In vitro efficacy of essential oils and extracts of *Schinus molle* L. against *Ctenocephalides felis felis*.

LILIAN C. DE S. O. BATISTA[1], YARA P. CID[1], ANA PAULA DE ALMEIDA[2], EDLENE R. PRUDÊNCIO[3], CRISTIANO J. RIGER[3], MARCO A. A. DE SOUZA[3], KATHERINE COUMENDOUROS[1] *and* DOUGLAS S. A. CHAVES[4]*

[1] *Department of Veterinary Parasitology, Federal Rural University of Rio de Janeiro, CEP 23897-000, Seropédica, Rio de Janeiro, Brazil*
[2] *Laboratório de Estudo Químico e Farmacológico de Produtos Naturais e Derivados LAEQUIFAR-PD, Universidade Severino Sombra (USS), Rio de Janeiro, Brazil*
[3] *Department of Chemistry, Federal Rural University of Rio de Janeiro, CEP 23897-000, Seropédica, Rio de Janeiro, Brazil*
[4] *Department of Pharmaceutical Science, Federal Rural University of Rio de Janeiro, CEP 23897-000, Seropédica, Rio de Janeiro, Brazil*

(*Received 16 October 2015; revised 29 December 2015; accepted 1 January 2016; first published online 18 February 2016*)

SUMMARY

Extracts and essential oils from plants are important natural sources of pesticides. These compounds are considered an alternative to control ectoparasites of veterinary importance. *Schinus molle*, an endemic species of Brazil, produces a high level of essential oil and several other compounds. The aim of this work was to determinate the chemical composition of extracts and essential oils of *S. molle* and further to evaluate the activity against eggs and adults of *Ctenocephalides felis felis*, a predominant flea that infests dogs and cats in Brazil. In an *in vitro* assay, the non-polar (*n*-hexane) extract showed 100% efficacy (800 μg cm^{-2}; LD_{50} = 524·80 μg cm^{-2}) at 24 and 48 h. Its major compound was lupenone (50·25%). Essential oils from fruits and leaves were evaluated, and had 100% efficacy against adult fleas at 800 μg cm^{-2} (LD_{50} = 353·95 μg cm^{-2}) and at 50 μg cm^{-2} (LD_{50} = 12·02 μg cm^{-2}), respectively. On the other hand, the essential oil from fruits and leaves was not active against flea eggs. This is the first study that reports the insecticidal effects of essential oils and extracts obtained from *Schinus molle* against *Ctenocephalides felis felis*.

Key words: Essential oil, Brazilian plants, cat flea, toxicity, *Saccharomyces cerevisiae*, triterpenes, sesquiterpenes, interdisciplinary study.

INTRODUCTION

Ctenocephalides felis felis (Bouché), the cat flea, is among the ectoparasites of cats and dogs with highest economic importance worldwide. In the USA and Europa, another flea subspecies (*Ctenocephalides canis* – dog flea) is found (Bitam *et al*. 2010). According to Dryden *et al*. (2013), when cat fleas acquire a host, they mate and start laying eggs within 24–48 h. Female cat fleas can produce 40–50 eggs per day.

The infestation by this flea causes several problems, such as allergic dermatitis and discomfort to pets and their owners. This flea has also been implicated in the transmission of bacteria such as *Rickettsia typhi, Rickettsia felis, Bartonella henselae* and other *Bartonella* species (Blagburn and Dryden, 2009). Annual spending to control fleas on pets is estimated at about US$ 1 billion in the USA and € 1·1 billion in Western Europe (Rust, 2005).

Cat fleas have become resistant to some conventional treatments, which are based on residual topical or oral medications. The first resistance reported for *C. felis felis* was in 1952 to dichloro-diphenyl-trichloroethane (DDT), followed by dieldrin in 1956. Strains of *C. felis felis* have also been found to be resistant to organophosphates, pyrethroids, pyrethrins, organochlorines, carbamates and fipronil (Coles and Dryden, 2014). Studies suggest there are three necessary conditions for evolution of resistance to occur: individuals in the population must differ genetically; genetic differences must produce a phenotypic difference; and the phenotypic difference must enhance survivability, transferring the resistance to the next generation (Coles and Dryden, 2014).

Studies have investigated many properties of plant-based repellents, such as essential oil of *Cinnamomum osmophloeum, Taiwania cryptomerioides, Plectranthus amboinicus, Calendula officinalis* L., *Allium sativum* L. and other plants (Stamopoulos *et al*. 2007; Lans *et al*. 2008; Ellse and Wall, 2013; Sparagano *et al*. 2013; Su *et al*. 2013). The use of plants and other natural sources as biological agents is increasing due

* Corresponding author. Departamento de Ciências Farmacêuticas (DCFar) – Laboratório de Química de Bioativos Naturais (LQBioN), Universidade Federal Rural do Rio de Janeiro, Instituto de Ciências Biológicas e da Saúde, Br 465, Km 7/sala 33, 23897-000 – Seropédica, *Rio de Janeiro*, Brazil. Tel./Fax: +55-21-2682-2807. E-mail: gnosy.ufrrj@gmail.com; chavesdsa@ufrrj.br

Parasitology (2016), **143**, 627–638.
doi:10.1017/S0031182016000081

to rising environmental concern among scientists and the public at large and the quest for healthier living habits, as well as the greater difficulty of treating certain diseases (Sousa *et al*. 2008; Souza-Moreira *et al*. 2010). Brazil is known for its great diversity of flora, and has many plants that are sources of natural therapeutic products.

Schinus molle L. (Anacardiaceae), popularly known as 'aroeira branca', is a native Brazilian species (Downer *et al*. 1988; Silva-Luz and Pirani, 2014). It is also known as felfelkazib or filfilrafie in Arabic, while in English it is known with various names including; pepper tree, California pepper tree, Chilean pepper tree, mastic tree, molle, pepper berry tree, weeping pepper, Peruvian mastic, pink pepper or Peruvian pepper tree (Ibrahim and Al-Naser, 2014). Its pepper fruits (*poivre-rose*) are greatly appreciated in European cooking (Cavalcanti *et al*. 2015). This native species occurs throughout the Midwest and South of Brazil, especially in coastal regions, where it has important economic and social value because in addition to use as a food seasoning, the essential oils are also used for medicinal and cosmetic purposes (Di Stasi and Hiruma-Lima, 2002).

The leaves contain essential oils, tannins, alkaloids and biflavonoids, which are used in folk medicine as antimicrobial, antifungal, antispasmodic, antipyretic and anti-inflammatory agents (Marongiu *et al*. 2004). Several studies have focused on identifying the components of this plant's extracts (Fourie *et al*. 2005; Lans *et al*. 2008; Sparagano *et al*. 2013). Furthermore, the essential oils and their components are reported to have strong repellent activity against several arthropods of veterinary importance (Ellse and Wall, 2013; Sparagano *et al*. 2013; Su *et al*. 2013). These highly volatile essential oils are an interesting option for the control of fleas on dogs and cats, considering that volatile compounds usually are safe for use on pets (although some can have adverse effects in dogs – Genovese *et al*. 2012; Addie *et al*. 2015) and humans and have selectivity to natural enemies, biodegradability, low cost and low environmental impact (Dayan *et al*. 2009).

Our goals were to evaluate the activity of the extracts and essential oils of *Schinus molle* L. against *C. felis felis* and to evaluate the cytotoxicity in eukaryotic cells.

MATERIALS AND METHODS

Plant material

The leaves and mature fruits of *S. molle* tree were collected during the summer period at Volta Redonda city, Rio de Janeiro, Brazil (GPS 22°31′36·23S; 44°04′31·62W). A specimen voucher was deposited in the herbarium of the Institute of Botany (UFRRJ, Brazil), and identified by Dr Pedro Germano Filho with the code RBR 35791. *Schinus molle* was dried at room temperature, protected from light and moisture.

Plant extracts

Extracts of leaves (100 g) from *S. molle* were obtained by sequential Soxhlet extraction with different solvents (1000 mL each) using increasing polarity (*n*-hexane, ethyl acetate and methanol). The extraction time was 2 h for each solvent. The resulting extracts were evaporated using rotatory evaporator affording hexanic (SmH), ethyl acetate (SmAe) and methanol (SmM) extracts.

Extraction, content (% w/w) and chemical characterization of the essential oil

Samples weighing 50 g of *S. molle,* consisting of dried aerial parts (leaves and fruits separately), were subjected to hydrodistillation using a Clevenger type apparatus. Aliquots of the essential oils (EO) at time intervals of 30, 60, 120, 180 and 240 min were taken to analyse the chemical composition for leaves, or 30, 60, 120, 240 and 360 min for fruits. The EO were dried over anhydrous sodium sulphate and concentrated with N_2 gas. The content (% w/w) of all fractions of EO collected was evaluated in triplicate and chemical analysis was performed by gas chromatography-mass spectrometry (GC-MS).

A gas chromatograph coupled to a mass spectrometer (Shimadzu QP-2010 Plus) was used for separation, detection and quantification the of *S. molle* EO components. The compounds were separated in a fused silica capillary column (5% diphenyl and 95% dimethylsiloxane), with 30 m × 0·25 mm (id) × 0·25 μm of film thickness. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}.

Injector and detector temperatures were set at 220 and 250 °C, respectively. Oven temperature was kept at 60 °C for 2 min, with increase of 5 °C min^{-1} to 110 °C followed by an increase of 3 °C min^{-1} to 150 °C, and finally, 15 °C min^{-1} to 290 °C, kept constant for 15 min. To separate and identify the substances, 1·0 μL of essential oil samples (10 μL mL^{-1}), in the defined times, were injected manually and in splitless mode. Mass spectra were obtained with a quadrupole detector operating at 70 eV, with mass range 40–400 *m*/*z* and a ratio of 0·5 scan s^{-1}.

The identification of EO was based on comparison of retention times (RT) against the data in the National Institute of Standards and Technology (NIST) database (2008) and retention index (RI) from the literature (Adams, 1995). RI was obtained based on co-injection of samples with a mixture of hydrocarbons, C8-C40 (Sigma-Aldrich, USA), and computed based on the literature (Van den Dool and Kratz, 1963).

Insecticidal activity in vitro *of* S. molle *on* C. felis felis *eggs*

The *in vitro* testing was realized using the filter paper impregnation method with a stock solution at a

https://doi.org/10.1017/S0031182016000081 Published online by Cambridge University Press

concentration of 200 mg mL^{-1} of the *S. molle* essential oils from fruits and leaves. Both oils were diluted in acetone, the solvent able to dissolve the oil. Acetone alone was used as the negative control.

Ten 1:2 serial dilutions were obtained from the stock solution, starting at a concentration of 800 μg cm^{-2}. Two repetitions were performed for each concentration, with filter paper strips measuring 10 cm^2 (1 cm wide and 10 cm long). Each strip was impregnated with 0·2 mL of the respective dilution, for a total of 20 strips, plus two for the negative control group, two for a positive control with phenylpyrazole (fipronil at 400 μg cm^{-2}) and two for another positive control using pyriproxyfen 400 μg cm^{-2} (an insect growth regulator). After the treatment, the strips were left in the open to dry for 30 min. The dried strips were then placed in test tubes containing 10 *C. felis felis* eggs along with a substrate necessary for larval development, consisting of sand, wheat bran and fecal material from adult fleas from a colony maintained since 1998 in the Laboratory for Experimental Chemotherapy in Veterinary Parasitology of Federal Rural University of Rio de Janeiro (UFRRJ). The tubes were sealed with non-woven tissue and rubber bands and kept in a climatized chamber at 28 ± 1 °C and relative humidity of 75 ± 10%. To evaluate the efficiency of each test dilution, the material in each tube was fixed in 70°GL alcohol 30 days after the incubation and then the number of adults that had emerged from the puparia were counted with the aid of a stereoscopic microscope.

The formula of Abbott (1987) was used to calculate the efficacy: Per cent efficacy = [(mean number of adult fleas of the control group – mean number of adult fleas from the treated group)/(mean number of adult fleas from the control group)] × 100.

In vitro *insecticidal activity of the extracts and essential oil of* S. molle *against* C. felis felis *adults*

In vitro insecticidal activity against *C. felis felis* adults was tested using the filter paper tests against unfed fleas obtained from a laboratory colony. The experiments followed the standards established by the Ethics Committee for Animal Use of the Institute of Biology (CEUA/IB).

Leaf extracts (SmH, SmAe and SmM) were diluted in *n*-hexane, ethyl acetate and methanol, respectively, and the final concentration ranging from 250 to 40 000 μg mL^{-1}, and negative controls were realized with each solvent. For the tests with EO using *S. molle* leaf extracts (EOL) and fruits (EOF), the concentration ranging from 78·125 to 40 000 μg mL^{-1} were obtained using acetone as solvent and negative control.

Strips of filter paper, 1 cm wide and 10 cm long, were impregnated with 0·2 mL of the each EO and extracts, and strips impregnated with acetone were used as negative control. Fipronil at 400 μg cm^{-2} was used as positive control. After drying at room temperature for 30 min, the strips were inserted into glass tubes containing 10 unfed adult cat fleas (five males and five females). The assay was conducted in duplicate as described by Batista *et al.* (2014). After application, the strips of paper had a final concentration ranging from 25 to 800 μg cm^{-2} for (SmH, SmAe and SmM) and 1·562–800 μg cm^{-2} of fruits and leaves EO.

The tubes were sealed with parafilm and rubber bands and kept in climatized chamber at 28 ± 1 °C and 75 ± 10% relative humidity. The motility evaluations were carried out at 10 min, 30 min, 1, 2, 24 and 48 h. Flea activity was monitored after gently tapping the glass tubes on a hard surface. Tubes where all fleas showed normal occasional jumps and coordinated movements were evaluated as zero efficacy. Fleas with uncoordinated movements or fleas that lay on one side showing only weak leg movements were counted as alive despite the fact that they would not be capable of infesting or feeding on a host. Fleas not moving at all after stimulation by tapping the tubes were counted as dead (Batista *et al.* 2014).

Normality of the data set was tested using the Shapiro–Wilk test. Statistical significance was set at 5% ($P < 0·05$). Mortality of adult flea at 24 and 48 h from treated and control groups were compared by the *t*-test using a significance level of $P < 0·05$. The estimate of LD_{50} (lethal dose – which killed 50% of the treated population) was done by probit analysis using Minitab® 16 (2013, Minitab Inc.).

Yeast strains and growth conditions

A wild strain of *Saccharomyces cerevisiae* BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) (Euroscarf, Frankfurt, Germany) was maintained on solid 2% yeast extract-peptone-dextrose (YPD) (1% yeast extract, 2% glucose, 2% peptone, and 2% agar). Cells were grown up to the middle exponential phase (about 10^6 cells mL^{-1}) in liquid 2% YPD medium using an orbital shaker at 28 °C and 160 rpm with the flask volume/medium ratio of 5/1 (Castro *et al.* 2008). The essential oils were solubilized in 1% dimethyl sulfoxide (DMSO), which was used as negative control.

Cell viability and mitochondrial damage

Cells were re-inoculated into fresh liquid medium containing 0, 78·125, 312·5 and 1250 μg mL^{-1} (1·562, 6·25 and 25·00 μg cm^{-2}, respectively) of fruit and leaf EO and incubated at 28 °C and 160 rpm. Initial cell concentration was 10^5 cells mL^{-1}. At 1, 6 and 24 h, cells from control tubes (1% DMSO) were diluted with 50 mM of phosphate buffer (pH 6·0) and plated on YPD plates and YPGLy; cells that were exposed to the EO of fruits and leaves were diluted in the same buffer and

https://doi.org/10.1017/S0031182016000081 Published online by Cambridge University Press

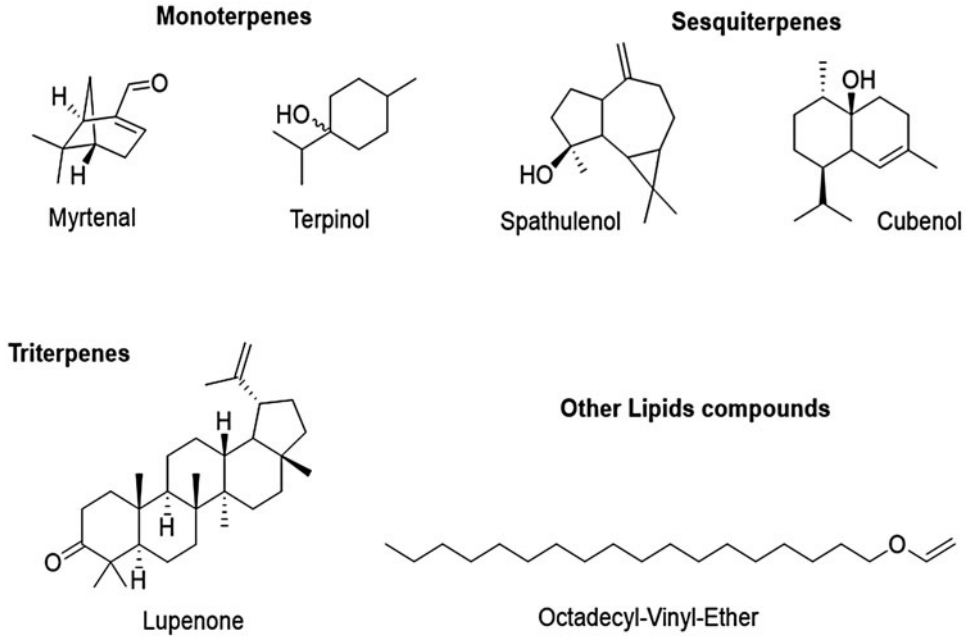

Fig. 1. Major substances obtained from *Schinus molle* L. Triterpenes and lipids were obtained by Soxhlet extraction (1 h); myrtenal, terpinol, spathulenol and cubenol (monoterpenes and sesquiterpenes, respectively) were obtained by hydrodistillation from leaves and fruits of *S. molle*.

plated on both YPD and YPGly plates (1% yeast extract, 4% glycerol, 2% peptone and 2% agar). YPGly was used to test the inability of cells to grow on an aerobic restricted medium (glycerol) due to the damage in the mitochondria. The assay was conducted in triplicate. The viable number of cells was determined after 72 h of growth. Viability was measured as percentage of viable cells, grown on YPD plates that survived after incubation with essential oils. The damage rates caused by essential oils were measured as the number of colonies counted on YPGly plates in relation to colonies counted on YPD plates (Jin *et al*. 1998).

RESULTS

Chemical analysis of plant extracts

The hexane extract (SmH) was found to contain eight identifiable compounds: spathulenol (8·9%), caryophyllene oxide (0·7%), 14-heptadecenal (1·3%), *n*-octadecanal (8·7%), *Z*-2-octadecen-1-ol (1·9%), octadecyl-vinyl-ether (21·5%), lupenone (50·5%), lupeol (2·9%), along with two unidentified compounds (3·6% together) (Fig. 1). These compounds were identified previously from plants of the genus *Schinus* and some of them in *S. molle* (Masateru *et al*. 2008; Carvalho *et al*. 2013). Analysis of methanol (SmM) and ethyl acetate (SmAe) extracts were not performed, considering that the samples were inactive in the biological model studied.

The hyperbolic distribution describing the extraction of *S. molle* essential oils in function of time showed maximum values equal to 0·99% (± 0·03) and 2·4% (± 0·04) for leaves and fruits, respectively.

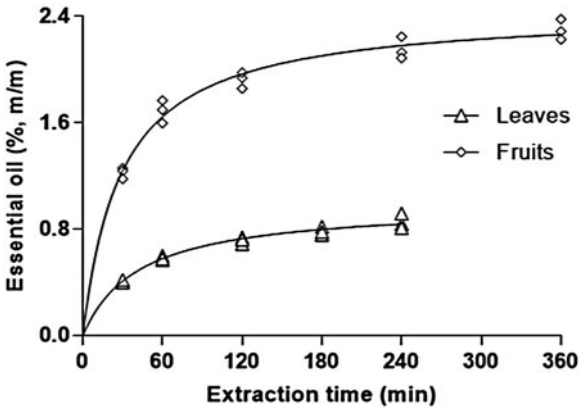

Fig. 2. % w/w of essential oil obtained by hydrodistillation of leaves and fruits dried, from *Schinus molle* L. in function of time (30, 60, 120, 180, 240 and 360 min).

The estimated extraction time was longer than 20 h (Fig. 2); but an initial asymptotic pattern was observed at 240 and 360 min for the leaves and fruits, respectively. The analysis of the essential oil from the *S. molle* leaves and fruits (EOL and EOF) showed differences in the composition and chemicals (Table 1 and Fig. 3). The main substances found in the leaves were spathulenol and cubenol (sesquiterpenes), while in the fruits they were 4-terpineol and myrtenal (monoterpenes) and the spathulenol.

In vitro *insecticidal activity of* S. molle *against eggs and adults of* C. felis felis

The activity of the essential oils from leaves at the concentration of 800 μg cm^{-2} was 10·53%, while efficacy

https://doi.org/10.1017/S0031182016000081 Published online by Cambridge University Press

Table 1. Chemical profile of *Schinus molle* essential oil obtained by hydrodistillation of leaves and fruits dried at room temperature protected from light and moisture

Order elution	Retention time	Substances	Kovats index	Retention index	EOL	EOF	Identification method
1	5·580	*α*-pinene	*939*	*935*	3·4	tr	*a, b*
2	6·881	Sabinene	*976*	*977*	0·6	–	*a, b*
3	7·048	*β*-pinene	*980*	*982*	6·7	tr	*a, b*
4	12·984	Linalool	*1098*	*1114*	–	1·0	*a, b*
5	14·108	Methyl octanoate	*1120*	*1133*	*tr*	4·3	*a, b*
6	14·283	*p*-menth-2-en-1-ol	*1121*	*1136*	*tr*	1·8	*b*
7	14·403	*α*-campholenal	*1125*	*1138*	1·1	tr	*a, b*
8	15·294	*trans*-pinocarveol	*1139*	*1154*	6·2	5·7	*a, b*
9	15·432	*cis*-*β*-terpineol	*1144*	*1156*	tr	2·0	*a*
10	15·703	*trans*-verbenol	*1144*	*1161*	2·8	tr	*b*
11	16·549	Pinocarvone	*1162*	*1176*	2·2	tr	*a*
12	17·293	*α*-phellandren-8-ol	*1166*	*1189*	1·6	4·6	*a*
13	17·521	4-terpineol	*1177*	*1193*	1·1	18·5	*a, b*
14	18·622	Myrtenal	*1193*	*1211*	5·1	20·9	*a, b*
15	18·853	Myrtenol	*1194*	*1215*	3·9	4·3	*a, b*
16	19·520	Verbenone	*1204*	*1226*	2·0	4·0	*a, b*
17	20·334	*cis*-carveol	*1217*	*1239*	0·8	–	*a, b*
18	31·406	*β*-caryophyllene	*1418*	*1425*	4·6	1·3	*a, b*
19	33·325	*α*-caryophyllene	*1454*	*1463*	0·8	tr	*a, b*
20	33·557	Alloaromadendrene	*1461*	*1467*	4·3	tr	*a, b*
21	34·634	*γ*-muurolene	*1477*	*1489*	2·3	2·3	*b*
22	35·407	Bicyclogermacrene	*1494*	*1505*	2·3	1·1	*b*
23	35·578	*α*-muurolene	*1499*	*1508*	1·4	tr	*a, b*
24	36·216	*trans*-*β*-guaiene	*1500*	*1523*	6·2	0·8	*b*
25	36·488	*γ*-cadinene	*1513*	*1529*	2·8	Tr	*a, b*
26	37·306	*α*-cadinene	*1538*	*1548*	0·9	Tr	*a, b*
27	39·250	Germacrenol	*1574*	*1592*	1·2	–	*a, b*
28	39·504	Spathulenol	*1576*	*1598*	11·7	14·5	*a, b*
29	39·809	Globulol	*1583*	*1607*	2·1	1·2	*a, b*
30	40·138	Gleenol	*1585*	*1618*	2·2	–	*b*
31	40·510	Ni		*1631*	1·3	–	
32	40·703	Humulene epoxide II	*1606*	*1638*	1·0	tr	*b*
33	40·880	(1, 10-di-epi)-cubenol	*1614*	*1644*	3·0	1·1	*b*
34	41·297	(1-epi)-cubenol	*1627*	*1658*	0·4	–	*b*
35	41·765	Cubenol	*1642*	*1674*	13·0	8·2	*a, b*
36	42·082	*α*-cadinol	*1653*	*1685*	1·0	2·4	*a, b*

OE, elution order; RT, retention time; KI, Kovats index; RIC, retention index calculated using alkanes series; identification: a – gas chromatography-mass spectrometry (GC-MS) (Nist Library, 98); b – mass spectrum of literature (Adams, 1995) with the aid of the RIC; less than 0·1% concentration (tr); not detected (–); not identified (ni).

at the concentration of 100 μg cm^{-2} was 5·26%. The other concentrations were not effective. The fruit essential oil had efficacy of 10·5% at concentrations of 800 and 50 μg cm^{-2}, and efficacy of 5·3% at concentrations of 200 and 3125 μg cm^{-2}. The positive controls, consisting of fipronil and pyriproxyfen applied at 400 μg cm^{-2}, were 100% effective, demonstrating that the method was employed correctly.

The mortality of *C. felis felis* caused by hexane (SmH), ethyl acetate (SmAe) and methanol (SmM) extracts from leaves was significantly different from the control group at 48 h [SmH ($P = 0{\cdot}0034$), SmM ($P = 0{\cdot}0062$) and SmAe ($P = 0{\cdot}0051$)]. The SmH extract showed efficacy of 100% at 800 μg cm^{-2}, while SmAe and SmH did not show a significant efficacy at 800 μg cm^{-2} (5 and 40%, respectively at 48 h). *Ctenocephalides felis felis* bioassays with different concentrations of the extracts of *S. molle* showed that only SmH extract had insecticidal activity. The LD_{50} values were 524·80 and 168·57 μg cm^{-2} after 24 and 48 h, respectively (Table 2).

The percentage (%) of mortality and efficacy of the two essential oils (EOL and EOF) against *C. felis felis* were determined by the filter paper test. The combined data and curve fitting statistics are reported in Table 3.

Efficacy for both EOL and EOF were observed at 24 and 48 h. EOF achieved 100% efficacy at the highest concentration tested (800 μg cm^{-2}), both at 24 and 48 h. Although at 24 h, the mortality induced by EOF was not significantly different from the control group ($P = 0{\cdot}0855$), it was significantly different at 48 h ($P = 0{\cdot}0201$). Leaf essential oil (EOL) achieved 100% efficacy at lower concentration (50 μg cm^{-2})

https://doi.org/10.1017/S0031182016000081 Published online by Cambridge University Press

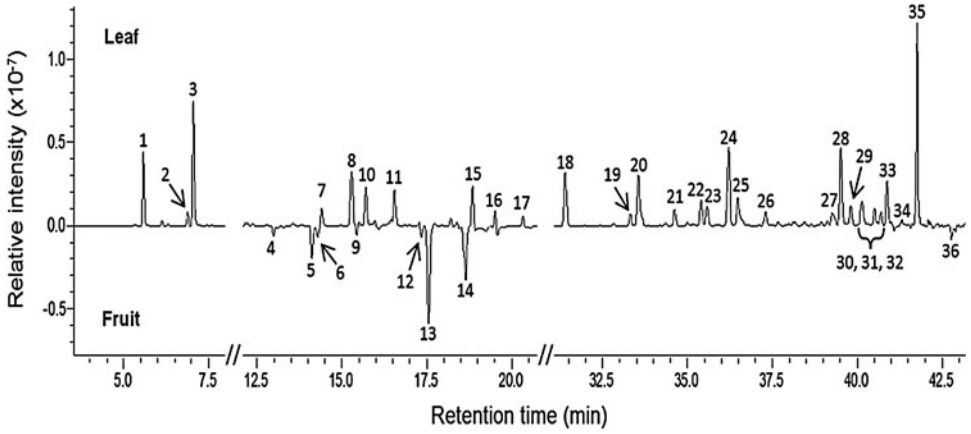

Fig. 3. Chemical profile of *Schinus molle* L. essential oil obtained by hydrodistillation of the leaves (above) and fruits (below), considering the difference (subtraction) of the same components (or same retention time). The numbers in order of elution (OE) are listed in Table 1.

Table 2. % Mortality of *Ctenocephalides felis felis* through filter paper test, using *Schinus molle* hexanic, methanolic and ethyl acetate extracts

	Schinus molle extract					
Extract dose (μg cm^{-2})	Hexanic (SmH)		Methanol (SmM)		Ethyl Acetate (SmAe)	
Time	24 h	48 h	24 h	48 h	24 h	48 h
800	75	100	10	40	0	5
400	25	55	20	40	0	15
200	35	65	10	35	5	20
100	0	25	5	20	0	20
50	0	10	20	35	20	40
25	10	20	25	40	5	25
LD_{50} (μg cm^{-2})	524·80	168·57	–	–	–	–
Slope	−4·35	−3·66	–	–	–	–
Normality test[a]	Passed	Passed	Passed	Passed	Passed	Passed
P values[b]	0·093	0·0034	0·125	0·0062	0·152	0·0051

[a] Normality (Shapiro–wilk).
[b] *P* values (mortality *vs*. control), *t*-test and probit analysis were performed for all data from both trials, using Minitab® 16 (2013, Minitab Inc., LEADTOOLS, LEAD Technologies, Inc.).
Fipronil at 400 μg cm^{-2} was used as positive control to ensure viability of the colony.

and were statistically different at 24 h ($P = 0{\cdot}003$) and 48 h ($P = 0{\cdot}002$) from the control group.

EOL and EOF gave negative slope values, indicating reliability improvement in probit analysis. EOF showed higher LD_{50} values (353·95 and 138·22 μg cm^{-2}) than for EOL (12·02 and 9·10 μg cm^{-2}) at 24 and 48 h. Therefore, according to the filter paper test, EOL was more effective against *C. felis felis* (with highest potency at 24 h) than EOF.

Insecticidal activity in vivo *in* S. cerevisiae *cells*

The results showed total cell death. In this case, because of the high toxicity it was not possible to detect mitochondrial damage. Additionally, cell cultures not exposed to oil (control) and exposed only to 1% DMSO were analysed, in both cases showing normal growth of colonies.

In accordance with the LD_{50} values shown in Table 3, the toxicity assays from the leaf oil were performed with concentrations near the LD_{50}: 1250 (25·00); 312·5 (6·25) and 78·125 μg mL^{-1} (1·562 μg cm^{-2}), respectively, C1, C2 and C3. Incubation for 1 and 2 h with the EOF or EOL did not show high toxicity to *S. cerevisiae* yeast cells at different concentrations (Fig. 4). Despite the significant results in the analysis of mitochondrial damage after cell exposure to EOF and EOL for 1 and 2 h at three different concentrations, there was no decrease in cell viability, showing that mitochondrial damage is not sufficient to cause cell death (Fig. 5).

DISCUSSION

Increasing research interest has been directed at essential oils of many plants to find new formulations

https://doi.org/10.1017/S0031182016000081 Published online by Cambridge University Press

Table 3. % Mortality of *Ctenocephalides felis felis* through filter paper test, and efficacy of *Schinus molle* fruits and leaves essential oil fruits (EOF) and leaves (EOL)

Essential oil Dose (μg cm^{-2})	*Schinus molle* Essential oil							
	Fruits (EOF) Mortality		Efficacy		Leaves (EOL) Mortality		Efficacy	
Time	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
800	100	100	100	100	100	100	100	100
400	35	70	35	70	100	100	100	100
200	40	70	40	70	100	100	100	100
100	0	10	0	10	100	100	100	100
50	5	10	5	10	100	100	100	100
25	0	15	0	15	50	55	47·4	52·6
12·5	20	30	20	30	40	45	36·8	42·1
6·25	0	0	0	0	45	45	42·1	42·1
3·125	5	5	5	5	15	35	10·5	31·6
1·562	0	5	0	5	10	15	5·3	10·5
LD_{50} (μg cm^{-2})	353·95	138·22	–	–	12·02	9·10	–	–
Slope	−3·16	−2·55	–	–	−1·73	−1·27	–	–
Normality test[a]	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
P values[b]	0·0855	0·0201	–	–	0·0003	0·0002	–	–

[a] Normality (Shapiro–wilk).
[b] *P* values (mortality *vs*. control), *t*-test and probit analysis were performed for all data from both trials, using Minitab® 16 (2013, Minitab Inc., LEADTOOLS, LEAD Technologies, Inc.).
Fipronil at 400 μg cm^{-2} was used as positive control to ensure viability of the colony.

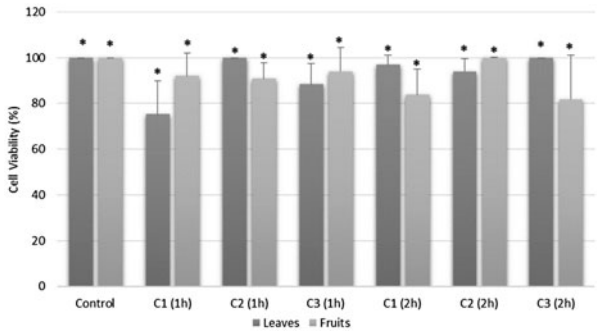

Fig. 4. Analysis of yeast survival after exposure to essential oils. *Saccharomyces cerevisiae* cells were submitted to (C1 = 1250 μg mL^{-1}), (C2 = 312·5 μg mL^{-1}) and (C3 = 78·125 μg mL^{-1}) during 1 and 2 h. The results represent the mean ± standard deviation of at least three independent experiments. Each group of results was separately analysed to determine statistical differences. Different symbols mean statistically different results at $P < 0·05$.

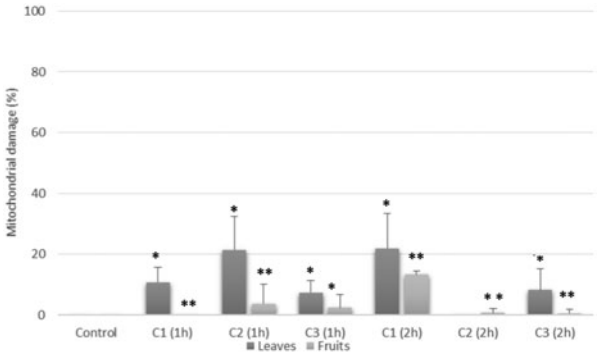

Fig. 5. Analysis of mitochondrial damage after exposure to essential oils. *Saccharomyces cerevisiae* cells were submitted to (C1 = 1250 μg mL^{-1}), (C2 = 312·5 μg mL^{-1}) and (C3 = 78·125 μg mL^{-1}) during 1 and 2 h. The results represent the mean ± standard deviation of at least three independent experiments. Each group of results was separately analysed to determine statistical differences. Different symbols mean statistically different results at $P < 0·05$.

for veterinary use and new active molecules, or even prototypes for the development of new drugs that are more effective and safer for use in veterinary medicine.

Different results have been reported regarding the content of essential oil extracted from *S. molle*, using different methods, such as supercritical fluid extraction (Barroso *et al*. 2011; Scopel *et al*. 2013), extraction from fresh plant parts (Murray *et al*. 2005; Benzi *et al*. 2009), soxhlet extraction (Silva *et al*. 2005), microwave extraction (Bousbia *et al*. 2009) and others. Only a few studies have been published using hydrodistillation, and the result of our study are similar to the published record (Barroso *et al*. 2011; Zahed *et al*. 2010; Gomes *et al*. 2013).

The chemical composition of the essential oils (Table 1) were consistent with the findings of other studies, except that the percentage of each compound, were different and we did not detect the presence of the monoterpenes myrcene, phellandrene and limonene in our samples (Murray *et al*. 2005; Silva *et al*. 2005; Benzi *et al*. 2009; Zahed *et al*.

https://doi.org/10.1017/S0031182016000081 Published online by Cambridge University Press

2010; Barroso *et al*. 2011; Gomes *et al*. 2013; Martins *et al*. 2014).

Abdel-Sattar *et al*. (2010) identified 65 compounds in the extracts, compared with 36 identified in this report. The GC-MS techniques were similar in both studies. The difference in the compounds identified can be explained by various factors described by Cavalcanti *et al*. (2015). Variations in essential oil contents from plant tissues can be related to different factors, some of them intrinsic and controlled by the plant's genetic traits. On the other hand, quantitative traits are susceptible to edaphoclimatic effects, such as seasonality, water availability and soil nutrients (Cavalcanti *et al*. 2015).

The composition of the essential oils can also be modulated by temperature, moisture, nutrient availability, light intensity, photoperiod and/or ecological interactions (Sangwan *et al*. 2001; Lima *et al*. 2003). Finally, it can be affected by other conditions, such as storage and whether the plant material used was dried or fresh (Jerković *et al*. 2001). The main factor that determines the concentration of each compound present in the essential oils is its genotype (Gomes *et al*. 2013).

Some non-polar compounds could be present in the genus *Schinus* and in SmH, such as fatty acids, triterpenes and steroids (Masateru *et al*. 2008). We evaluated the hexane extract's chemical composition and found a high concentration of lupenone (50·2%), a steroidal triterpene. Thus, the toxic action of the extract could be related to this triterpenoid, but there are no published reports attributing toxic action to lupenone. Therefore, further studies to evaluate the action of lupenone against *C. felis felis* are warranted.

Methanol (SmM) and ethyl acetate (SmAe) extracts were not analysed since the samples were inactive in the biological model studied. SmAe and SmM, showed a nonlinear response with maximum efficacy of 5 and 40% at 48 h, respectively. This nonlinear response may be based on the synergism between the compounds present in the extracts. However, it is known that extracts of *S. molle* of medium to high polarity contain phenolic acids, flavonoids, biflavones and terpenes (Masateru *et al*. 2008), along with gums, alkaloids and tannins (Ferrero *et al*. 2006). Few studies have reported the action of these secondary metabolites in the control of infestation by fleas. Phenolic (phenolic acids, flavonoids and tannins) and gums have not been evaluated, however, monoterpenes and sesquiterpenes are known as agents used to control fleas; Insecticidal use of monoterpene limonene has been successfully applied for the control of insect parasitoids of pet animals. Weekly application of (+)-limonene reduced flea infestations by 80% in cat, and was toxic to all life stages of the cat flea, with no adverse effects on liver and kidney function (Ibrahim *et al*. 2001). Custonolide (sesquiterpene) have been reported as insecticidal agents, shown to be highly effective, however, these compounds have not been evaluated against the fleas (Vattikonda *et al*. 2015).

Historically it is known that alkaloids have insecticidal activity (nicotinic acid and its derivatives) but efficacy against fleas has not been evaluated extensively. Macrocyclic lactones; such as avermectin, selamectin and spinosyns (polyketides) have been used to control fleas and were derived from natural products (Mertz and Raymond, 1990; Zakson-Aiken *et al*. 2001).

We evaluated efficacy of extraction at 3 and 6 h for EOL and EOF, respectively, which gave maximum values of 0·86% ± 0·05 for EOL and 2·3% ± 0·07 for EOF, results similar to those calculated for the time of 20 h (0·99% ± 0·03 for EOL and 2·4% ± 0·04 for EOF). Therefore, the time of 3 h for EOL and 6 h for EOF were ideal, close to the maximum values estimated for extraction of *S. molle* essential oils by hydrodistillation (Fig. 2).

Studies evaluating the activity of plant extracts or essential oils on inhibition of the life cycle of *C. felis felis*, or the activity of *S. molle* on the eggs of other insects have not been published. Akinneye *et al*. (2006), evaluating the insecticidal potential of powders made from the root bark, stem bark and leaves of *Cleisthopholis patens*, found that the root and stem bark powders inhibited the development of eggs to adults of the Indian mealmoth (*Plodia interpunctella*).

Shojaaddini *et al*. (2008) tested the essential oil of *Carum copticum* by fumigation for control of *P. interpunctella* and observed that the adult insects were approximately 500 times more susceptible than the other life phases. They also noted that the last-instar larvae were more susceptible than the eggs. In general, all the development phases were affected by the essential oil of this plant, which caused morality of eggs, larvae, pupae and adults of 80, 90, 90 and 100%, respectively.

Lans *et al*. (2008) thoroughly saturated pets infested with fleas with an infusion obtained from *Melissa officinalis* (10 and 18% w/v) for 30 min, and then allowed each animal's coat to air dry. They also used other formulations, such as spray of *Citrus* spp. (decoction), *Lavandula officinalis* (decoction), *Thuja plicata* and *Juniperus communis* (essential oil) against the fleas (Lans *et al*. 2008). For all tests, efficient repellency was observed 24 h after treatment, but they did not report on the percentage of infestation after treatment; therefore, efficacy could not be determined.

We observed greater efficacy of *S. molle* on *C. felis felis* adults than on the immature forms. In the test to evaluate the activity of the *S. molle* fruit oil on *C. felis felis*, of the 10 eggs exposed to the essential oil, an average of 9·2 fleas reached the adult stage, while the corresponding result for the leaf oil was 9·5

https://doi.org/10.1017/S0031182016000081 Published online by Cambridge University Press

adults. In sharp contrast, both essential oils were 100% effective against adult fleas. This result is similar to that found by Shojaaddini *et al*. (2008), who also observed greater sensitivity of adult insects compared with immature forms.

Baldin *et al*. (2008) noted high activity of a powder made from *Ruta graveolens* in inhibiting egg-adult development of the Mexican bean weevil (*Zabrotes subfasciatus*). Baldin *et al*. (2009) found increase in the egg-adult development period of *Acanthoscelides obtectus*, another bean weevil species, when the beans were treated with powder made from *Arnica montana*. Raja and John William (2008) investigating the volatile oils of *Cymbopogon winterianus, Eculyptus citrodora, Cymbopogon flexuosus, Vetiveria zizanioides* and *Cymbopogon Martini*, observed that these species had ovicidal activity against the cowpea beetle (*Callosobruchus maculatus*), reducing adult emergence by up to 88%. Souza and Trovão (2009) studying dried extracts of *Cnidoscolus quercifolius* to protect corn against the maize beetle (*Sitophilus zeamais*), observed ovicidal and larvicidal activity.

Our results differ from these studies, in that the essential oil from *S. molle* was not able to kill the immature forms of *C. felis felis* and did not cause an increase in the flea's development period.

Carneiro *et al*. (2011) investigated the action of an extract of *Annona coriacea* on eggs and newly hatched nymphs of the insect *Rhodnius neglectus*, using two application methods, topical and contact. They evaluated concentrations of 25, 50, 100 and 200 mg mL^{-1}. The extract was able to inhibit the egg hatching by up to 90% when applied topically, while in the contact method, the mortality was up to 96·6% of nymphs at the highest concentration, possible after contact with residue of the extract on impregnated paper. The topical application method was efficient in inhibiting egg hatching and the contact method was sufficient to inhibit development of recently hatched nymphs. The contact method was similar to that used by us, where 10 eggs for each repetition were placed in contact with an impregnated filter paper. However, we did not observe any change in the biological cycle of *C. felis felis*: the immature forms developed within the expected interval, whereby the adults emerged 30 days after exposure of the eggs to the oil, both from the fruits and leaves, impregnated on filter paper.

The choice of pyriproxyfen as positive control was because it is a juvenile hormone analog of insects, and it interrupts the embryonic development of *C. felis felis* (Rust, 2005). It does not kill the insect directly. Instead, it acts on the growth and development of its immature stages (Graf, 1993). In a study conducted by Batista *et al*. (2012) using a combination of 0·05% pyriproxyfen and 0·04% cyfluthrin (a pyrethroid) on immature forms of *C. felis felis*, the authors found average efficacy of 98·8% in interrupting the egg-adult development during 182 days.

Fipronil was also used as a positive control. It is a pyrazole insecticide (Cole *et al*. 1993) with good selective activity against insects (Narahashi *et al*. 2010). It is approved globally for treatment and control of infestations by fleas and ticks on dogs and cats (Taylor, 2001). The drug interferes in the insect's neuromodulation, leading to death by hyperexcitation (Rauh *et al*. 1990). Both positive controls used in this study were 100% effective in interrupting the egg-adult cycle.

There is a growing body of evidence indicating the potential value of essential oils as control agents against a range of arthropod ectoparasites, particularly lice, mites and ticks. However, results published regarding fleas are scant and not conclusive and there are no specific studies to evaluate the efficacy of whole essential oils against fleas (Ellse and Wall, 2013).

A recent bibliographical search only identified four papers that describe the activity of essential oil against the cat flea (Ibrahim *et al*. 2001; Lans *et al*. 2008; Genovese *et al*. 2012; Su *et al*. 2013). These studies generally show repellent activity but no adulticide activity.

Results of a new method for analysing repellents in large scale showed the essential oil of *Cinnamomum osmophloeum* (leaf), *Taiwania cryptomerioides* (heartwood) and *Plectranthus amboinicus* (leaf) had dose-dependent repellent activity against cat fleas. The repellency activities were 97·6% for EO of *C. osmophloeum* and 90·6% for EO of *P. amboinicus* during 4 and 8 h, respectively (Su *et al*. 2013).

Essential oils of *Schinus molle* (fruit and leaf) showed insecticidal activity against *Trogoderma granarium* (khapra beetle) and *Tribolium castaneum* (red flour beetle) (Abdel-Sattar *et al*. 2010). Data on the cumulative mortality during 6 days of exposure revealed that the fruit oil caused 50% mortality at 250 μL and 93·3% mortality at 1000 μL, while that obtained from leaves was less toxic. The mortality of *T. granarium* was 50% at 500 μL and 68·8% at 1000 μL. These results are important considering that the mortality increased with longer exposure time (Abdel-Sattar *et al*. 2010).

Other studies show that the essential oils of *Schinus molle* were efficient as a repellent at 176·0, 70·0 and 35·38 μg cm^{-2}, causing 100, 95 and 82·5% mortality against *Triatoma infestans,* respectively (Chopa *et al*. 2006). Thus, our results allow inferring that the essential oils from the leaves and fruit of *Schinus molle* can have repellent activity against the cat flea, since the leaf extract caused 100% mortality at 50 μg cm^{-2} concentration.

Assays with *Saccharomyces cerevisiae* yeast cells in the presence of the essential oils obtained from fruit and leaves did not show toxicity levels near the LD_{50} found in our study. This seems confusing given the strong antifungal activity of essential oils, such as of *Cinnamomum zeylanicum* on the pathogenic

https://doi.org/10.1017/S0031182016000081 Published online by Cambridge University Press

filamentous fungus *Fusarium keratitis* (Homa *et al.* 2015), or of *Melaleuca alternifolia* on the fungus *Botrytis cinerea* (Yu *et al.* 2015).

In a study of the antifungal action of the essential oil of *Schinus lentiscifolius*, Gehrke *et al.* (2013) evaluated the toxicity of the crude extract and hexane fraction on *S. cerevisiae*, finding minimum inhibitory concentration values of 125 and 250 $\mu g\ mL^{-1}$ and minimum lethal concentration values above 500 and 500 $\mu g\ mL^{-1}$ for the crude extract and hexane fraction, respectively. The results of another study of exposure of different fungi (*Aspergillus niger, Aspergillus oryzae, Fusarium oxysporum, Rhizophus oryzae* and *Rhyzophus stolonifer*) to essential oil from fruits and leaves of *S. molle* revealed minimum inhibitory concentrations between 500 and 1000 $\mu g\ mL^{-1}$ (Martins *et al.* 2014). These data are in accordance with those found in this study, because the oil extracted from the fruit at the concentration of 12 500 $\mu g\ mL^{-1}$ caused zero cell survival. However, we observed that lower concentrations of the leaf and fruit essential oil (hexane fraction) did not significantly reduce the cell survival, a finding also corroborated by the low impairment of the mitochondrial functions (Figs 4 and 5).

Therefore, although cells of *Saccharomyces cerevisiae* are unicellular, and thus much more sensitive to the action of the two *Schinus molle* essential oils, lower concentrations might not be toxic to the yeast, but still toxic to fleas. It is important to stress that *S. cerevisiae* cells are widely used in models to study cells of higher mammals, because they have very similar and homologous cell structures, metabolism and macromolecules (Khurana and Lindquist, 2011; Ribeiro *et al.* 2015).

Another point in favour of a larger study of the use of essential oils of *Schinus* for prevention of ectoparasites is the possibility of topical use, preventing internal organs' exposure to their cytotoxic action at higher concentrations.

In conclusion, the insecticidal properties of essential oils and extracts obtained by three methods (decoction, infusion and maceration) were evaluated. Our data show that non-polar extracts are the most active against *C. felis felis*. Therefore, the study of polar extracts probably would not lead to the discovery of a substance that could be used to treat flea infestations.

We can also conclude that the essential oil of *Schinus molle* is an important source of chemical constituents that may have potential use in veterinary medicine. The essential oils, which are rich in terpenes (monoterpenes and sesquiterpenes) did not have toxic effects at lower concentrations on *S. cerevisiae* cells or cause change in plasmatic membranes and mitochondrial damage, thus allowing the possible inclusion of these oils in formulations that can be applied directly to the animal.

This is the first study reporting the *in vitro* insecticidal effects of essential oils and extracts obtained from *S. molle*. Major oil compounds (myrtenal, terpineol, spathulenol and cubenol) and the hexane extract (lupenone) should be evaluated for use as natural pesticides. These fractions may have possible value as candidates for alternative control of fleas. However, further studies are necessary to evaluate the pulicide activity under *in vivo* conditions. The processes of isolating these compounds are being evaluated with the intention of conducting *in vivo* testing.

ACKNOWLEDGEMENTS

We thank Dr Pedro G. Filho (IB, UFRRJ/Brazil) to collaborate in this study.

FINANCIAL SUPPORT

Financial support from FAPERJ (E-26/110·746/2012 to D.S.A.C) and CAPES (to L.L.C.S.O.B. and E.R.P.) is gratefully acknowledged.

REFERENCES

Abdel-Sattar, E., Zaitoun, A. A., Farag, M. A., Gayed, S. H. and Harraz, F. M. (2010). Chemical composition, insecticidal and insect repellent activity of *Schinus molle* L. leaf and fruit essential oils against *Trogoderma granarium* and *Tribolium castaneum*. *Natural Products Research* **24**, 226–235.

Abbott, W. S. (1987). A method of computing the effectiveness of an insecticide. *Journal of the American Mosquito Control Association* **3**, 302–303.

Adams, R. P. (1995). *Identification of Essential Oil Components by Gas Chromatography-Mass Spectroscopy*. Corp., Carol Stream, USA.

Addie, D. D., Boucraut-Baralon, C., Egberink, H., Frymus, T., Gruffydd-Jones, T., Hartmann, K., Horzinek, K. C., Hosie, M. J., Lloret, A., Lutz, H., Marsilio, F., Pennisi, M. G., Radford, A. D., Thiry, E., Truyen, U. and Möstl, K. (2015). Disinfectant choices in veterinary practices, shelters and households ABCD guidelines on safe and effective disinfection for feline environments. *Journal of Feline Medicine and Surgery* **17**, 594–605.

Akinneye, J. O., Adedire, C. O. and Arannilewa, S. T. (2006). Potential of Cleisthopholis patens Elliot as a maize protectant against the stored product moth, Plodia. Interpunctella (Hubner) (Lepidoptera; Pyralidae). *African Journal of Biotechnology* **5**, 2510–2515.

Baldin, E. L. L., Pereira, J. M., Dal Pogetto, M. H. F. A., Christovam, R. S. and Caetano, A. C. (2008). Efeitos de pós vegetais sobre "*Zabrotes subfasciatus*" Bohemann (Coleoptera: Bruchidae) em grãos de feijão armazenado. *Boletin de Sanidad Vegetal de Plagas* **34**, 177–185.

Baldin, E. L. L., Prado, J. P. M., Christovam, R. S. and Dal Pogetto, M. H. F. A. (2009). Uso de pós de origem vegetal no controle de *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae) em grãos de feijoeiro. *BioAssay* **4**, 1–6.

Barroso, M. S. T., Villanueva, G., Lucas, A. M., Perez, G. P. and Vargas, R. M. F. (2011). Supercritical fluid extraction of volatile and non-volatile compounds from *Schinus molle* L.. *Brazilian Journal of Chemical Engineering* **28**, 305–312.

Batista, L. C. S. O., Vieira, V. P. C., Correia, T. T., Santos, E. C. F., Fazio-Junior, P. I., Florencio, C. N., Carneiro, M. B., Scott, F. B. and Coumendouros, K. (2012). Eficácia *in vitro* de uma formulação aerossol de piriproxifen e ciflutrina no controle de *Ctenocephalides felis felis* (Bouché, 1835) (Siphonaptera: Pulicidae). *Revista Brasileira de Medicina Veterinária* **34**(Suppl. 1), 41–45.

Batista, L. C. S. O., Cid, Y. P., Magalhaes, V. S., Chaves, D. S. A. and Coumendouros, K. (2014). Bioprospecção de extratos de jaborandi contra *Ctenocephalides felis felis, Rhipicephalus sanguineus* e *Rhipicephalus microplus*. *Revista Brasileira de Medicina Veterinária* **35**, 113–118.

Benzi, V. S., Stefanazzi, N. and Ferrero, A. A. (2009). Biological activity of essential oils from leaves and fruits of pepper tree (*Schinus molle* L.) to control rice weevil (*Sitophilus oryzae* L.). *Chilean Journal of Agricultural Research* **69**, 154–159.

Bitam, I., Dittmar, K., Parola, P., Whiting, M. F. and Raoult, D. (2010). Fleas and flea-borne diseases. *International Journal of Infectious Diseases* **14**, e667–e676.

https://doi.org/10.1017/S0031182016000081 Published online by Cambridge University Press

Blagburn, B. L. and Dryden, M. W. (2009). Biology, treatment, and control of flea and tick infestations. *Veterinary Clinics of North America: Small Animal Practice* **39**, 1173–1200.

Bousbia, N., Vian, M. A., Ferhat, M. A., Petitcolas, E., Meklati, B. Y. and Chemat, F. (2009). Comparison of two isolation methods for essential oil from rosemary leaves: Hydrodistillation and microwave hydrodiffusion and gravity. *Food Chemistry* **114**, 355–362.

Carneiro, A. P., Pereira, M. J. B. and Galbiati, C. (2011). Efeito biocida de Annonacoriacea Mart 1841 sobre ovos e ninfas do vetor *Rhodnius neglectus* Lent 1954. *Neotropical Biology and Conservation* **6**, 131–136.

Carvalho, M. G., Melo, A. G. N., Aragão, C. F. S., Raffin, F. N. and Moura, T. F. A. L. (2013). *Schinus terebinthifolius* Raddi: chemical composition, biological properties and toxicity. *Revista Brasileira de Plantas Mediciais* **15**, 158–169.

Castro, F. A., Mariani, D., Panek, A. D., Eleutherio, E. C. A. and Pereira, M. D. (2008). Cytotoxicity mechanism of two naphthoquinones (menadione and plumbagin) in *Saccharomyces cerevisiae*. *PLoS ONE* **3**, e3999.

Cavalcanti, A. S., Alves, M. S., da Silva, L. C. P., Patrocínio, D. S., Sanches, M. N., Chaves, D. S. A. and Souza, M. A. A. (2015). Volatiles composition and extraction kinetics from *Schinus terebinthifolius* and *Schinus molle* leaves and fruit. *Brazilian Journal of Pharmacognosy* **25**, 356–362. http://dx.doi.org/10.1016/j.bjp.2015.07.003

Chopa, C. S., Alzogaray, R. and Ferrero, A. (2006). Repellency assays with *Schinus molle* var. areira (L.) (Anacardiaceae) Essential oils against *Blattella germanica* L. (Blattodea: Blattellidae). *BioAssay* **1**, 1–3.

Cole, L. M., Nicholson, R. A. and Casida, J. E. (1993). Action of phenylpyrazole insecticides at the GABA-gated chloride channel. *Pesticide Biochemistry and Physiology* **46**, 47–54.

Coles, T. B. and Dryden, M. W. (2014). Insecticide/acaricide resistance in fleas and ticks infesting dogs and cats. *Parasites & Vectors* **7**, 1–10.

Dayan, F. E., Cantrell, C. L. and Duke, S. O. (2009). Natural products in crop protection. *Bioorganic & Medicinal Chemistry* **17**, 4022–4034.

Di Stasi, L. C. and Hiruma-Lima, C. A. (2002). *Plantas Medicinais na Amazônia e na Mata Atlântica*, 2nd Edn. Editora UNESP, São Paulo, 604p.

Dryden, M. W., Payne, P. A., Smith, V., Heaney, K. and Sun, F. (2013). Efficacy of indoxacarb applied to cats against the adult cat flea, *Ctenocephalides felis*, flea eggs and adult flea emergence. *Parasites & Vectors* **6**, 1–6.

Downer, J. A., Svihra, P., Molinar, R. H., Fraser, J. B. and Koehler, C. S. (1988). New psyllid pest of California pepper tree. *California Agriculture* **42**, 30–31.

Ellse, L. and Wall, R. (2013). The use of essential oils in veterinary ectoparasite control: a review. *Medical and Veterinary Entomology* **28**, 233–243.

Ferrero, A. A., Werdin Gonzalez, J. O. and Sanchez Chopa, C. (2006). Biological activity of *Schinus molle* on *Triatoma infestans*. *Fitoterapia* **77**, 381–383.

Fourie, J. J., Fourie, L. J. and Horak, I. G. (2005). Efficacy of orally administered powdered aloe juice (Aloe ferox) against ticks on cattle and ticks and fleas on dogs. *Journal of the South African Veterinary Association* **76**, 193–196.

Graf, J. F. (1993). The role of insect growth regulators in arthropod control. *Parasitology Today* **9**, 471–474.

Gehrke, I. T. S., Neto, A. T., Pedroso, M., Mostardeiro, C. P., Da Cruz, I. B. M., Silva, U. F., Ilha, V., Dalcol, I. I. and Morel, A. F. (2013). Antimicrobial activity of *Schinus lentiscifolius* (Anacardiaceae). *Journal of Ethnopharmacology* **148**, 486–491.

Genovese, A. G., McLean, M. K. and Khan, S. A. (2012). Adverse reactions from essential oil-containing natural flea products exempted from Environmental Protection Agency regulations in dogs and cats. *Journal of Veterinary Emergency and Critical Care* (*San Antonio*) **22**, 470–475.

Gomes, V., Agostini, G., Agostini, F., Atti dos Santos, A. C. and Rossato, M. (2013). Variation in the essential oils composition in Brazilian populations of *Schinus molle* L. (Anacardiaceae). *Biochemical Systematics and Ecology* **48**, 222–227.

Homa, M., Fekete, I. P., Böszörményi, A., Singh, Y. R. B., Selvam, K. P., Shobana, C. S., Manikandan, P., Kredics, L., Vágvölgyi, C. and Galgóczy, L. (2015). Antifungal effect of essential oils against *Fusarium* keratitis isolates. *Planta Medica* **81**, 1277–1284.

Ibrahim, B. and Al-Naser, Z. (2014). Analysis of fruits *Schinus molle* extractions and the efficacy in inhibition of growth the fungi in laboratory. *International Journal of ChemTech Research* **6**, 2799–2806.

Ibrahim, M., Kainulainen, P., Aflatuni, A., Tiilikkala, K. and Holopainen, J. K. (2001). Insecticidal, repellent, antimicrobial activity and phytotoxicity of essential oils: with special reference to limonene and its suitability for control of insect pests. *Agricultural and Food Science in Finland* **10**, 243–259.

Jerković, I., Mastelić, J. and Miloš, M. (2001). The impact of both the season of collection and drying on the volatile constituents of *Origanum vulgare* L. ssp. hirtum grown wild in Croatia. *International Journal of Food Science & Technology* **36**, 649–654.

Jin, T., Lu, J. and Nordberg, M. (1998). Toxicokinetics and biochemistry of cadmium with special emphasis on the role of metallothionein. *Neurotoxicology* **19**, 529–535.

Khurana, V. and Lindquist, S. (2011). Modelling neurodegeneration in *Saccharomyces cerevisiae*: why cook with baker's yeast? *Nature Reviews Neuroscience* **11**, 436–449.

Lans, C., Turner, N. and Khan, T. (2008). Medicinal plant treatments for fleas and ear problems of cats and dogs in British Columbia, Canada. *Parasitology Research* **103**, 889–898.

Lima, H. R., Kaplan, M. A. C. and Cruz, A. V. M. (2003). Influencia dos fatores abióticos na produção e variabilidade de terpenóides em plantas. *Floresta e Ambiente* **10**, 71–77.

Marongiu, B., Porcedda, A. P. S., Casu, R. and Pierucci, P. (2004). Chemical composition of the oil and supercritical CO_2 extract of *Schinus molle* L.. *Flavour and Fragrance Journal* **19**, 554–558.

Martins, M. R., Arantes, S., Candeias, F., Tinoco, M. T. and Cruz-Morais, J. (2014). Antioxidant, antimicrobial and toxicological properties of *Schinus molle* L. essential oils. *Journal of Ethnopharmacology* **151**, 485–492.

Masateru, O. N. O., Yamashita, M., Mori, K., Masuoka, C., Eto, M., Kinj, J., Ikeda, T., Yoshimitsu, H. and Nohara, T. (2008). Sesquiterpenoids, Triterpenoids, and flavonoids from the fruits of *Schinus molle*. *Food Science and Technology Research* **14**, 499–508.

Mertz, F. and Raymond, C. Y. (1990). "*Saccharopolyspora spinosa* sp. nov. Isolated from soil Collected in a Sugar Mill Rum Still". *International Journal of Systematic Bacteriology* **40**, 34–39.

Murray, A. P., Frontera, M. A., Tomas, M. A. and Mulet, M. C. (2005). Gas chromatography-mass spectrometry study of the essential oils of *Schinus longifolia* (Lindl.) Speg., *Schinus fasciculata*(Griseb.) I. M. Johnst., and *Schinus areira* L. *Zeitschrift für Naturforschung* **60**, 25–29. PMID: 15787239.

Narahashi, T., Zhao, X., Ikeda, T., Salgado, V. L. and Yeh, J. Z. (2010). Glutamate-activated chloride channels: unique fipronil targets present in insects but not in mammals. *Pesticide Biochemistry Physiology* **97**, 149–152.

Raja, M. and John William, J. (2008). Impact of volatile oils of plants against the Cowpea Beetle *Callosobruchus maculates* (FAB) (Coleoptera: Bruchidae). *International Journal of Integrative Biology* **2**, 62–64.

Rauh, J. J., Lummis, S. C. R. and Sattelle, D. B. (1990). Pharmacological and biochemical properties of insect GABA receptors. *Trends in Pharmacological Science* **11**, 325–329.

Ribeiro, T. P., Fernandes, C., Melo, K. V., Ferreira, S. S., Lessa, J. A., Franco, R. W. A., Schenk, G., Pereira, M. D. and Horn, A., Jr. (2015). Iron, copper, and manganese complexes with *in vitro* superoxide dismutase and/or catalase activities that keep *Saccharomyces cerevisiae* cells alive under severe oxidative stress. *Free Radical Biology and Medicine* **80**, 67–76.

Rust, M. K. (2005). Advances in the control of *Ctenocephalides felis* (cat flea) on cats and dogs. *Trends in Parasitology* **21**, 232–236.

Sangwan, N. S., Farooqi, A. H. A., Shabih, F. and Sangwan, R. S. (2001). Regulation of essential oil production in plants. *Plant Growth Regulation* **34**, 3–21.

Scopel, R., Neto, R. G., Falcão, M. A., Cassel, E. and Vargas, R. M. F. (2013). Supercritical CO_2 extraction of *Schinus molle* L with co-solvents: mathematical modeling and antimicrobial applications. *Brazilian Archives of Biology and Technology* **56**, 513–519.

Shojaaddini, M., Moharramipour, S. and Sahaf, B. Z. (2008). Fumigant toxicity of essential oil from *Carumcopticum* against Indian meal moth, *Plodia interpunctella*. *Journal of Plant Protection Research* **48**, 411–419.

Silva, C. G., Herdeiro, R. S., Mathias, C. J., Panek, A. D., Silveira, C. S., Rodrigues, V. P., Renno, M. N., Falcao, D. Q., Cerqueira, D. M., Minto, A. B., Nogueira, F. L., Quaresma, C. H., Silva, J. F., Menezes, F. S. and Eleutherio, E. C. A. (2005). Evaluation of antioxidant activity of Brazilian plants. *Pharmacological Research* **52**, 229–233.

Silva-Luz, C. L. and Pirani, J. R. (2014). Anacardiaceae in Lista de Espécies da Flora do Brasil. http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB4398. Jardim Botânico do Rio de Janeiro.

Sousa, F. C. F., Melo, C. T. V., Citó, M. C. O., Félix, F. H. C., Vasconcelos, S. M. M., Fonteles, M. M. F., Filho, J. M. B. and Viana, G. S. B. (2008). Medicinal plants and their bioactive constituents: a scientific review of bioactivity and potential benefits in the anxiety disorders in animal models. *Brazilian Journal of Pharmacognosy* **18**, 642–654.

Souza, M. C. C. and Trovão, D. M. B. M. (2009). Bioatividade do extrato seco de plantas da caatinga e do Nim (*Azadiractha indica*) sobre *Sitophilus zeamais* Mots em milho armazenado. *Revista Verde de Agroecologia e Desenvolvimento Sustentável* **4**, 120–124.

Souza-Moreira, T. M., Salgado, H. R. N. and Pietro, R. C. L. R. (2010). Brazil in the context of plants and derivates quality control. *Brazilian Journal of Pharmacognosy* **20**, 435–440.

Sparagano, O., Khallaayoune, K., Duvallet, G., Nayak, S. and George, D. (2013). Comparing terpenes from plant essential oils as pesticides for the poultry red mite (*Dermanyssus gallinae*). *Transboundary and Emerging Diseases* **60**(Suppl. 2), 150–153.

Stamopoulos, D. C., Damos, P. and Karagianidou, G. (2007). Bioactivity of five monoterpenoid vapours to *Tribolium confusum* (du Val) (Coleoptera: Tenebrionidae). *Journal of Stored Products Research* **43**, 571–577.

Su, L. C., Huang, C. G., Chang, S. T., Yang, S. H., Hsu, S. H., Wu, W. J. and Huang, R. N. (2013). An improved bioassay facilitates the screening of repellents against cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae). *Pest Management Science* **70**, 264–270.

Taylor, M. A. (2001). Recent developments in ectoparasiticides. *The Veterinary Journal* **161**, 253–268.

Van den Dool, H. and Kratz, P. D. (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography* **11**, 463–471.

Vattikonda, S. R., Amanchi, N. R. and Sangam, S. R. (2015). Effect of Costunolide a plant product of *Saussurea lappa* on feeding behaviour of *Papilio demoleus* L. (Lepidoptera: Papilionidae) Larvae. *Research Journal of Recent Sciences* **4**, 55–58.

Yu, D., Wang, J., Shao, X., Xu, F. and Wang, H. (2015). Antifungal modes of action of tea tree oil and its two characteristic components against *Botrytis cinerea*. *Journal Applied Microbiology* **119**, 1253–1262.

Zahed, N., Hosni, K., Brahim, N. B., Kallel, M. and Sebei, H. (2010). Allelopathic effect of *Schinus molle* essential oils on wheat germination. *Acta Physiologiae Plantarum* **32**, 1221–1227.

Zakson-Aiken, M., Gregory, L. M., Meinke, P. T. and Shoop, W. L. (2001). Systemic activity of the avermectins against the cat flea (Siphonaptera: Pulicidae). *Journal of Medical Entomology* **38**, 576–580. http://dx.doi.org/10.1603/0022-2585-38.4.576.