Parasitology

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In vitro activity of essential oils against adult and immature stages of *Ctenocephalides felis felis*

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Research Article

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Abstract

Essential oils (EOs) are considered a new class of ecological products aimed at the control of insects for industrial and domestic use; however, there still is a lack of studies involving the control of fleas. *Ctenocephalides felis felis*, the most observed parasite in dogs and cats, is associated with several diseases. The aim of this study was to evaluate the *in vitro* activity, the establishment of LC_{50} and toxicity of EOs from *Alpinia zerumbet* (Pers.) B. L. Burtt & R. M. Sm, *Cinnamomum* spp., *Laurus nobilis* L., *Mentha spicata* L., *Ocimum gratissimum* L. and *Cymbopogon nardus* (L.) Rendle against immature stages and adults of *C. felis felis*. Bioassay results suggest that the method of evaluation was able to perform a pre-screening of the activity of several EOs, including the discriminatory evaluation of flea stages by their LC_{50}. *Ocimum gratissimum* EO was the most effective in the *in vitro* assays against all flea stages, presenting adulticide ($LC_{50} = 5.85\,\mu g\,cm^{-2}$), ovicidal ($LC_{50} = 1.79\,\mu g\,cm^{-2}$) and larvicidal ($LC_{50} = 1.21\,\mu g\,cm^{-2}$) mortality at low doses. It also presented an excellent profile in a toxicological eukaryotic model. These findings may support studies involving the development of non-toxic products for the control of fleas in dogs and cats.

Introduction

Increased human–pet interactions lead to concerns related to the prevention and treatment of ectoparasites' infestations, among other issues. Therefore, the search for new active compounds with ectoparasiticide activity has great relevance. On the other hand, the overuse of these products is associated with numerous side-effects, such as resistance and environmental pollution (Sadaria *et al*., 2017; Teerlink *et al*., 2017) and has been a matter of concern for both scientists and the public in recent years (Tripathi and Mishra, 2017). In this scenario, the use of natural products could be an excellent alternative to synthetic compounds as a mean to reduce the negative impact to human health and environment. Some medicinal plants (*Artemisia vulgaris*, *Citrus x limon*, *Juniperus communis*, *Lavundula officinalis*, *Melissa officinalis* and *Thujaplicata*) had their uses as ethnoveterinary insecticides against fleas in cat and dogs already reported (Lans *et al*., 2008). Efforts all over the world have been performed in an attempt to develop prospects for essential oils (EOs) for insect control (Bakkali *et al*., 2008).

An EO is a complex mixture of compounds, which may be obtained from different plant organs (Cavalcanti *et al*., 2015) and may be extracted by hydrodistillation. Their chemical composition is based mainly on terpenes (mono and sesquiterpenes) and/or phenylpropanoids. EOs have shown to be very promising due to their insecticidal potential, due to the different bioactive compounds present in them. However, the major body of research on EOs describes their activity against mosquitoes and ticks (Benelli and Pavella, 2018). The use of EOs extracted from plants for the control of veterinary ectoparasites received peculiar attention since they show high efficacy, multiple mechanisms of action and low toxicity on non-target vertebrates, including aquatic ones (Ellse and Wall, 2014). Mite mortality using EOs of *Cinnamomum zeylanicum* (Na *et al*., 2011), *Laurus novocanariensis* (Macchioni *et al*., 2006) and *Cymbopogon nardus* (Magi *et al*., 2006) has been reported. Tick and flies mortality has been described using two different *Mentha* species, *M. longifolia* (Koc *et al*., 2012) and *M. piperita* (Morey and Khandagle, 2012), respectively. *Cymbopogon nardus* EO has also been used for many years as an insect repellent (Zaridah *et al*., 2003). However, there is a lack of studies involving

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Table 1. Main information about the plant species used in this study

Scientific name[a]	Common name	Botanic family	Part used	Specimen voucher
Alpinia zerumbet (Pers.) B. L. Burtt& R. M. Sm	Shellflower	*Zingiberaceae*	Leaves	RBR44875
Cymbopogon nardus (L.) Rendle	Citronella	Poaceae	Leaves	RBR44848
Ocimum gratissimum L.	Clove basil	Lamiaceae	Leaves	RBR36382
Mentha spicata L.	Spearmint	Lamiaceae	Leaves	CBPM 096
Laurus nobilis L.	Bay lurel	Lauraceae	Leaves	RBR 42612
Cinnamomum spp.	Cinnamom	Lauraceae	Stem	Commercial sample

[a]The scientific names were proposed according to The Plant List 2019 (http://www.theplantlist.org) and Reflora 2020 (http://floradobrasil.jbrj.gov.br/reflora).

EO and fleas (Ellse and Wall, 2014), both related to insecticidal activity or repellency, as well as the relationship between EO composition and its activity (Benelli and Pavella, 2018).

Ctenocephalides felis felis (Bouché, 1835), the cat flea, is an ectoparasite of warm-blooded hosts, which affects mostly mammals in general. It is currently widespread around the world, with a preference for temperate regions (Lehane, 2005). It is the most important ectoparasite in dogs and cats (Dryden, 1993), due to its vector competence and geographical distribution (Linardi and Santos, 2012). Its biological cycle can be divided into the following stages: egg, three larval stages, inactive pupae and adult (Blagburn and Dryden, 2009). *Ctenocephalides felis felis* is frequently associated as a vector or an intermediate host of bacteria, protozoa and helminths (Rust and Dryden, 1997; Avelar *et al.*, 2011; ESCCAP, 2015). Additionally, it promotes irritation especially in dogs and cats, such as allergic dermatitis, the most common veterinary dermatologic condition in the world (Carlotti and Jacobs, 2000). The goals of the flea control are to provide adulticidal effectiveness, eliminating the adult fleas on all the animals in the house as well as environmental life-stage control, eliminating immature fleas in the environment (Halos *et al.*, 2014). For example, previously published results pointed to the flea activity of the *S. molle* EO (Batista *et al.*, 2016) that led to the formulation of products based on that EO with verified efficacy for the treatment of fleas in cats and dogs (de Almeida *et al.*, 2016).

Based on this information, and in the search for new and less aggressive insecticides to humans, animals and the environment, aligned with the one health concept, the aim of this study was to evaluate the *in vitro* activity and to establish the LC_{50} of several EOs. In this way, *Alpinia zerumbet*, *Cinnamomum* spp., *Laurus nobilis*, *Mentha spicata*, *Ocimum gratissimum* and *C. nardus* EOs were tested against immature stages (eggs and larvae) and adults of *C. felis felis*. Some of them also had their toxicity evaluated against *Saccharomyces cerevisiae* yeast cells, unicellular eukaryotic organism with great orthology to mammalian cells; especially with regards to the macromolecules, organelles and cellular metabolism (Fikry *et al.*, 2019).

Material and methods

Plant material

Leaves of *A. zerumbet* (Pers.) B. L. Burtt & R. M. Sm, *C. nardus* (L.) Rendle, *Ocimum gratissimum* L., *M. spicata* L. and *L. nobilis* L. were collected at the Botanical garden of the Universidade Federal Rural do Rio de Janeiro (GPS 22°31′36.23S; 44° 04′31.62W), dried in an over chamber at 37°C for 72 h and manually pulverized. All specimen vouchers (Table 1) were deposited in the Herbarium of the Institute of Botany (UFRRJ, Brazil). Stems of *Cinnamomum* spp. were purchased commercially from the company (Marca do Sabor®, Nova Friburgo/Rio de Janeiro state).

Extraction, content and chemical characterization of the essential oils

EOs from both dried leaves and *Cinnamomum* spp. stems were obtained by hydrodistillation in a Clevenger apparatus for 3 h and dried over anhydrous Na_2SO_4. GC analysis was carried out on a Hewlett-Packard 5890 II (Palo Alto, USA) apparatus equipped with flame ionization detection (FID) and a split/splitless injector. Substances were separated into the fused silica capillary column HP-5 (30 m × 0.25 mm i.d., 0.25 μm, Agilent J &W). The oven, injector and detector temperatures were programmed as reported by Adams (1995). Helium was used as the carrier gas (1 mL min^{-1}). Injected volume was 1 μL on a 1:20 split ratio. Percentage of EO compounds was calculated from the relative area of each peak analysed by GC-FID. EOs were also analysed on a GC/MS QP-2010 Plus (Shimadzu, JPN). Carrier gas flow, capillary column and temperature conditions for GC/MS analysis were the same as those described for GC/FID and reported by Adams (1995). Mass spectrometer operating conditions were ionization voltage at 70 eV and mass range 40–400 *m/z* and 0.5 scan/s. The compounds retention index was calculated based on co-injection of samples with a C_8-C_{20} hydrocarbon mixture as reported by Van Den Dool and Kratz (1963). Constituents were identified by comparison of their mass spectra with the NIST library (2008) and with those reported by Adams (1995).

In vitro *activity of essential oils against* Ctenocephalides felis felis

Bioassays were performed using the filter paper impregnation method. Stock solutions at a concentration of 200 mg mL^{-1} of EOs from *A. zerumbet*, *Cinnamomum* spp., *L. nobilis*, *M. spicata*, *O. gratissimum* and *C. nardus* were prepared using acetone as a diluent, which was also used as a negative control. Fipronil at 8 μg cm^{-2} was used as a positive control.

Serial dilutions (1:2) were performed from stock solutions allowing for 10 solutions in a concentration range varying from 40 000 to 78.125 μg mL^{-1}. Each concentration was evaluated in duplicate, 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 reaching final concentrations in the range of 800–1.5625 μg cm^{-2}. After the treatment, the strips were left in the open to dry for 30 min.

Mortality of adult stage

In vitro insecticidal activity against *C. felis felis* adults was tested using the filter paper tests against unfed fleas obtained from the laboratory colony. The impregnated and dried strips were inserted into glass tubes containing 10 unfed adult cat fleas (five males and five females). The tubes were sealed with non-woven tissue and rubber bands and kept in the climatized chamber at 28 ± 1°C and 75 ± 10% relative humidity. The evaluation criterion used

was motility, any flea that presented minimal movement was considered alive. The mean number of live adult fleas per concentration was evaluated at 24 and 48 h using a stereoscopic microscope. The tests were performed in duplicates for each concentration.

Mortality of immature stages (egg and larvae)

In vitro activity of the EOs against immature stages of *C. felis felis* was tested using the filter paper tests against fleas' eggs obtained from the laboratory colony. The impregnated and 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. 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%. The evaluation criterion used was egg hatching, where each hatching egg was considered alive. For the larvicidal test, the same procedure was performed using 10 *C. felis felis* larvae per tube. The evaluation criterion used was motility, any larva that presented minimal movement was considered alive. The mean number of live eggs and larvae per concentration was evaluated in periods of mainly 24 h with the help of a stereoscopic microscope. The tests were performed in duplicates for each concentration.

Efficacy evaluation and LC_{50} establishment

The Abbott's formula (1987) was used to calculate the efficacy: per cent efficacy = [(mean number of fleas (adult, egg or larvae) of the control group – mean number of fleas (adult, egg or larvae) from the treated group)/(mean number of fleas (adult, egg or larvae) from the control group)] × 100.

The calculation of LC_{50} (concentration that kills 50% of the treated population) for both mature and immature stages was performed by probit analysis using Minitab® 16 (2013, Minitab Inc. LEADTOOLS, LEAD Technologies, Inc., State College, PA, USA). Statistical significance was set at 5% ($P < 0.05$).

Cell viability

The *S. cerevisiae* strain used in this study was BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) acquired from Euroscarf (Frankfurt, Germany). Stock solution of the yeast strain was maintained on solid 2% YPD (1% yeast extract, 2% glucose, 2% peptone and 2% agar) at refrigerated temperature. Media components were obtained from Difco (EUA). A stock solution of EOs (2000 $\mu g\,mL^{-1}$) was prepared with DMSO at 50%. For all experiments, cells were cultivated in liquid 2% YPD using an orbital shaker at 28°C and 160 rpm, until growth to stationary phase (4.0 mg dry weight per mL), measured by optical density at 570 nm. Only two EOs were analysed in this experiment with yeasts, the one with the best results (*O. gratissimum*) and one of the worst results (*C. nardus*) in the *in vitro* bioassays. Thymol and fipronil were used as parameters in the cell viability assay. Cells were treated for 24 and 48 h with *O. gratissimum* and *C. nardus* at 10 and 100 $\mu g\,mL^{-1}$ concentration for each EO and 100 $\mu g\,mL^{-1}$ for thymol or fipronil (three independent experiments at least). The control (no EO addition) was used as the basal value. After incubation, an equivalent volume corresponding to 4 μg cells was collected, diluted (1000×) in buffer phosphate (50 mm, pH 6.0), plated on YPD 2%, incubated at 28°C/72 h and the colonies were counted (de Sá *et al.*, 2013).

Results

Content and chemical characterization of the essential oils

The analysis of the EOs from the studied species showed differences in their content and chemical composition (Table 2). The major compounds (Fig. 1) found in the studied species were: 4-terpineol (22.1%) and eucalyptol (17.5%) in *A. zerumbet*; carvone (83.3%) in *M. spicata*; citronellal (45.8%), geraniol (22.3%) and citronellol (11.4%) in *C. nardus*; eugenol (74.5%) and eucalyptol (14.8%) in *O. gratissimum*; eucalyptol (19.2%), linalool (18.4%) and α-terpineol acetate (13.5%) in *L. nobilis* and (*E*)-cinnamaldehyde (91.7%) in *Cinnamomum* spp.

Mortality of adult stage

All EOs tested presented activity against the mature stage of *C. felis felis* in the concentration range tested. The negative control (acetone) was 0% effective and the positive control (fipronil at 8 $\mu g\,cm^{-2}$) was 100% effective, demonstrating that the method was employed correctly. The best efficacy results were found for the EO from *O. gratissimum*, which achieved 100% of efficacy in the concentration of 25 $\mu g\,cm^{-2}$. *Cinnamomum* spp. also presented good results with 100% of efficacy at 200 $\mu g\,cm^{-2}$. The other EOs presented 100% of efficacy only at the maximum concentration tested (800 $\mu g\,cm^{-2}$), except for *M. spicata* that achieved a maximum of 75% of efficacy at the concentration range tested (Table 3).

Mortality of immature stages (egg and larvae)

Immature stages were more sensitive to EOs when compared with mature stage, achieving 100% of efficacy in lower concentrations for all EOs tested. *Ocimum gratissimum* and *Cinnamomum* spp. EOs also presented the best results for immature forms with 100% of efficacy in the concentration of 12.5 and 6.25 $\mu g\,cm^{-2}$, respectively, against eggs and larvae. *Cymbopogon nardus* achieved 100% of efficacy at higher concentrations (400 $\mu g\,cm^{-2}$) and the other EOs presented 100% of efficacy only at the maximum concentration tested (800 $\mu g\,cm^{-2}$) (Table 4). The negative control (acetone) was 0% effective and the positive control (fipronil at 8 $\mu g\,cm^{-2}$) was 100% effective, demonstrating that the method was employed correctly.

LC_{50} estimative

Lc_{50} and slope values of EOs for all stages evaluated are demonstrated in Table 5. *Alpinia zerumbet*, *L. nobilis*, *M. spicata* and *C. nardus* EOs presented LC_{50} values for adult stage varying between 412.09 and 597.56 $\mu g\,cm^{-2}$ after 24 h and between 380.09 and 486.05 $\mu g\,cm^{-2}$ after 48 h of exposure. *Cinnamomum* spp. and *O. gratissimum* EOs presented LC_{50} values at different concentration ranges of the other EOs evaluated for both 24 and 48 h of exposure, presenting relative potency of 10 and 100-fold higher, respectively.

LC_{50} values found for the immature stages varied between 1.79 and 30.39 $\mu g\,cm^{-2}$ and 0.43 and 12.57 $\mu g\,cm^{-2}$ for eggs and larvae, respectively, demonstrating a greater sensitivity of the larva stage to EOs (Table 5).

Cell viability

The results observed for the viability cell assay with *O. gratissimum* and *C. nardus* EOs on yeast cells showed no toxicity at the tested concentrations of 10 and 100 $\mu g\,mL^{-1}$ after 24 h of exposure (Fig. 2A). Fipronil and thymol were also used at the concentration of 100 $\mu g\,mL^{-1}$. Fipronil, a synthetic compound widely used in flea combat, showed a statistically similar result to both oils evaluated; however, thymol was proven to be more toxic to the BY4741 strain. Thymol (2-isopropyl-5-methyl-phenol), a known natural repellent, found abundantly in oregano and thyme EOs, has antibacterial and antifungal properties

Table 2. Essential oils chemical profile from plant species obtained by hydrodistillation

OE	Compounds	AI_T	AZ	MS	CN	OG	LN	C
1	α-thujone	*924*	1.6	–	0.1	–	–	–
2	α-pinene	*932*	1.0	–	–	–	0.9	–
3	Sabinene	*969*	11.5	–	–	–	2.9	–
4	β-pinene	*974*	2.5	–	–	–	1.3	–
5	α-terpinene	*1014*	2.4	–	–	–	–	–
6	*o*-cymene	*1022*	3.3	–	–	–	–	–
7	limonene	*1024*	–	1.1	0.9	–	0.7	–
8	eucalyptol	*1026*	17.5	–	–	14.8	19.2	0,2
9	γ-terpinene	*1054*	10.9	–	–	–	–	–
10	*cis*-sabinene hydrate	*1065*	3.3	–	–	–	–	–
11	terpinolene	*1086*	1.7	–	–	–	–	–
12	linalool	*1095*	–	0.6	–	0.6	18.4	0,3
13	*trans*-sabinene hydrate	*1098*	3.8	–	–	–	0.9	–
14	*cis*-*p*-menth-2-en-1-ole	*1118*	1.1	–	–	–	–	–
15	citronellal	*1148*	–	–	45.8	–	–	–
16	δ-terpineol	*1162*	–	–	–	–	1.0	–
17	4-terpineol	*1174*	22.1	–	–	–	4.4	0,4
18	α-terpineol	*1186*	1.9	–	–	–	9.3	0,2
19	citronellol	*1228*	–	–	11.4	–	–	–
20	carvone	*1239*	–	83.3	–	–	–	–
21	geraniol	*1249*	–	–	22.3	–	–	–
22	geranial	*1264*	0.3	6.2	–	–	–	–
23	(*E*)-cinnamaldehyde	*1267*	–	–	–	–	–	91,7
24	α-terpineol acetate	*1346*	–	–	–	–	13.5	–
25	citronellol acetate	*1350*	–	–	–	–	3.0	–
26	eugenol	*1356*	–	–	2.5	74.5	1.2	1,0
27	geraniol acetate	*1379*	–	–	3.0	–	–	–
28	methyleugenol	*1403*	–	–	–	–	6.3	–
29	α-*cis*-bergamotene	*1411*	–	–	–	–	–	0,2
30	β-caryophyllene	*1417*	4.9	1.7	–	2.6	–	0,5
31	(*E*)-cinnamyl acetate	*1443*	–	–	–	–	–	3,4
32	α-humulene	*1452*	0.7	0.3	–	–	0.6	–
33	γ-gurjunene	*1477*	–	–	1.5	–	–	–
34	germacrene D	*1484*	–	2.0	–	1.6	–	–
35	δ-selinene	*1491*	–	–	–	3.5	–	–
36	α-selinene	*1498*	–	–	–	1.2	–	–
37	α-(*E*,*E*)-farnesene	*1505*	–	–	0.4	–	–	–
38	elemol	*1548*	–	–	2.6	–	–	–
39	germacrene D-4-ol	*1574*	–	–	1.9	–	–	–
40	caryophyllene oxide	*1582*	2.9	1.0	–	–	2.2	–
41	*epi*-α-muurolol	*1642*	–	–	1.2	–	–	–
42	β-eudesmol	*1649*	–	–	–	–	1.8	–
43	α-cadinol	*1652*	–	0.3	4.0	–	–	–
	Monoterpenes hydrocarbons		34,8	1.1	1.0	–	5.8	–
	Monoterpenes oxygenated		49,9	90.1	82.4	15.4	69.6	1.1
	Sesquiterpenes hydrocarbons		5,7	4.0	1.9	8.9	0.6	0.7

(*Continued*)

Table 2. (*Continued.*)

OE	Compounds	AI_T	AZ	MS	CN	OG	LN	C
	Sesquiterpenes oxygenated		2,9	1.3	9.7	–	3.9	–
	Phenylpropanoid		–	–	2.5	74.5	7.4	96.1
	Total		93,3	96.5	97.5	98.8	87.4	97.9

The chemical composition was analysed by GC-MS and organized in the table by order of elution (EO) in the chromatographic column. The concentration (%) was calculated based on the total area of the peak by GC-FID. Tabulated arithmetic index (AI_T). Not detected (–). Essential oil of *Alpinia zerumbet* (AZ), *Mentha spicata* (MS), *Cymbopogon nardus* (CN), *Ocimum gratissimum* (OG), *Laurus nobilis* (LN) and *Cinnamomum* spp. (C).

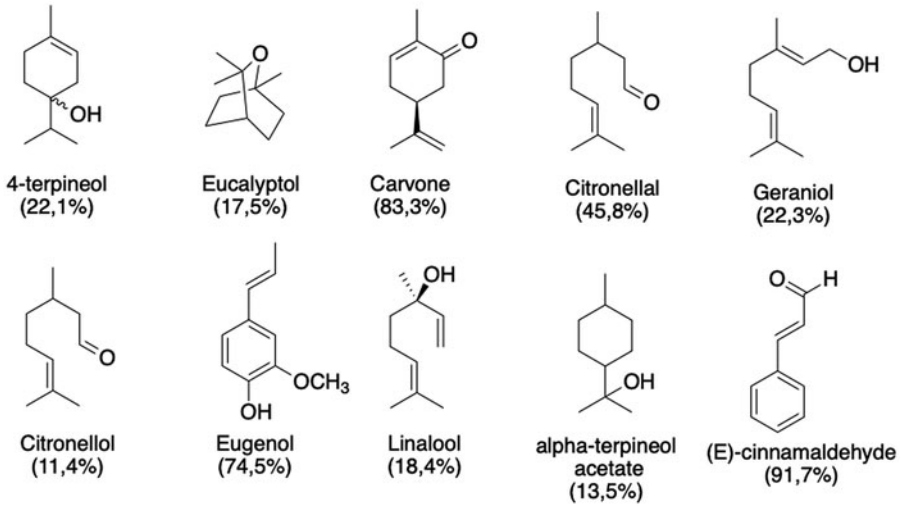

Fig. 1. Major compounds identified in the essential oil of the studied plant species.

Table 3. Essential oils *in vitro* activity through filter paper test (% mortality) against mature stage (adults) of *Ctenocephalides felis felis* after 24 and 48 h

Essential oils ($\mu g\,cm^{-2}$)	AZ		C		LN		MS		OG		CN	
Time (hours)	24	48	24	48	24	48	24	48	24	48	24	48
800	90	100	100	100	100	100	60	75	100	100	100	100
400	15	20	100	100	40	50	30	45	100	100	40	50
200	5	5	100	100	0	0	25	30	100	100	20	25
100	0	5	35	65	0	0	10	20	100	100	5	15
50	0	5	25	40	0	5	0	5	100	100	0	5
25	5	5	5	5	0	0	0	0	100	100	5	10
12.5	0	0	15	25	0	0	0	0	65	90	10	10
6.25	0	0	10	15	0	0	0	0	65	75	5	15
3.125	0	0	5	10	0	10	0	0	30	35	15	20
1.562	0	0	0	5	0	0	0	0	0	0	10	25

Essential oil of *Alpinia zerumbet* (AZ), *Mentha spicata* (MS), *Cymbopogon nardus* (CN), *Ocimum gratissimum* (OG), *Laurus nobilis* (LN) and *Cinnamomum* spp. (C).

(Marchese *et al.*, 2016). The results (Fig. 2A and B) showed its higher toxicity compared to the EOs evaluated.

In the period of 48 h of exposure to yeasts, there was a decrease in cell viability in the treatment with *C. nardus* at the concentration of $100\,\mu g\,mL^{-1}$; while *O. gratissimum* EO remained non-toxic to the cells. This reveals that besides *O. gratissimum* being the most effective in the *in vitro* assays, it also presents an excellent result in a eukaryotic model, making it promising for the tests in higher mammals. It is important to emphasize the high sensitivity of this assay, since direct exposure of the substances to the cells occurs, increasing the probability of toxicity when compared to topical use in animals. Fipronil maintained the same profile of results in 48 h of incubation; however, there was an increase in toxicity with thymol.

Preliminary tests on the toxicity of compounds with high potential for topical use in animals are important and necessary. In our case, we used the direct exposure of *O. gratissimum* and *C. nardus* to *S. cerevisiae* cells. This cell type has been widely used for the evaluation of toxicity of substances, including assays with EOs (Zhang *et al.*, 2017; Armijos *et al.*, 2018).

Discussion

Our species showed classical chemotype classification (CT) according to the data published in the literature; *A. zerumbet* CT eucalyptol (*syn.* 1,8-cineole) (Pinto *et al.*, 2009), *M. spicata* CT carvone (Morcia *et al.*, 2016), *C. nardus* CT citronellal (Weng *et al.*, 2015), *O. gratissimum* CT eugenol (Chimnoi

Table 4. Essential oils *in vitro* activity through filter paper test (% mortality) against immature stages (eggs and larvae) of *Ctenocephalides felis felis*

Essential oils ($\mu g\,cm^{-2}$)	AZ		C		LN		MS		OG		CN	
	Egg	Larvae	Egg	Larvae	Egg	Larvae	Egg	Larvae	Egg	Larvae	Egg	Larvae
800	100	100	100	100	100	100	100	100	100	100	100	100
400	69	74	100	100	95	95	64	64	100	100	100	100
200	79	84	100	100	79	90	48	64	100	100	74	84
100	69	74	100	100	79	84	48	53	100	100	43	48
50	53	53	100	100	69	95	48	53	100	100	53	58
25	43	53	100	100	90	95	48	53	100	100	48	53
12.5	53	53	100	100	74	84	33	38	100	100	48	53
6.25	43	48	100	100	54	90	43	53	84	95	43	48
3.125	38	38	84	95	38	43	23	32	74	90	38	43
1.562	32	43	38	89	54	64	38	48	43	58	38	43

Essential oil of *Alpinia zerumbet* (AZ), *Mentha spicata* (MS), *Cymbopogon nardus* (CN), *Ocimum gratissimum* (OG), *Laurus nobilis* (LN) and *Cinnamomum* spp. (C).

Table 5. LC_{50} ($\mu g\,cm^{-2}$) establishment and slope of essential oils against mature (adults) and immature stages (eggs and larvae) of *Ctenocephalides felis felis*

Essential oil	Flea stage	LC_{50} ($\mu g\,cm^{-2}$) (95% CI)	Slope (S.E.)	χ^2
Alpinia zerumbet	Adult (24 h)	553.31 (405.42–874.22)	337.80 (2.95)	0.091
	Adult (48 h)	456.27 (330.04–722.87)	172.10 (2.44)	0.105
	Egg	13.07 (5.07–26.29)	4.05 (1.30)	0.483
	Larvae	7.29 (2.08–15.38)	3.72 (1.30)	0.503
Cinnamomu spp.	Adult (24 h)	67.87 (49.80–94.54)	70.07 (1.65)	0.269
	Adult (48 h)	41.87 (29.89–59.66)	37.20 (1.51)	0.156
	Egg	1.80 (1.27–2.22)	44 626.21 (17.59)	1.000
	Larvae	0.43 (0.15–1.00)	142.59 (22.68)	1.000
Laurus nobilis	Adult (24 h)	412.09 (n.f.)	79.43 (2.22)	1.000
	Adult (48 h)	454.88 (289.91–869.10)	28.96 (1.67)	1.000
	Egg	2.41 (0.47–5.76)	4.33 (1.34)	0.262
	Larvae	0.52 (0.1–1.83)	4.31 (1.41)	0.100
Mentha spicata	Adult (24 h)	597.56 (406.13–1160.17)	77.22 (2.44)	0.987
	Adult (48 h)	380.09 (269.74–609.94)	74.71 (2.10)	0.995
	Egg	30.39 (11.20–78.52)	2.98 (1.29)	0.111
	Larvae	12.57 (2.62–33.08)	2.67 (1.28)	0.143
Ocimum gratissimum	Adult (24 h)	5.85 (4.47–7.54)	651.51 (2.75)	0.438
	Adult (48 h)	4.49 (3.54–5.62)	4752.81 (4.18)	0.951
	Egg	1.79 (0.94–2.50)	275.75 (3.78)	0.995
	Larvae	1.21 (0.35–1.80)	463.70 (6.70)	1.000
Cymbopogon nardus	Adult (24 h)	597.05 (276.56–1204.19)	6.22 (1.37)	0.179
	Adult (48 h)	486.05 (190.29–868.60)	3.80 (1.31)	0.205
	Egg	11.98 (4.88–23.32)	4.40 (1.31)	0.207
	Larvae	7.32 (2.50–14.76)	4.36 (1.32)	0.260

Probit analyses were performed for all data using Minitab® 16 (2013, Minitab Inc., LEADTOOLS, LEAD Technologies, Inc.); LC_{50} ($\mu g\,cm^{-2}$) (95% CI): 50% lethal concentration values together with their 95% confidence interval; Slope (S.E.): slope of the concentration curve and standard error; χ^2: goodness of fit test as accuracy of data fitting to probit analysis. Values showed no significant heterogeneity at the level of $P \geqslant 0.05$); n.f., not found.

et al., 2018), *L. nobilis* CT 1,8 cineole (Merghni *et al.*, 2015) and *Cinnamomum* sp. CT (*E*)-cinnamaldehyde (Jeyaratnama *et al.*, 2016).

The bioassay results suggest that the method of evaluation of insecticidal activity was able to perform a pre-screening of several EOs and to estimate the LC_{50} values for both mature and immature flea stages. Moreover, the results showed that immature stages (eggs and larvae) presented greater sensitivity to all EOs evaluated.

Ocimum gratissimum EO (74.5% of eugenol) exhibited great insecticidal activity against adult fleas ($LC_{50} = 5.85\,\mu g\,cm^{-2}$),

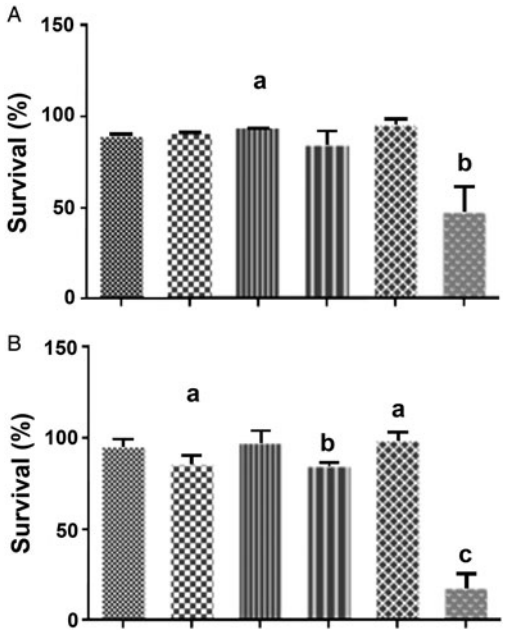

Fig. 2. Cell viability after incubation with *O. gratissimum* (OG) and *C. nardus* (CN) essential oils at 10 or 100 $\mu g\ mL^{-1}$ for 24 h (A) and 48 h (B). Results are the average from, at least, three independent experiments. Statistical significance was calculated by analysis of variance (ANOVA) followed by Tukey post-test. *P* values <0.05 (*$P < 0.05$) were considered significant. Fipronil (Fip) and thymol (Tym) were used as positive controls. Different letters mean statistically different results.

with relative potency up to 100-fold higher when compared to the other EOs evaluated in this work and also more potent than previously reported by Batista *et al.* (2016) with *Schinus molle* L. EO. This EO also showed great results for larvicidal (LC_{50} = 1.21 $\mu g\ cm^{-2}$) and ovicidal (LC_{50} = 1.79 $\mu g\ cm^{-2}$) activities. EOs containing eugenol have had their mortality (Yones *et al.*, 2016) and repellence (Iwamatsu *et al.*, 2016) activity against *P. humanus capitis* already described. Eugenol itself had its insecticide and repellence activity against *Sitophilus zeamais* (Huang *et al.*, 2002), *Dinoderus bifloveatus* (Ojimelukwe and Adler, 2000), *Ixodes ricinus* (Bissinger and Roe, 2010) and *C. maculatus* (Ajayi *et al.*, 2014) described nevertheless its activity in flea mortality had not been reported yet.

Cinnamomum spp. EO [91.7% of (*E*)-cinnamaldehyde] showed 10-fold higher adulticide mortality compared to the remaining EOs (LC_{50} = 67 $\mu g\ cm^{-2}$) and also great results for larvicidal (LC_{50} = 0.43 $\mu g\ cm^{-2}$) and ovicidal (LC_{50} = 1.80 $\mu g\ cm^{-2}$) activities. Cinnamaldehyde insecticide activity and repellence efficacy against cats and dogs ectoparasites have already been reported (Tripathi and Mishra, 2017). EOs containing (*E*)-cinnamaldehyde as their major compound have had their mortality activity against head and body lice already described (Yones *et al.*, 2016).

Eucalyptol and Linalool, compounds of *L. nobilis* EO, have their insecticide and repellent activity described for several insects (Aggarwall *et al.*, 2001; Toloza *et al.*, 2006; Sfara *et al.*, 2009) including against fleas (Hink *et al.*, 1998); however, our results show good activity only against immature forms, not achieving such great activity for adults.

Citronellal (CT of *C. nardus*) is a popular insect repellent in formulations that have been used for many years (Zaridah *et al.*, 2003). Despite its recognized repellency, *C. nardus* EO did not achieve the best mortality results both against mature and immature stages in our study. Moreover, it caused a decrease in *S. cerevisiae* cell viability at higher concentrations (100 $\mu g\ mL^{-1}$).

Therefore, some EOs such as *O. gratissimum* and *Cinnamomum* spp. demonstrated the activity against different stages of fleas' maturity. Although these are encouraging results, further studies including *in vivo* assays must be performed to evaluate pulicide activity. Further studies must also be performed with major oil compounds such as eugenol, (E)-cinnamaldehyde, linalool and eucalyptol. Insecticide activity of these compounds both isolated and in association (synergistic effect) should be evaluated to explore its uses as possible candidates for alternative control of fleas.

Conclusion

Ocimum gratissimum EO was the most effective in the *in vitro* assay against all flea stages and also presented an excellent result in the toxicological assay using a eukaryotic model, making it promising for further tests using higher mammals. These results are promising as they point out to the development of alternative herbal products for flea control, minimizing the use of synthetic products.

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Ethical standards. The experiments followed the standards established by the Ethics Committee for Animal Use of the Institute of Veterinary (CEUA/IV n° 091/14). Fleas (adults, eggs and larvae) used in the experiment were obtained from a colony maintained since 1998 in the Laboratory for Experimental Chemotherapy in Veterinary Parasitology of Federal Rural University of Rio de Janeiro (UFRRJ).

Conflict of interest. None.

References

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

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

Aggarwal KK, Tripathi AK, Prajapati V and Kumar S (2001) Toxicity of 1,8-cineole towards three species of stored product coleopterans. *Insect Science and Its Application* **21**, 155–160.

Ajayi OE, Arthur GA and Henry YF (2014) Fumigation toxicity of essential oil monoterpenes to *Callosobruchus maculatus* (Coleoptera: Chrysomelidae: Bruchinae). *Journal of Insects* **2014**, 1–7. doi.org/10.1155/2014/917212.

Armijos C, Valarezo E, Cartuche L, Zaragoza T, Finzi PV, Mellerio GG and Vidari G (2018) Chemical composition and antimicrobial activity of *Myrcianthesfragrans* essential oil, a natural aromatizer of the traditional Ecuadorian beverage colada morada. *Journal of Ethnopharmacol* **225**, 319–326.

Avelar DM, Melo MN and Linardi PM (2011) Morphology and growth characteristics of cultured *Leptomonas ctenocephali* from *Ctenocephalides felis felis* (Siphonaptera: Pulicidae) of dogs in Brazil. *Veterinary Parasitology* **80**, 394–398.

Bakkali F, Averbeck S, Averbeck D and Idaomar M (2008) Biological effects of essential oils – a review. *Food and Chemical Toxicology* **46**, 446–475.

Batista LCSO, Cid YP, Almeida AP, Prudêncio ER, Riger CJ, Souza MAA, Coumendouros K and Chaves DSA (2016) In vitro efficacy of essential oils and extracts of *Schinus molle* L. against *Ctenocephalides felis felis*. *Parasitology* **143**, 627–638.

Benelli G and Pavella R (2018) Beyond mosquitoes – essential oil toxicity and repellency against bloodsucking insects. *Industrial Crops & Products* **117**, 382–392.

Bissinger BW and Roe RM (2010) Tick repellents: past, present, and future. *Pesticide Biochemistry and Physiology* **96**, 63–79.

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

Carlotti DN and Jacobs DE (2000) Therapy, control and prevention of flea allergy dermatitis in dogs and cats. *Veterinary Dermatology* **11**, 83–98.

Cavalcanti A, de Alves MS, da Silva LCP, dos Patrocínio DS, Mirza Nalesso Sanches MN, de Chaves DSA and de Souza MAA (2015) Volatiles

composition and extraction kinetics from *Schinus terebinthifolius* and *Schinus molle* leaves and fruit. *Brazilian Journal of Pharmacognosy* **25**, 356–362.

Chimnoi N, Reuk-Ngam N, Chuysinuan P, Khlaychan P, Khunnawutmanotham N, Chokchaichamnankit D, Thamniyom W, Klayraung S, Mahidol C and Techasakul S (2018) Characterization of essential oil from *Ocimum gratissimum* leaves: antibacterial and mode of action against selected gastroenteritis pathogens. *Microbiology Pathogens* **118**, 290–300.

de Almeida AP, Chaves DSA, Coumendouros K, Batista, LC de SO, Rosado LHG, de Souza MAA and Cid YP (2016) Composições farmacêuticas para o tratamento e profilaxia das infestações causadas por ectoparasitas contendo óleo essencial de *Schinus molle L.* Titular Universidade Federal Rural do Rio de Janeiro BR 10 2016 028710 3. Depósito: 07 dez. 2016.

de Sá RA, de Castro FA, Eleutherio EC, de Souza RM, da Silva JF and Pereira MD (2013) Brazilian Propolis protects *Saccharomyces cerevisiae* cells against oxidative stress. *Brazilian Journal of Microbiology* **44**, 993–1000.

Dryden MW (1993) Biology of fleas of dogs and cats. *Compendium of Continuing Education for the Practising Veterinarian* **15**, 569–579.

Ellse L and Wall R (2014) The use of essential oils in veterinary ectoparasite control: a review. *Medical and Veterinary Entomology* **28**, 233–243.

ESCCAP (2015) *European Scientific Counsel Companion Animal Parasites, Control of Ectoparasites in Dogs and Cats*, *guidelines 03*, 4th Edn. Malvern, Worcestershire, England: ESCCAP.

Fikry S, Khalil N and Salama O (2019) Chemical profiling, biostatic and biocidal dynamics of *Origanum vulgare* L. essential oil. *AMB Express* **9**, 41.

Halos L, Beugnet F, Cardoso L, Farkas R, Franc M, Guillot J, Pfister K and Wall R (2014) Flea control failure? Myths and realities. *Trends in Parasitology* **30**, 228–233.

Hink WF, Liberati TA and Collart MG (1998) Toxicity of linalool to life stages of the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae), and its efficacy in carpet and on animals. *Journal of Medical Entomology* **25**, 1–4.

Huang Y, Shuit-Hung Ho SH, Lee HC and Yap YL (2002) Insecticidal properties of eugenol, isoeugenol and methyleugenol and their effects on nutrition of *Sitophilus zeamais* Motsch (Coleoptera: Curculionidae) and Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae). *Journal of Stored Products Research* **38**, 403–412.

Iwamatsu T, Miyamoto D, Mitsuno H, Yoshioka Y, Fujii T, Sakurai T, Ishikawa Y and Kanzaki R (2016) Identification of repellent odorants to the body louse, *Pediculus Humanus corporis*, in clove essential oil. *Parasitology Research* **115**, 1659–1666.

Jeyaratnama N, Noura AH, Kanthasamya R, Nourb AH, Yuvaraj AR and Akindoyo JO (2016) Essential oil from *Cinnamomum cassia* bark through hydrodistillation and advanced microwave assisted hydrodistillation. *Industrial Crops and Products* **92**, 57–66.

Koc S, Oz E, Aydin L and Cetin H (2012) Acaricidal activity of the essential oils from three *Lamiaceae* plant species on *Rhipicephalus Turanicus Pom.* (*Acari: Ixodidae*). *Parasitology Research* **111**, 1863–1865.

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.

Lehane MJ (2005) *The Biology of Blood-Sucking in Insects, Second Edition*. UK: Cambridge University Press.

Linardi PM and Santos JLC (2012) *Ctenocephalides felis felis* vs. *Ctenocephalides canis* (Siphonaptera: Pulicidae): some issues in correctly identify these species. *Revista Brasileira de Parasitologia Veterinária* **21**, 345–354.

Macchioni F, Perrucci S, Cioni P, Morelli L, Castilho P and Cecchi F (2006) Composition and acaricidal activity of *Laurus novocanariensis* and *Laurus nobilis* essential oils against *Psoroptes cuniculi*. *Journal of Essential Oil Research* **18**, 111–114.

Magi E, Jarvis T and Miller I (2006) Effects of different plant products against pig mange mites. *Acta Veterinaria Brno* **75**, 283–287.

Marchese A, Orhan IE, Daglia M, Barbieri R, Di Lorenzo A, Nabavi SF, Gortzi O, Izadi M and Nabavi SM (2016) Antibacterial and antifungal activities of thymol: a brief review of the literature. *Food Chemistry* **210**, 402–414.

Merghni A, Marzouki H, Hentati H, Aouni M and Mastouri M (2015) Antibacterial and antibiofilm activities of *Laurus nobilis* L. essential oil against *Staphylococcus aureus* strains associated with oral infections. *Pathology Biology (Paris)* **S0369–8114**, 101–107.

Morcia C, Tumino G, Ghizzoni R and Terzi V (2016) Carvone (Mentha spicata L.) oil. In *Essential Oils in Food Preservation, Flavor and Safety*. Amsterdam, The Netherlands: Elsevier, pp. 309–316.

Morey RA and Khandagle AJ (2012) Bioefficacy of essential oils of medicinal plants against housefly, *Musca domestica L. Parasitology Research* **111**, 1799–1805.

Na YE, Kim SI, Bang HS, Kim BS and Ahn YJ (2011) Fumigant toxicity of *cassia* and *cinnamon oils* and *cinnamaldehyde* and structurally related compounds to *Dermanyssusgallinae (Acari:Dermanyssidae)*. *Veterinary Parasitology* **178**, 324–329.

Ojimelukwe PC and Adler C (2000) Toxicity and repellent effects of eugenol, thymol, linalool, menthol and other pure compounds on Dinoderus bifloveatus (Coleoptera: Bostrichidae). *Journal of Sustainable Agriculture and Environment* **2**, 47–54.

Pinto NV, Assreuy AM, Coelho-de-Souza AN, Ceccato VM, Magalhães PJ, Lahlou S and Leal-Cardiso JH (2009) Endothelium-dependent vasorelaxant effects of the essential oil from aerial parts of *Alpinia zerumbet* and its main constituent 1,8-cineole in rats. *Phytomedicine* **16**, 1151–1155.

Rust M and Dryden M (1997) The biology, ecology and management of the cat flea. *Annual Review of Entomology* **42**, 451–473.

Sadaria AM, Sutton R, Moran KD, Teerlink J, Brown JV and Halden RU (2017) Passage of fiproles and imidacloprid from urban pest control uses through wastewater treatment plants in northern California, USA. *Environmental and Toxicology Chemistry* **36**, 1473–1482.

Sfara V, Zerba EN and Alzogaray RA (2009) Fumigant insecticidal activity and repellent effect of five essential oils and seven monoterpenes on first-instar nymphs of *Rhodnius prolixus*. *Journal of Medical Entomology* **46**, 511–515.

Teerlink J, Hernandez J and Budd R (2017) Fipronil washoff to municipal wastewater from dogs treated with spot-on products. *Science Total Environmental* **599–600**, 960–966.

Toloza AC, Zygadlo J, Mougabure Cueto G, Biurrun F, Zerba E and Picollo MI (2006) Fumigant and repellent properties of essential oils and component compounds against permethrin-resistant *Pediculus humanus* capitis (Anoplura: Pediculidae) from Argentina. *Journal of Medical Entomology* **43**, 889–895.

Tripathi A and Mishra AK (2017) Knowledge and passive adaptation to climate change: an example from Indian farmers. *Climate Risk Management* **16**, 195–207.

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

Weng DCJ, Latip J, Hasbullah SA and Sastrohamidjojo H (2015) Optimal extraction and evaluation on the oil content of citronella oil extracted from *Cymbopogon nardus*. *Malaysian Journal of Analytical Sciences* **19**, 71–76.

Yones DA, Bakir HY and Bayoumi SAL (2016) Chemical composition and efficacy of some selected plant oils against *Pediculus humanus capitis in vitro*. *Parasitology Research* **115**, 3209–3218.

Zaridah MZ, Nor Azah MA, Abu Said A and Mohd Faridz Z (2003) Larvicidal properties of citronellal and *Cymbopogon nardus* essential oils from two different localities. *Tropical Biomedicine* **20**, 169–174.

Zhang L, Yang Z, Chen D, Huang Z, Li Y, Lan X, Su P, Pan W, Zhou W, Zheng X and Du Z (2017) Variation on composition and bioactivity of essential oils of four common curcuma herbs. *Chemistry & Biodiversity* **14**, 11.