leaf blade, angular collenchyma is present (Fig. 2c). Phenolic compounds are met in the epidermal, subepidermal (Fig. 2a–f) and in the mesophyll cells (Fig. 2a–d) and react positively with ferric chloride (Fig. 2c) and potassium dichromate (Fig. 2f).

In contrast to the presence of isobilateral mesophyll in *S. molle*, dorsiventral mesophylls have been found in *S. longifolia* var. *longifolia* (Perrotta and Arambarri, 2004), *S. terebinthifolius* (Duarte et al., 2006), *S. fasciculatus* var. *fasciculatus* (Delbón et al., 2010) and *S. polygamus* (Dias et al., 2013). This characteristic is a good anatomical marker for *S. molle* identification.

The midrib, in cross-section, is flat on both sides (Fig. 2d). Different shapes were observed in different species of *Schinus*, such as biconvex in *S. fasciculatus* var. *fasciculatus* (Delbón et al., 2010) and in *S. terebenthifolius* (Duarte et al., 2006), and biconvex with keel-shaped adaxially in *Schinus weinmanniifolia* Mart. ex Engl. (Arambarri et al., 2008). Midrib shape can also be useful to differentiate *S. molle* from other species of *Schinus*.

The epidermis is uniseriate and covered by a thick (Fig. 2c, f) and striate cuticle. The palisade parenchyma is substituted by some layers of angular collenchyma on both sides, yet well-developed on the abaxial side. There are two large secretory ducts, but other smaller ones can occur near them (Fig. 2d, e). They measure 50 × 100 μm on average and contain VOs. There are at least three vascular bundles not well-organized (Fig. 2d). Several druses and prismatic crystals are evident in the ground parenchyma.

Phenolic compounds are also found in the epidermal and subepidermal cells (Fig. 2d–f), in the epithelium of the ducts and around the vascular bundles (Fig. 2d–f). As also observed in the blade, phenolic compounds reacted positively with ferric chloride (Fig. 2c) and potassium dichromate (Fig. 2f) solutions.

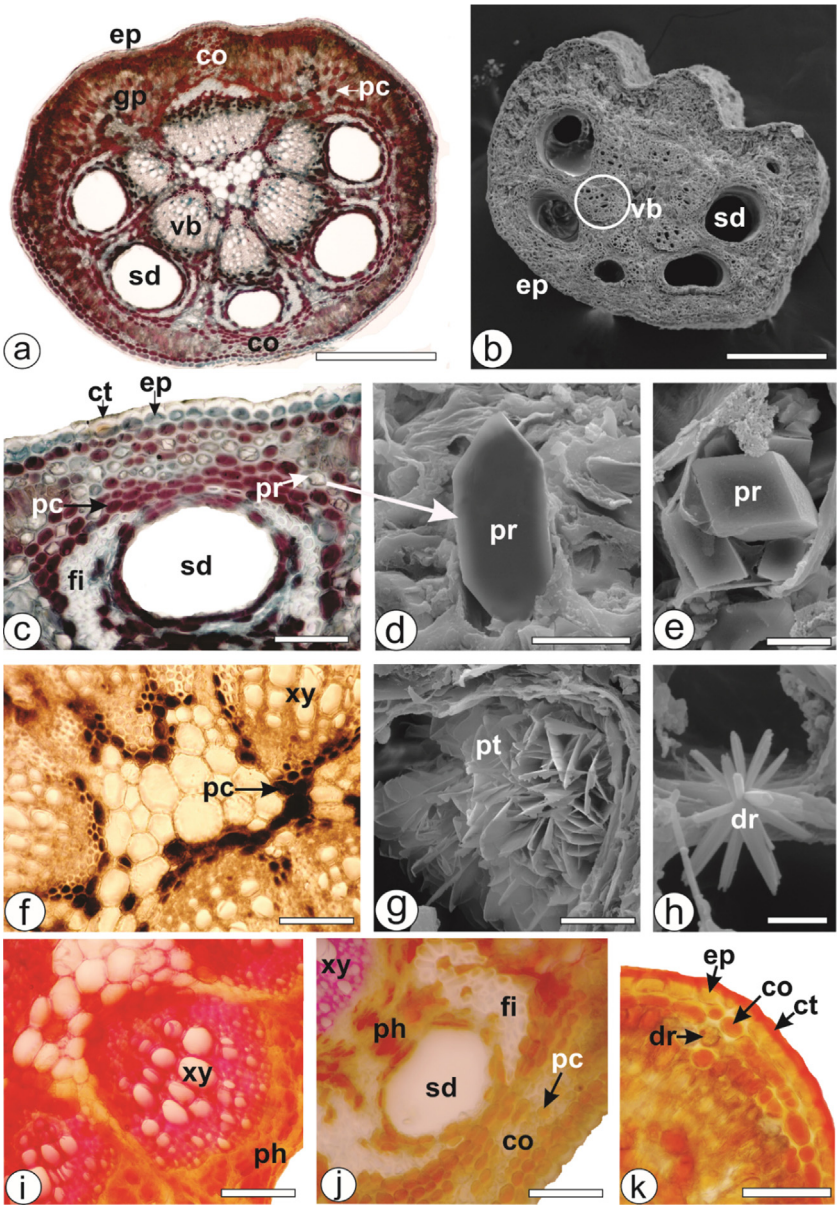

Fig. 3. Petiole anatomy of *Schinus molle* [a, c, i, j, k: light microscopy; b, d, e, g, h: FESEM]. a, b, c, f, i, j, k: Transverse sections; d, e: solitary prismatic crystals; g, h: druses of prismatic crystals [co: collenchyma; ct: cuticle; dr: druses; ep: epidermis; fi: fibers; gp: ground parenchyma; pc: phenolic compounds; ph: phloem; pr: prismatic crystal; pt: platy aggregation cluster; sd: secretory duct; vb: vascular bundle; xy: xylem]. Scale bars: a, b = 200 μm; c, f, i, j, k = 50 μm; d, g = 10 μm; e = 5 μm; h = 1 μm.

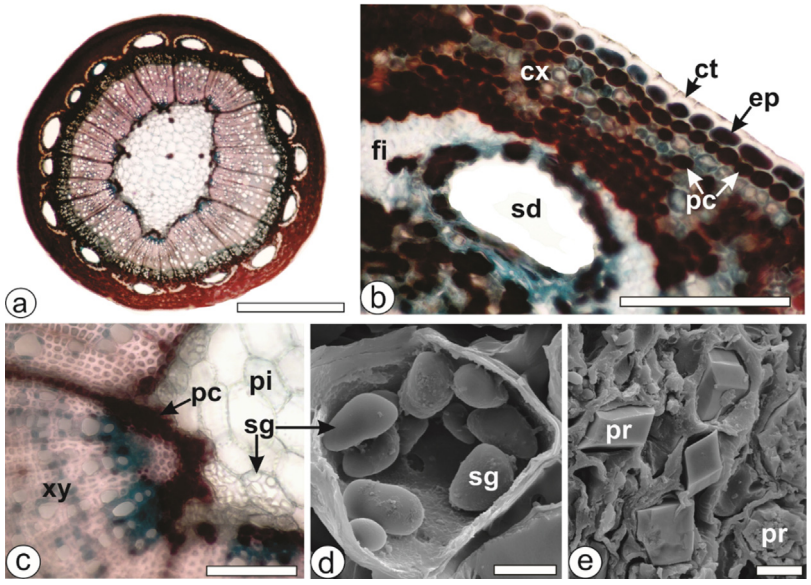

Fig. 4. Stem anatomy of *Schinus molle* [a–c: light microscopy; d, e: FESEM]. a–c: Transverse sections of the stem; d: starch grains, e: prismatic crystals [cx: cortex; ct: cuticle; ep: epidermis; fi: fiber; pc: phenolic compounds; pi: pith; pr: prismatic crystal; sd: secretory duct; sg: starch grains; xy: xylem]. Scale bar: a = 500 μm, b = 100 μm, c = 50 μm, d, e = 10 μm.

The cross-section of the petiole, in the middle and distal parts, has almost round shape with a slight convexity on the adaxial side (Fig. 3a). However, in the proximal region, petiole shows two concavities on the adaxial side (Fig. 3b). *Schinus longifolia* var. *longifolia* (Perrotta and Arambarri, 2004) presented flat convex, whereas *S. polygamus* showed biconvex shape (Dias et al., 2013).

The petiole epidermis has the same characteristics as that of the leaf blade (Fig. 3c) and reacted positively with Sudan III in the histochemical test (Fig. 3k). A subepidermal layer can be observed. Beneath the subepidermis, several layers of chlorenchyma are present and there are discontinuous patches of angular collenchyma (Fig. 3a), especially toward vascular bundles. The vascular system is formed by five collateral vascular bundles arranged in U-shape and a dorsal bundle, the largest of them (Fig. 3a). Because of the arrangement of the vascular bundle, a medulla is formed (Figs. 3a, f) in the center of the midrib. Lignin is found in the xylem to react with phloroglucinol/HCl (Fig. 3i). In *S. longifolia* var. *longifolia*, five vascular bundles were organized in an open arc (Perrotta and Arambarri, 2004).

In the petiole, each vascular bundle in the open arc presents one secretory duct in the phloem. They are more circular than in the midrib and show different sizes (Fig. 3a–c). A discontinuous fiber ring surrounds the secretory ducts (Fig. 3a, c, j) and react with phloroglucinol/HCl (Fig. 3j). Druses (Fig. 3h, k) and prismatic crystals in different shapes, such as hexagon (Fig. 3d), rectangle, diamond (Fig. 3e) and platy aggregation cluster (Fig. 3f) were found scattered in the petiole.

In transection, the stem presented a circular shape (Fig. 4a). The epidermis appears in a single layer with thick cuticle. The cortex is formed by up to ten layers of angular collenchyma cells filled with phenolic compounds. About twenty secretory ducts, localized in the phloem, are organized in a circle. Ducts have the features described for those of midrib and petiole and are adjoined by perivascular fiber caps (Fig. 4a, b). The vascular cylinder presents phloem outward and xylem inward. Lignification was detected in the perivascular fiber caps and xylem, using the phloroglucinol/HCl test.

The pith is composed of parenchymatous cells with thin walls (Fig. 4a). Starch grains have ovate shape, rarely rounded, appear compound aggregates of various granules in the perimedullary region, measuring 10×5 μm on the average (Fig. 4d). Phenolic compounds are also located in the perimedullary region (Fig. 4c). Druses and prismatic crystals as described above for mesophyll, midrib and petiole are also found scattered in the stem (Fig. 4e).

In the present study, the crystals were analyzed for their elemental composition (Fig. 5). The EDS spectra showed prominent peaks for carbon (38.9 and 12.6%), oxygen (41.7 and 53.9%) and calcium (19.3 and 33.5%) for prismatic (Fig. 5a) and druses (Fig. 5b), respectively. Spectrum of platy aggregation cluster (Fig. 5c) showed carbon (24.3%), oxygen (50.4%), calcium (20.3%) and magnesium (5.0%). The major unlabeled peaks represent gold element used in coating the samples.

The presence or absence of crystals and their type and chemical composition is helpful in plant taxonomy. Crystals of calcium oxalate are designed from endogenously synthesized oxalic acid and calcium taken from the environment. Crystallization produces crystals with definite shapes in specific tissues or organs in certain plants (Meric, 2009). Crystals have been identified in some studies as calcium oxalate by using EDS (Andrade et al., 2017; Migacz et al., 2018; Santos et al., 2018). Although calcium oxalate is more common in plants, calcium sulfate, magnesium oxalate, magnesium oxalate and other elements, such as potassium and silicon can also be found in the crystals (He et al., 2012).

Yield and chemical composition of volatile oil (VO)

Hydrodistillation of the leaves of *S. molle* yielded 0.78% v/w of a viscous and pale yellow VO with a touch of pungent fragrance. Similar characteristics were observed for this species by Hamdan et al. (2016). Different yields of VO of *S. molle* were found in the literature, such as 1.25% for aerial parts (Simionatto et al., 2011), 1.1% for leaves (Martins et al., 2014; Zahed et al., 2010) and 0.91% for fruits (Martins et al., 2014).

The GC–MS analysis of the VO (Table 1) revealed identification of 26 compounds corresponding to 97.1% of the total number of compounds in the VO. A comparison of the chemical groups in the VO of *S. molle* presents a largest fraction of monoterpenes (52.8%), of which 38.8% are monoterpenes hydrocarbons.

Volatile oils of aerial parts of *S. molle* have been studied and some authors have reported that the major compounds of the

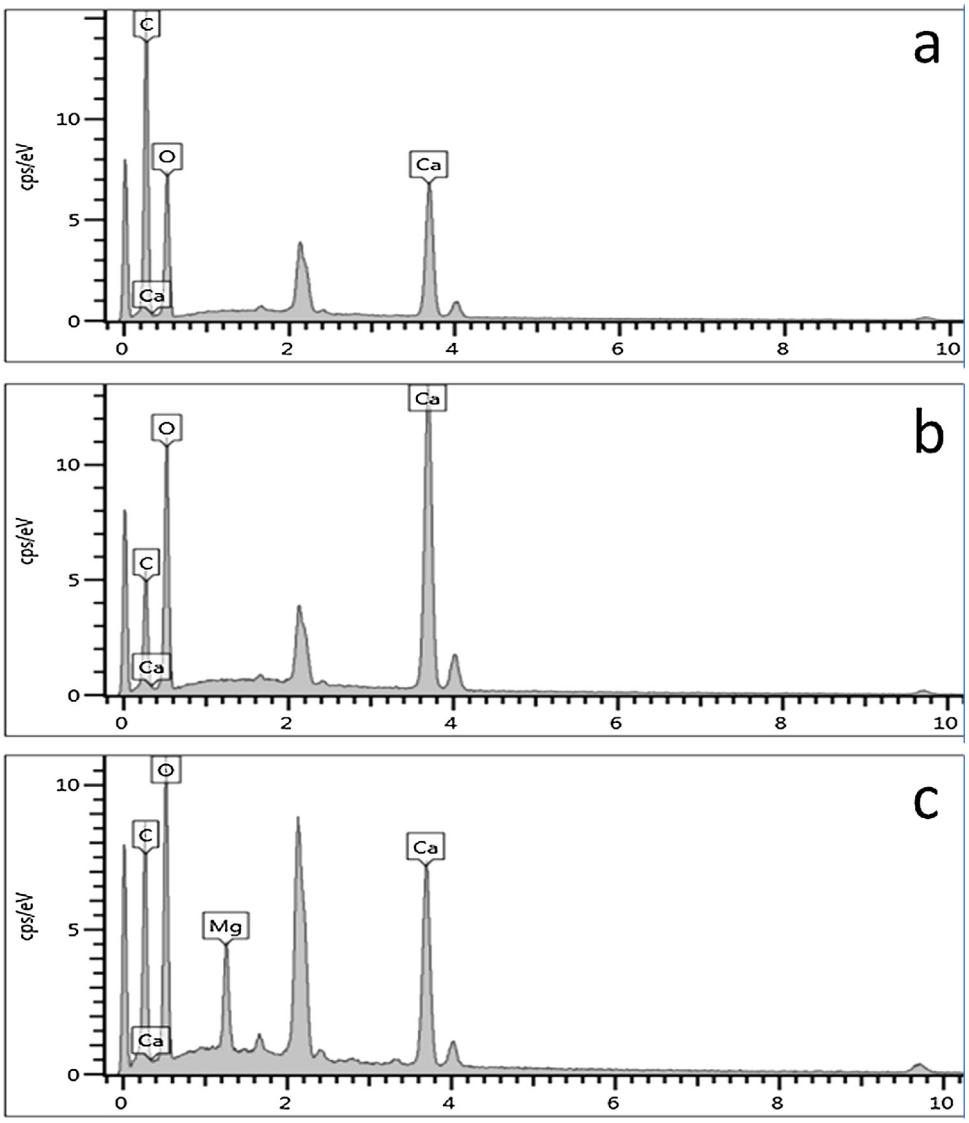

Fig. 5. EDS spectrum of prismatic crystal (a), druse (b) and platy aggregation cluster (c) of *Schinus molle*.

VO from leaves and fruits are monoterpenes (Abdel-Sattar et al., 2010; Gomes et al., 2013). However, sesquiterpenes were the main components in different studies by Simionatto et al. (2011) and Cavalcanti et al. (2015).

The main components of VO of *S. molle* were the monoterpenes β-pinene (14.7%), α-pinene (14.1%), limonene (9.4%) and the sesquiterpene muurolol (11.8%). There are significant differences in the chemical composition of VO from the leaves of this species collected in different locations within Brazil. Gomes et al. (2013) analyzed eleven Brazilian populations of *S. molle* from Rio Grande do Sul, and classified the samples in four groups based on their VO composition. These groups were characterized by the presence of the compounds sabinene, α and β-pinene, α-cadinol and myrcene.

Muhd et al. (2015) found bicyclogermacrene (20.5%), β-caryophyllene (19.7%) and spathulenol (19.2%) as the major compounds in VO from leaves of *S. molle* collected in Santa Maria, Brazil. Cavalcanti et al. (2015) reported cubenol (27.1%), caryophyllene oxide (15.3%) and spathulenol (12.4%) as the major components of VO from the leaves of *S. molle* collected in Rio de Janeiro, Brazil.

The differences in VO chemical compositions within species may be due to variations in the environmental and edaphic factors, methods and parts of the plant used for VO extraction, process employed for drying the plant material or storage conditions (Gobbo-Neto and Lopes, 2007; Lemos et al., 2012; Saulle et al., 2018). Although the VO chemical composition is often associated to phenological and ecological influences it is necessary to investigate if these variations may be related to different chemotypes.

All the components of VO could act synergistically and could be responsible for VO biological activities (Cimanga et al., 2002). Among the major compounds present in VO of *S. molle*, α and β-pinene presented antimicrobial, insecticidal and fungicidal activities, although their isomers and enantiomers can show different activities (Silva et al., 2012), and muurolol shows strong antifungal activity against *Lenzites betulina*, *Pycnoporus coccineus*, *Trametes versicolor* and *Laetiporus sulphureus* (Cheng et al., 2004). Limonene showed insecticidal activity against insect parasitoids of pet animals, reducing flea infestations by 80% in cats and being toxic to all life stages of the cat flea. No adverse effects on liver and kidney functions in cats were observed (Ibrahim et al., 2001).

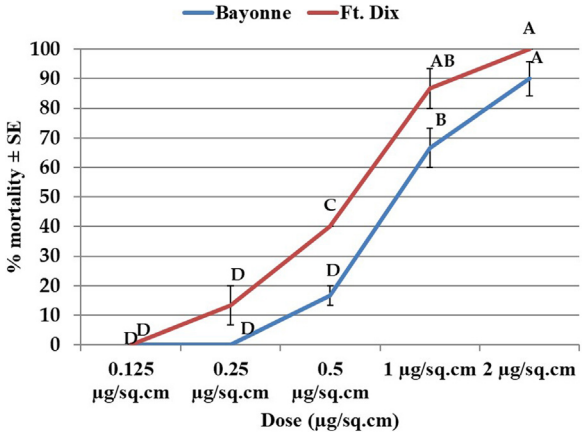

Fig. 6. Percent mean (±SE) mortality of adult bed bugs against various doses of volatile oil of *Schinus molle* 24 h after the treatment in vapor toxicity test. Different letters in the graphs indicate significant difference (two-factor ANOVA, $p < 0.05$; Tukey's HSD: three replicates with 10 bed bugs/replicate) JMP 10.0. DDVP (Standard) produced 100% mortality in both the strains at 0.05% (1 µg/125 sq. cm) concentration. Lethality, probit analysis SAS® 9.3 Bayonne: $LD_{50, 90}$ (µg/125 sq .cm), (95%CI): 53.9 (45.33–64.31), LD_{90}: 110.5 (88.56–157.42), Chi. Sq. (46.37), df (13); Ft. Dix: LD_{50}: 33.2 (27.9–39.54), LD_{90}: 68.7 (55.17–96.52), Chi. Sqi. (51.56), df (13).

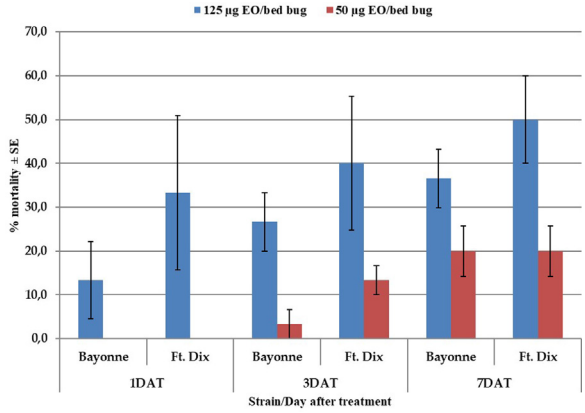

Fig. 7. Percent mean (±SE) mortality of adult bed bugs against two doses of volatile oil of *Schinus molle* when applied topically. Means and std. error calculated in JMP 10. Deltamethrin (Standard) produced 100% mortality in Bayonne strain and 65% in Ft. Dix at 0.0024 µg/bug. DAT (day after treatment).

Insecticidal studies

The results revealed that VO of *S. molle* is toxic to bed bugs as a fumigant although its effect was weak when the VO was applied topically and not active in residual application. Vapors of the VO of *S. molle* are toxic to bed bug (Fig. 6). The VO produced 100.0 ± 0.00% (Ft. Dix) and 90.0 ± 5.77% (Bayonne) of mortality at 2 µg VO/sq. cm (250 µg/125 sq. cm) 24 h after the treatment. In the Bayonne strain, mortality was 66.7 ± 6.67%, 16.7 ± 3.33% at 2 µg and 1 µg/sq. cm, respectively while in Ft. Dix strain, mortality was 86.7 ± 6.67%, 40.0 ± 0.00%, and 13.3 ± 6.67% at 2 µg, 1 µg, 0.5 µg VO/sq. cm. No mortality was observed at 0.125 µg/sq. cm in both the strains.

The benefits of botanical insecticides, mainly VO, may comprise the synergistic properties of its compounds, lower production prices and eco-friendly compounds for insect control (Yunes et al., 2001). Several VO derived from plants are classified as 'minimum risk' insecticides that require no registration with the US Environmental Protection Agency. Thus, they have become attractive in formulations of pest control products (Feldlaufer and Ulrich, 2015).

In that sense, this exploratory study was devoted to inquire about the insecticidal potential of VO of *S. molle* against bed bugs because of increasing demands for information about effective control tactics and their public health risks especially in relation to eco-friendly approaches such as natural volatile oils.

The VO of *S. molle* topically applied on the dorsal side of the adult bed bug did not cause significant mortality (Fig. 7). Percent mortality reached 36.6 ± 6.67% (Bayonne) and 50.0 ± 10% (Ft. Dix) 7 days after the treatment at 125 µg VO/bed bug. In the residual bioassay, 10% of the bugs died after 7 days of exposure at the highest concentration of 100 µg/cm^2. Considering these results, the VO of *S. molle* was weak when applied topically and did not possess any residual action.

The insecticidal investigations of the VO of *S. molle* demonstrated it to be a strong fumigant. The concentration of 2 µg/sq. cm (250 µg/125 sq. cm) in 24 h exposure time produced 100% mortality. The fumigation toxicity may be due to highly volatile major compounds, such as β-pinene, α-pinene, muurolol and limonene. We will further investigate these compounds in a follow up study. To the best of our knowledge, this is the first report on insecticidal activity *S. molle* VO against bed bugs.

Fumigant activity of VO of *S. terebinthifolia* and its isolated constituents were tested against *Rhyzopertha dominica* and the properties of the pure VO and artificial mixtures suggested that the action of these VO cannot be attributed only to the individual toxicity of each compound, but rather to the content of each component and interactions (synergistic and antagonistic effects) among the components (Nascimento et al., 2018). The same reasoning could be used to the *S. molle* in which the possible interaction among the volatile components might explain the fumigation effect observed. This information could be applied to the integrated management program of bed bugs.

Conclusions

The main anatomical features of *S. molle* are highlighted in this study, which was performed to provide more information about the standardization of the species in order to support the quality control of this vegetable material. The following characteristics are helpful when conducting the quality control procedure: isobilateral and amphistomatic leaves; anomocytic and cyclocytic stomata; capitate glandular and conical non-glandular trichomes; flat midrib shape on both sides; two large secretory ducts in the midrib; petiole with 5 collateral vascular bundles arranged in U-shape and one dorsal bundle; circular stem, and perivascular fiber caps around the ducts.

The histochemical tests showed the presence of phenolic compounds in the epidermis, subepidermal layer and mesophyll in the leaf blade; epidermis, ground parenchyma and around the ducts in the midrib and petiole; and in the epidermis, cortex and perimedullary region of the stem. Starch grains are oval and rounded, occur in compound aggregates. Lignified elements were met in the perivascular fiber caps and the xylem.

The volatile oil presents a high fraction of monoterpenes (52.8%), of which 38.8% are monoterpene hydrocarbons. The main components of VO are β-pinene (14.7%), α-pinene (14.1%), limonene (9.4%) and muurolol (11.8%). There is significant variability in the chemical composition of VO described is this study as well as reported in the literature.

Schinus molle volatile oil did not show good activity when applied directly (topically) or as residues on filter paper, whereas exhibited strong fumigation toxicity against bed bugs. This finding paves the way for further *in vivo* investigations intended at developing a safe and active phytoinsecticide.

Authors' contributions

CDM collected the plant, extracted the volatile oil and carried out the anatomical analysis. VPA performed the anatomical analysis. RZS supervised the anatomical analysis. JUR carried out the insecticidal assays. JMB carried out the anatomy analysis and wrote the manuscript. BHLNSM and EKM performed the volatile oil analysis. VR carried out the anatomical analysis. JMB and PVF created the project and supervised the laboratory work. VR and IAK provided critical reading and insightful recommendations of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

CDM thanks to the Araucaria Foundation for the scholarship. The authors would like to thank the Electron Microscopy Center of the c-LABMU at the State University of Ponta Grossa for providing the FESEM images and EDS spectra. The bed bug research is supported by USDA-Discovery & Development of Natural Products based insect management for medical, veterinary & Urban (58-6066-6-043).

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