Insecticidal and repellent activity of essential oils from Copaifera reticulata, Citrus paradisi, Lavandula hybrida and Salvia sclarea against immature and adult stages of Ctenocephalides felis felis

Emily Andressa Santos Lima
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
Diefrey Ribeiro Campos
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
Eduardo Fellipe Melo Santos Soares
edu.fellipe@live.com
Universidade Federal Rural do Rio de Janeiro https://orcid.org/0000-0001-6057-9649
Anna Beatriz Ribeiro Fortunato
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
Taynara Monsores e Silva
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
Nayana de Figueiredo Pereira
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
Douglas Siqueira de Almeida Chaves
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
Yara Peluso Cid
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro
Katherina Coumendouros
Federal Rural University of Rio de Janeiro: Universidade Federal Rural do Rio de Janeiro

Research Article

Keywords: Flea control, Ectoparasites, Natural insecticides, Pulicidae, Vectors

Posted Date: April 29th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4139452/v1

Version of Record: A version of this preprint was published at Acta Parasitologica on August 15th, 2024. See the published version at https://doi.org/10.1007/s11686-024-00874-3.

Abstract

Purpose The flea Ctenocephalides felis (Siphonaptera: Pulicidae), parasitizes dogs and cats globally, acting as a vector for various pathogens affecting both animals and humans. Growing interest in environmentally friendly, plant-based products prompted this study. The aim of the study was to determine the chemical composition of essential oils (EOs) from Copaifera reticulata, Citrus paradisi, Lavandula hybrida and Salvia sclarea, assessing their insecticidal and repellent properties, determining lethal concentrations (LC50 and LC90), and evaluating residual efficacy in vitro against Ctenocephalides felis felis. Methods Gas Chromatography with Flame Ionization Detector analyzed EO composition. In vitro tests involved preparing EO solutions at various concentrations. Ten specimens from each life stage (egg, larva, pupa, adult) were used for insecticidal activity assessment. For immature stages, Petri dishes were used. Mortality percentage was calculated using (number of dead insects X 100) / number of incubated insects. Probit analysis calculated LC50 values with a 95% confidence interval. Results Major EO constituents were β-caryophyllene (EOCR), linalool (EOLH), linalyl acetate (EOSS), and limonene (EOCP). LC50 values were obtained for all stages except for the essential oil of C. paradisi. All oils showed repellent activity at 800 µg/cm2. OECR exhibited greater residual efficacy. Conclusion Each EO demonstrated superior insecticidal activity against specific C. felis felis stages.

Introduction

Ctenocephalides felis felis (Bouché), the cat flea, is considered the major ectoparasite of cats and dogs worldwide. Of cosmopolitan distribution, causing discomfort to pets and their owners, fleas are associated with several diseases, such as flea allergic dermatitis (FAD), cat-scratch fever, and could be involved in the transmission of feline leukemia, viruses (myxomatosis), bacterial diseases (murine typhus, bartonellosis, salmonellosis), protozoa (trypanosomiasis) and helminthiasis [1–4].

The global annual spending on pet flea control is approximately US$ 15 billion, mainly relying on chemical insecticides [5]. Brazil, the world's largest consumer of pesticides since 2008, uses over 700 thousand tons of synthetic products annually [6]. Due to environmental concerns, there is a growing demand for less aggressive methods to control ectoparasites [7]. Essential oils (EO) have emerged as a promising alternative, offering a biodegradable and less polluting option for veterinary ectoparasite control [8, 9]. Despite lower effectiveness in environmental applications, EO is gaining traction in veterinary medicine, especially for controlling parasites like *C. felis felis*. [10–12].

Copaifera spp, part of the Caesalpinaceae *F* subfamily [13], produces an essential oil rich in sesquiterpenes like β-caryophyllene, α-humulene, and α-copaene [14], demonstrating notable *in vitro* activity against *Aedes aegypti* [15]. *Lavandula hybrida* EO, with main components including linalool and linalyl acetate, shows acaricidal and repellent properties against *Rhipicephalus (Boophilus) microplus* [16, 17]. *Salvia sclarea* L. EO, composed by linalyl acetate as major component [18], exhibits larvacidal activity against *Culex pipiens* [19] and *Aedes albopictus* [20]. *Citrus paradisi* L. EO, containing limonene, α-pinene, and β-pinene [21] has potential larvicidal and fumigant effects on *Culex pipiens* [22].

In the demand for new and less aggressive insecticides to the environment, humans, and animals, the aim of this study was to evaluate *in vitro* at the first time the insecticidal and repellent activity of essential oils from *C. reticulata, C. paradisi, L. hybrida* and *S. sclarea* against immature and adult stages of *C. felis felis.*

Material and Methods

Origin of fleas

The cat flea *C. felis felis* and their respective stages (eggs, larvae, pupae, and adults) used in the experiment were obtained from the experimental colony maintained at Laboratory of Experimental Chemotherapy in Veterinary Parasitology of Federal Rural University of Rio de Janeiro (UFRRJ), which was approved by the Commission for Ethics in the Use of Animals of the Veterinary Institute of UFRRJ (CEUA-IV-UFRRJ) with protocol 4313110419.

Essential oils

Essential oils from the leaves of *C. reticulata* (Fabaceae), from the peel of the fruits of *C. paradisi* (Rutaceae), the flowers of *L. hybrida* (Lamiaceae), and the flowers and leaves of *S. sclarea* (Lamiaceae) were purchased from Via Aroma Indústria de

Aromatizadores de Ambientes LTDA (Porto Alegre) and kept in amber bottles protected in a freezer at -20ºC until the moment of chromatographic analysis and biological analyzes with the purpose to curb the degradation of the chemical compounds.

Chemical composition analysis

Chemical composition analysis of essential oils was carried out by gas chromatography using a flame ionization detector (FID) and a split/split-less injector used to detect and separate the constituents of *C. reticulata, C. paradisi, S. sclarea*, and *L. hybrida.* The compounds were separated in HP-fused silica (30 m × 0.25 mm i.d.; film thickness, 0.25 m; Agilent J & W, California, United States). The carrier gas used was helium (1 $mL.min^{-1}$), and the injected volume was 1 µl at a 1:20 division ratio. EO was analyzed using GC-MS QP-2010 Plus (Shimadzu, Japan). The injector, oven, and detector temperatures were determined according to previous studies [23]. The percentage of EO compounds was calculated from the relative area of each peak analyzed by GC-FID. Furthermore, the carrier gas flow, temperature conditions, and capillary column used for GC/MS analysis were the same as those described for GC/FID [23]. Operating conditions of the mass spectrometer were: ionization voltage, 70 eV; mass range, 40–400 m/z, and 0.5 $scan.s^{-1}$. The compound retention index was calculated based on the co-injection of samples with a combination of C_8–C_{20} hydrocarbons. Compounds were distinguished by comparing their mass spectra with the NIST-Mass Spectrometry Data Center library and data from Adams [23].

Bioassays

Samples preparations

Direct dilution was performed from the EOs or stock solution to obtain the desired concentration range using acetone (99%) as diluent. For insecticidal activity bioassay the concentration range for each EO in each stage are described in Table 1. For the repellency test, three concentrations were performed for the four EOs which were 40000; 20000 and 5000 $µg.mL^{-1}$ that corresponded to 800; 400 and 100 $µg.cm^{-2}$ after strip impregnation.

For the placebo control, acetone alone was used, for the negative control paper filter discs or strips did not receive any type of impregnation, and for positive control of insecticidal activity, fipronil (8 $µg.cm^{-2}$) was used for the stages of larva, pupa, and adult, and pyriproxyfen (8 $µg.cm^{-2}$) for eggs. For the repellency test, the positive control was N,N-diethyl-m-toluamide (DEET) (40000 $µg.mL^{-1}$ and 800 $µg.cm^{-2}$).

To evaluate the adulticidal activity, filter paper strips with an area of 10 cm² (1x10cm) Whatman nº1 (80g) were used impregnated with 0.200 mL of each solution. To evaluate ovicidal, larvicidal, and pupicidal activity, discs of the same filter paper with an area of 23.76 cm² impregnated with 0.470 mL of each solution were used. The repellent activity tests were made using strips of the same filter paper with an area of 7 cm² (0.7 x 10 cm). After impregnation, the material remained on the bench for a period of 30 minutes for complete evaporation of the acetone before performing the test.

Table 1
Concentration ranges for *in vitro* insecticidal activity bioassays for each essential oil in each stage.

	Concentration range	
Stage	**µg.mL^{-1}**	**µg.cm^{-2}**
***Copaifera reticulata* Essential Oil**		
Egg	1250; 2500; 5000; 10000; 20000; 40000	25; 50; 100; 200; 400; 800
Larva	5000; 7500; 10000; 15000; 20000; 30000	110; 165; 220; 330; 440; 660
Pupa	10000; 15000; 20000; 25000; 30000; 40000	200; 300; 400; 500; 600; 800
Adult	10000; 20000; 25000; 30000; 35000; 40000	150; 200; 300; 400; 600; 800
BCI	2500; 5000; 7500; 10000; 15000	30; 50; 100; 150; 200; 300
Lavandula hybrida Essential Oil		
Egg	2000; 6000; 8000; 10000; 15000; 20000	40; 120; 160; 200; 300; 400
Larva	4000; 6000; 7000; 8000; 9000; 10000	90; 130; 150; 180; 200; 220
Pupa	15000; 20000; 30000; 35000; 40000; 50000	300; 400; 600; 700; 800; 1000
Adult	10000; 20000; 25000; 30000; 35000; 40000	200; 400; 500; 600; 700; 800
BCI	250; 1000; 2000; 6000; 8000; 15000	5; 20; 40; 120; 160; 300
Salvia sclarea Essential Oil		
Egg	5000; 10000; 15000; 20000; 30000; 40000	100; 200; 300; 400; 600; 800
Larva	4000; 5000; 6000; 8000; 9000; 10000	80; 100; 120; 160; 180; 200
Pupa	5000; 10000; 20000; 40000; 60000; 80000	100; 200; 400; 800; 1200; 1600
Adult	20000; 30000; 40000; 50000; 60000; 80000	400; 600; 800; 1.000; 1.200; 1.600
BCI	500; 1250; 2500; 5000; 10000; 20000	10; 25; 50; 100; 200; 300
Citrus paradisi Essential Oil		
All stages	50000; 60000; 70000; 80000; 90000; 100000	1000; 1200; 1400; 1600; 1800; 2000
BCI = Biological Cycle Inhibition		
Insecticidal activity (Mortality)		

To evaluate the adulticide activity of EOs, for each repetition, 10 adult fleas, not fed, with 14 days old, five males and five females were placed in a test tube (1 x 10cm) with a filter paper tape impregnated with the different concentrations of EOs. For the evaluation of the activity against the immature stages, were used: 10 eggs aged less than 24 hours, 10 larvae aged between five to seven days (L3), and 10 pupae aged 10 days old. These were arranged in plastic Petri dishes (60 x 15cm) that contained the filter paper disc inside impregnated with different concentrations of EOs. Following the test, the material was incubated in climate-controlled chambers with demand oxygen biochemistry with controlled temperature and relative humidity (27 ± 1°C; 75 ± 10%). The evaluation period for adult fleas and larvae was 24 hours, for eggs 72 hours, and for pupae, it was 15 days after incubation. The motility criterion used for adult fleas and larvae was the movement, which, any minimal activity presented by the insect, characterizes it as alive. The eggs that did not hatch larvae were considered dead. For pupae, it was considered as dead individuals, pupae that did not emerge as adult fleas.

Biological cycle inhibition

For analysis of the biological cycle inhibition (BCI) of *C. felis felis*, 10 eggs (aged less than 24 hours) were arranged in plastic Petri plates (60 x 15cm) that contained inside the filter paper disk impregnated with the different OEs concentrations. It was added 0.5 g

of a larval diet based on wheat bran, dehydrated cattle blood and washed sand, in the proportion of 1:1:5, as described by Correia et al. (2003). Petri's plates were maintained in temperature of 27 ± 1°C and relative humidity 75 ± 10% for a period of 30 days. Any egg was considered dead if was not capable of generating an adult flea.

Residual efficacy

The residual efficacy of the essential oils against adults of *C. felis felis* was determined by means of the filter paper impregnation technique [24] against unfed adult fleas. Strips impregnated and dried were inserted into glass tubes containing 10 unfed adult cat fleas, which five males and five females. Tubes were sealed and kept in a climatized chamber at 28 ± 1°C and relative humidity of 75 ± 10%. Every 24h, fleas were counted (dead and alive) and all fleas were replaced with 10 new fleas. The tests were performed in sextuplicate, using the first concentration responsible for 100% mortality. The evaluation was carried out until a percentage of mortality equal to zero was obtained.

Repellent activity

To evaluate *in vitro* repellent activity of EOs the technique to determine the flea susceptibility or resistance to insecticides was the same as Su et al. [25]. Glass test tubes (1,4 x 25 cm) and two long strips of filter paper were used. The filter paper strips were impregnated and after drying, a transparent double-sided adhesive tape was glued longitudinally on the two strips of impregnated filter paper. In each tube, two strips were inserted vertically into the tube and parallel to each other, one impregnated with the diluent (99% acetone) and the other impregnated with the EO (or positive control), which five flea couples were inserted and waited for a period of 30 minutes. After 30 minutes, the quantitative record was performed analyzing the distribution of the specimens in the two strips of filter paper (impregnated with diluent and with the EO) present inside the test tube, determining the percentage of repellency. For the persistence evaluation, the specimens were removed from the tubes and new couples were introduced to perform new evaluations at times of 3, 6, 12, 24, and 48 h, posteriorly, calculating the repellency rate.

Data Analysis

In the tests in which the insecticidal activity was evaluated, the number of live and dead individuals was counted and the percentage of mortality was calculated for each concentration using the formula described by Abbott [26]: percentage efficacy = (number of dead insects in the treated group–number of dead insects in the control group) × 100 / (100–number of dead insects in the control group). LC_{50} and LC_{90} values were calculated for each evaluation using probit analysis through the statistical program RStudio. Team software (2020, RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA, USA), with a 95% confidence interval ($p < 0.05$). In the repellency tests, after accounting the distribution of fleas on the tapes, it was determined the percentage of repellency from the formula described by WHO [27]: Repellency (%) = (Number of individuals on placebo strip - Number of individuals on EO strip / Number of individuals on placebo strip) x 100.

Results

Chemical Composition of Essential Oils

GC-MS analysis shown that *C. reticulata* EO mainly comprised β-caryophyllene (48%), α-humulene (8.65%) and α-copaene (7.87%). *Lavandula hybrida* EO comprised 27.8% of linalool, 19.29% acetate linalyl, and 13.48% camphor. *Salvia sclarea* EO contained linalyl acetate (51.37%) and linalool (21.13%). *Citrus paradisi* EO showed limonene (91.42%) as its major constituent. All results of EOs composition are described in Table 2.

Table 2
Chemical composition results of essential oils of *Citrus paradisi, Copaifera reticulata, Lavandula hybrida* and *Salvia sclarea.*

Chemical Composition (%)				
Compounds	**EOCP**	**EOCR**	**EOLH**	**EOSS**
(*Z*)-salvene	- - -	- - -	- - -	0.070
(*E*)-salvene	- - -	- - -	- - -	0.040
n-hexanol	- - -	- - -	- - -	0.040
α-pinene	**1.080**	0.450	1.77	0.070
β-pinene	0.820	- - -	- - -	0.090
Myrcene	**3.760**	- - -	- - -	1.180
Limonene	**91.420**	0.340	**5.61**	0.660
(*Z*)-*β*-ocimene	- - -	- - -	- - -	0.540
(Z)-*β*-ocimene <(E)-β->	0.080	- - -	- - -	0.480
p-mentha-2,4(8)-diene	- - -	- - -	- - -	0.220
Linalool	- - -	- - -	**27.81**	**21.130**
Terpinen-4-ol	- - -	- - -	2.16	0.050
α-terpineol	- - -	- - -	0.35	**5.170**
γ-terpineol	- - -	- - -	- - -	0.020
Nerol	- - -	- - -	- - -	0.620
Linalool acetate	- - -	- - -	**19.29**	**51.370**
Linalool acetate < dihydro->	- - -	- - -	0.32	0.320
Geranyl formate	- - -	- - -	- - -	0.130
Neryl acetate	- - -	- - -	- - -	1.760
α-copaene	- - -	**7.870**	- - -	0.690
Geranyl acetate	- - -	- - -	0.79	**3.610**
β-bourbonene	- - -	- - -	- - -	0.130
(*E*)-caryophyllene	- - -	- - -	- - -	**3.340**
β-copaene	- - -	- - -	- - -	0.060
α-humulene	- - -	**8.650**	- - -	0.060
Germacrene D	- - -	- - -	- - -	**3.880**
Germacrene B	- - -	1.260	- - -	- - -
Bicyclogermacrene	- - -	- - -	- - -	0.690
(*E,E*)-farnesene	- - -	- - -	- - -	0.030
δ-cadinene	- - -	2.800	- - -	0.100
Caryophyllene oxide	- - -	0.800	- - -	0.110
1,8-cineole	- - -	0.820	- - -	- - -
δ-elemene	- - -	0.420	- - -	- - -

Chemical Composition (%)				
Compounds	**EOCP**	**EOCR**	**EOLH**	**EOSS**
α-cubebene	- - -	0.680	- - -	- - -
β-elemene	- - -	1.210	- - -	- - -
β-caryophyllene	- - -	**48.820**	- - -	- - -
α-trans-bergamotene	- - -	**7.020**	- - -	- - -
Caryophyllene < 9-epi-(E)->	- - -	0.510	- - -	- - -
β-chamigrene	- - -	2.060	- - -	- - -
Muurolene < γ->	- - -	**7.150**	- - -	- - -
β-selinene	- - -	0,520	- - -	- - -
α-selinene	- - -	0,950	- - -	- - -
α-muurolene	- - -	0,830	- - -	- - -
β-bisabolene	- - -	4,160	- - -	- - -
(E)-cadina-1,4-diene	- - -	0.510	- - -	- - -
(Z)-limonene oxide	0.130	- - -	- - -	- - -
Hexyl acetate	- - -	- - -	0.28	- - -
1,4-cineole	- - -	- - -	0.46	- - -
1,8-cineole	- - -	- - -	**9.17**	- - -
p-cymene	- - -	- - -	0.71	- - -
γ-terpinene	- - -	- - -	0.26	- - -
Terpinolene	- - -	- - -	0.7	- - -
Camphor	- - -	- - -	**13.48**	- - -
Isoborneol	- - -	- - -	2.03	- - -
Borneol	- - -	- - -	3.48	- - -
Lavandulyl acetate	- - -	- - -	1.22	- - -
trans-caryophyllene	- - -	- - -	**7.29**	- - -
n.i.	2.410	2.170	2.820	3.340
Total identified (%)	98.7	100	100	94.8

EOCP = essential oil of *Citrus paradisi;* EOCR = essential oil of *Copaifera reticulata;* EOLH = essential oil of *Lavandula hybrida;* EOSS = essential oil of *Salvia sclarea*. n.i. = not identified.

Insecticidal Activity and Biological Cycle Inhibition

Citrus paradisi EO achieved 25% of mortality against eggs and larvae stages and no mortality against pupae and adults at the maximum concentration (2000 μg.cm^{-2}) tested. BCI was also not observed at the concentration range evaluated. Since mortality above 50% was achieved, LC_{50} and LC_{90} were not calculated to this EO. Mortality of *C. reticulata*, *L. hybrida*, *S. sclarea* and *C. paradisi* EOs against each stage of *C. felis felis* are described in Table 3.

Table 3
Mortality rate of essential oils of *C. reticula* against immature and mature stages of *Ctenocephalides felis felis*

Copaifera reticulata Essential Oil									
Eggs		**Larvae**		**Pupae**		**Adults**		**BCI**	
Conc. (µg.cm⁻²)	**Mortality (%)**	**Conc. (µg.cm⁻²)**	**Mortality (%)**	**Conc. (µg.cm⁻²)**	**Mortality (%)**	**Conc. (µg.cm⁻²)**	**Mortality (%)**	**Conc. (µg.cm⁻²)**	**Mortality (%)**
Neg. Control	5.0	**Neg. Control**	0	**Neg. Control**	12.2	**Neg. Control**	0	**Neg. Control**	25.3
Placebo	0	**Placebo**	0	**Placebo**	11.7	**Placebo**	1.6	**Placebo**	21.5
25	0	**110**	6.7	**200**	34.0	**150**	0	**30**	0
50	0	**165**	30.0	**300**	59.6	**200**	16.7	**50**	1.7
100	0	**220**	51.7	**400**	78.7	**300**	43.3	**100**	13.6
200	0	**330**	78.3	**500**	80.8	**400**	43.3	**150**	52.3
400	15.0	**440**	83.3	**600**	93.6	**600**	83.3	**200**	72.7
800	15.0	**660**	96.7	**800**	100.0	**800**	100.0	**300**	100.0
Lavandula hybrida Essential Oil									
Eggs		**Larvae**		**Pupae**		**Adults**		**BCI**	
Conc. (µg.cm⁻²)	**Mortality (%)**	**Conc. (µg.cm⁻²)**	**Mortality (%)**	**Conc. (µg.cm⁻²)**	**Mortality (%)**	**Conc. (µg.cm⁻²)**	**Mortality (%)**	**Conc. (µg.cm⁻²)**	**Mortality (%)**
40	7.0	**90**	0	**300**	8.5	**150**	10.0	**5**	3.8
120	15.8	**130**	35.0	**400**	25.4	**200**	16.7	**20**	18.9
160	38.6	**150**	71.2	**600**	40.7	**300**	21.7	**40**	34.0
200	82.5	**180**	90.0	**700**	61.0	**400**	60.0	**120**	62.3
300	98.3	**200**	90.2	**800**	76.3	**600**	85.5	**160**	88.7
400	100.0	**220**	100.0	**1000**	100.0	**800**	100.0	**300**	100.0
Salvia sclarea Essential Oil									
Eggs		**Larvae**		**Pupae**		**Adults**		**BCI**	
Conc. (µg.cm⁻²)	**Mortality (%)**	**Conc. (µg.cm⁻²)**	**Mortality (%)**	**Conc. (µg.cm⁻²)**	**Mortality (%)**	**Conc. (µg.cm⁻²)**	**Mortality (%)**	**Conc. (µg.cm⁻²)**	**Mortality (%)**
40	7.0	**90**	0	**300**	8.5	**150**	10.0	**5**	3.8
120	15.8	**130**	35.0	**400**	25.4	**200**	16.7	**20**	18.9
160	38.6	**150**	71.2	**600**	40.7	**300**	21.7	**40**	34.0
200	82.5	**180**	90.0	**700**	61.0	**400**	60.0	**120**	62.3
300	98.3	**200**	90.2	**800**	76.3	**600**	85.5	**160**	88.7
400	100.0	**220**	100.0	**1000**	100.0	**800**	100.0	**300**	100.0
Pos. Control	100.0	**Pos. Control**	100.0	**Pos. Control**	100.0	**Pos. Control**	100.0	**Pos.**	100.0

Copaifera reticulata Essential Oil									
Eggs		Larvae		Pupae		Adults		BCI	
Conc. (µg.cm⁻²)	Mortality (%)	Conc. (µg.cm⁻²)	Mortality (%)	Conc. (µg.cm⁻²)	Mortality (%)	Conc. (µg.cm⁻²)	Mortality (%)	Conc. (µg.cm⁻²)	Mortality (%)
								Control	

Neg. Control = Negative control group; Pos. Control = Positive control group; BCI = Inhibition of development. Fipronil (8 µg.cm^{-2}) was used for positive control for biological cycle inhibition.

Copaifera reticulata EO presented 96.7% mortality at concentrations of 660 µg.cm^{-2} against larvae, and 100% mortality at a concentration of 800 µg.cm^{-2} against pupal and adult stages, however, no ovicidal activity was observed. *Lavandula hybrida* EO showed a 100% mortality rate at concentrations of 400, 220, 800 and, 1000 µg.cm^{-2} against eggs, larvae, adult and pupae, respectively. *Salvia sclarea* EO achieved 100% mortality at concentrations of 800 µg.cm^{-2} against eggs, 98.4% at 200 µg.cm^{-2} against larvae, and, at a concentration of 1600 µg.cm^{-2} against pupae and adults. Regarding the BCI, it was possible to observe inhibition of development at the highest concentration (300 µg.cm^{-2}) for the EOs of *C. reticulata, L. hybrida* and *S. sclarea.*

LC_{50} and LC_{90} estimative

Determining the lethal concentration values, as seen in Table 4, to *C. reticulata* EO, LC_{50} for larvae was 230.6 µg.cm^{-2}. For pupae, the LC_{50} was 223.1 µg.cm^{-2} and for adults it was 462.5 µg.cm^{-2}. For CL_{90}, 477.3 µg.cm^{-2} was obtained for larvae, for pupae 533.2 µg.cm^{-2} and adults 695.8 µg.cm^{-2}. As it was not observed ovicidal activity, LC_{50} and LC_{90} were not obtained.

For *L. hybrida* EO, for eggs the LC_{50} was 171.4 µg.cm^{-2}, for larvae 142.0 µg.cm^{-2}. For pupae the LC_{50} was 672.9 µg.cm^{-2} and for adults it was 559.3 µg.cm^{-2}. The LC_{90} obtained for eggs was 281.9 µg.cm^{-2}, for larvae it was 188.4 µg.cm^{-2}, for pupae it was obtained 961.3 µg.cm^{-2} and adults 748.3 µg.cm^{-2}.

For *S. sclarea* EO, the LC_{50} for eggs was 390.2 µg.cm^{-2}, for the larval stage was 103.7 µg.cm^{-2}. For pupal stage the LC_{50} was 891.0 µg.cm^{-2} and for adults it was 559.3 µg.cm^{-2}. The LC_{90} obtained for eggs was 390.2 µg.cm^{-2}, for larvae was 168.0 µg.cm^{-2}. For pupae it was 1483.2 µg.cm^{-2}, and adults 1290.6 µg.cm^{-2}.

Table 4
Lethal Concentrations 50 and 90 values for the different stages of *Ctenocephalifes felis felis* after exposure to *Copaifera reticulata, Lavandula hybrida* and *Salvia sclarea* essential oils.

Stages	**Confidence Interval (95%)**		**Slope (ER)**	**R^2**	**Chi-squared**	***p***
	Lethal Concentration (Minimum – Maximum)					
***Copaifera reticulata* Essential Oil**						
Larvae	**LC_{50}**	230.6 (208.0–253.6)	4.78 (0.50)	0.919	4.779	0.146
	LC_{90}	477.3 (425.0–551.0)				
Pupae	**LC_{50}**	223.1 (190.5–252.6)	3.39 (0.98)	0.924	11.419	0.878
	LC_{90}	533.2 (463.9–643.6				
Adults	**LC_{50}**	462.5 (419.4–507.4)	7.22 (1.51)	0.970	2.258	0.311
	LC_{90}	695.8 (595.8–718.4)				
BCI	**LC_{50}**	100.0 (80.3–112.0)	3.1 (1.11)	0.973	13.012	0.989
	LC_{90}	262.8 (207.8–389.6)				
Lavandula hybrida Essential Oil						
Stages	**Confidence Interval (95%)**		**Slope (ER)**	**R^2**	**Chi-squared**	**p**
	Lethal Concentration (Minimum – Maximum)					
Eggs	**LC_{50}**	171.4 (116.1–193.0)	4.0 (0.25)	0.951	29.313	0.999
	LC_{90}	281.9 (240.1–352.3)				
Larvae	**LC_{50}**	142.0 (133.9–149.2)	10.4 (3.01)	0.919	11.328	0.921
	LC_{90}	188.4 (177.9–204.1)				
Pupae	**LC_{50}**	672.9 (580.2–708.0)	5.7 (1.23)	0.975	13.532	0.991
	LC_{90}	961.3 (846.4–1199.0)				
Adults	**LC_{50}**	559.3 (520.9–596.3)	10.1 (1.54)	0.965	10.345	1
	LC_{90}	748.3 (688.7–857.7)				
BCI	**LC_{50}**	47.8 (37.2–58.4)	2.2 (1.32)	0.934	19.747	0.997
	LC_{90}	183.8 (144.9–256.5)				
Salvia sclarea Essential Oil						
Stages	**Confidence Interval (95%)**		**Slope (ER)**	**R^2**	**Chi-squared**	**p**
	Lethal Concentration (Minimum – Maximum)					
Eggs	**LC_{50}**	390.2 (346.1–438.3)	4.85 (0.74)	0.989	9.749	0.955
	LC_{90}	716.6 (609.7–923.6)				
Larvae	**LC_{50}**	103.7 (94.5–112.1)	7.01 (0.88)	0.975	5.566	0.766

CL_{50} = Lethal concentration 50; CL_{90} = Lethal concentration 90; BCI = Inhibition of development

Copaifera reticulata Essential Oil						
Stages	**Confidence Interval (95%)**		**Slope (ER)**	**R^2**	**Chi-squared**	***p***
	Lethal Concentration (Minimum – Maximum)					
	LC_{90}	168.0 (142.3–187.1)				
Pupae	**LC_{50}**	475.0 (364.9–589.0)	2.45 (0.50)	0.997	14.286	0.994
	LC_{90}	1483.2 (1279.5–1573.9)				
Adults	**LC_{50}**	891.0 (823.0–958.6)	7.97 (0.66)	0.995	2.754	0.400
	LC_{90}	1290.6 (1171.1–1502.0)				
BCI	**LC_{50}**	75,0 (60.3–91.8)	2.9 (0.28)	0.850	4.435	0.963
	LC_{90}	210.5 (161.7–313.1)				
CL_{50} = Lethal concentration 50; CL_{90} = Lethal concentration 90; BCI = Inhibition of development						

The comparative LC_{50} and LC_{90} between the EOs for ovicidal, larvicidal, pupicidal and adulticidal activity is present in Fig. 1. Comparing LC_{50} of *the C. reticulata* EO among the different stages, it was perceptible the following order of potency: pupae (LC_{50} = 223.12 µg.cm^{-2}) > larva (LC_{50} = 230.64 µg.cm^{-2}) > adult (LC_{50} = 462.45 µg.cm^{-2}), noticing that this EO was 2.07 times more potent for pupae and 2.01 times more potent for larva when compared to the adult stage.

The lavandin EO comparison among LC_{50} stages demonstrated the following order of potency: larvae (LC_{50} = 142.04 µg.cm^{-2}) > eggs (LC_{50} = 171.44 µg.cm^{-2}) > adults (LC_{50} = 559.3 µg.cm^{-2}) > pupae (LC_{50} = 672.90 µg.cm^{-2}). For this OE the least susceptible stage was the pupal. This EO was 3.9 times more potent for eggs, 4.73 times more potent for larvae when compared to pupae.

Salvia sclarea EO was more effective in the following order: larvae (LC_{50} = 103.73 µg.cm^{-2}) > egg (LC_{50} = 390.16 µg.cm^{-2}) > pupae (LC_{50} = 474.97 µg.cm^{-2}) > adult (LC_{50} = 891.04 µg.cm^{-2}). The relative potency indicates the EO is 8.58 times more potent for the larval stage, and 2.28 times more potent for the egg stage. The ratio between the LC_{50} of the pupal and adult stages was less than 2, so it is possible to infer there is no potency ratio between these two stages. When comparing exposure time, the relative potency ratio was lower than 2.

Regarding the relative potency, it was possible to verify that the larval stage was 2.22 times less susceptible to *C. reticulata* EO when compared to the others. *Lavandula hybrida* EO when compared to *S. sclarea* EO and *C. reticulata* EO, it was noted that the ratio was less than 2, therefore, there is no difference between them for the larval stage.

For the pupal stage, *C. reticulata* EO presented (Fig. 1) the lowest LC_{50} value (223.1 µg.cm^{-2}), followed by the EO of *S. sclarea* (475.0 µg.cm^{-2}) and *L. hybrida* EO (672.9 µg.cm^{-2}). It was possible to verify that this stage was 3.01 times less susceptible to *C. reticulata* EO when compared to *L. hybrida* EO, and 2.12 times compared to *S. sclarea*.

Concerning the adulticidal activity, it was possible to notice (Fig. 1) that the highest LC_{50} value was the *S. sclarea* EO (891.1 µg.cm$^{-2)}$ followed by the *L. hybrida* EO (559.3 µg.cm^{-2}) and the lowest LC_{50} value was *C. reticulata* EO (462.5 µg.cm^{-2}). It was also possible to verify that for this stage the ratio was less than 2 for all EOs, therefore, there is no difference for the adult stage.

The LC_{50} and LC_{90} values for BCI were respectively 100.0 and 262.8 µg.cm^{-2} for *C. reticulata* EO, 47.8 µg.cm^{-2} and 183.8 µg.cm^{-2} for *L. hybrida* EO, and 75.0 µg.cm^{-2} and 210.5 µg.cm^{-2} for *S. sclarea* EO. When comparing the LC_{50} values (Fig. 1), we observed an ascending order of LC_{50} for BCI: *L. hybrida* (47.8 µg.cm^{-2}) < *S. sclarea* (75.0 µg.cm^{-2}) < *C. reticulata* (100.0 µg.cm^{-2}). The *L. hybrida* EO was 2.09 times more potent than that of *C. reticulata* EO.

Residual efficacy

Residual efficacy was not calculated for *C. paradisi* EO as it did not exhibit adulticidal activity at a concentration of 2000 $\mu g.cm^{-2}$ (100000 $\mu g.mL^{-1}$). However, for *C. reticulata* and *L. hybrida* EOs, a concentration of 800 $\mu g.cm^{-2}$ (40000 $\mu g.mL^{-1}$) was employed to assess residual efficacy. *Copaifera reticulata* EO achieved 100% mortality solely on the first day, declining to zero by day 21, indicative of a gradual decrease in efficacy, and presenting a slope value of -4,49. In contrast, *L. hybrida* EO recorded 100% mortality on the first day of analysis, followed by a reduction to zero after six days of exposure, presenting a value of slope of -21,66. Regarding *S. sclarea EO*, a concentration of 1600 $\mu g.cm^{-2}$ (80,000 $\mu g/mL^{-1}$) was applied, resulting in 100% mortality after 24 hours of exposure. However, the residual efficacy declined to zero by the eleventh day following the challenge, presenting a value of slope of -10,89. Additionally, a positive control (fipronil at 8 $\mu g.cm^2$ – 400 $\mu g.mL^{-1}$) demonstrated a residual efficacy of 99% on the twenty-second day after the challenge. Negative control and placebo experiments did not observe any mortality during the analyses.

Repellency activity

Essential oils repellency activity was evaluated against *C. felis felis* as it is observed in Fig. 3.

For the *C. reticulata* EO, the repellency percentage (RP) range observed for the concentration of 800 $\mu g.cm^{-2}$ was 90 to 18.8%, for 400 $\mu g.cm^{-2}$ was 78.0 to 15.2%, 1h and 48h respectively, and for 100 $\mu g.cm^{-2}$, RP was less than 30%.

In the evaluation of *L. hybrida* EO activity, the RP range observed for the concentration of 800 $\mu g.cm^{-2}$ was 86.8 to 12.5%, for 400 $\mu g.cm^{-2}$ the RP was 60.5 to 18.2% in the evaluations of 1h and 48h respectively. To the concentration of 100 $\mu g.cm^2$ the RP was less than 30%.

Salvia sclarea EO showed a RP range for the concentration of 800 $\mu g/cm^2$ of 92.9 to 46.2%, for 400 $\mu g.cm^{-2}$ the RP range was 60.5 to 23.5%, in the evaluations of 1 and 48h respectively. Concentration of 100 $\mu g.cm^{-2}$ the RP was less than 20%.

For the *C. paradisi* EO the RP observed for the concentration of 800 $\mu g.cm^{-2}$ was lower than 60% from 1h assessment and 30% from the 48h assessment. At the concentration of 400 $\mu g.cm^{-2}$ the RP was less than 50% in the first evaluation and 28.6% in the 48h evaluation. Concentration of 100 $\mu g.cm^{-2}$ the RP was less than 30%.

Also, positive control group (DEET) presented percentages of repellency for the concentration in a range of 800 $\mu g.cm^{-2}$ of 100 to 66.7% evaluations at 1h and 48h respectively. For the concentration of 400 $\mu g.cm^{-2}$ the repellency percentage range was 93.3 to 13.3% in the evaluations of 1h and 48h respectively. For the concentration of 100 $\mu g.cm^{-2}$ the RP was less than 40%.

Discussion

Our findings in chemical composition analysis of EOs (Table 2) corroborate to previous studies found in literature, as *C. reticulata* EO, which Junior et al. [14], detected 78.2% of sesquiterpenes and β-caryophyllene presented with 40.9% in its composition, as one of the major components. Our findings in GC of the *L. hybrida* EO corresponds to previous articles, which was identified linalool (33.2%), linalyl acetate (29.7%), camphor (7.1%) and 1,8-cineole (7.6%) [16], as well as Robu et al. [28], that found same chemical components. *Salvia sclarea* EO composition was previously determined with linalyl acetate (56.04%) and linalool (20.49%) as major constituents [18, 29]. Regarding the *C. paradisi* EO, Uysal et al. [21] in their studies reported that the limonene component was also observed as the major constituent (91.5%), being compatible with the results obtained in the present study. It was found a similarity between *L. hybrida* and *S. sclarea* EOs in their composition, and both are different to *C. reticulata* and *C. paradisi* EOs.

Based on the results of this study, no insecticidal activity nor BCI activity of *C. paradisi* EO was observed for any stage of the flea *C. felis felis* at the evaluated concentrations. However, *C. paradisi* EO insecticidal activity has already been reported for several insects [30, 31] being associated with its major component d-limonene [32]. However, it is possible that higher concentrations allow the detection of a range of mortality for this EO.

Lavandula hybrida EO demonstrated activity against eggs, larvae, pupae and adults of *C. felis felis* (Table 3). The ovicidal and larvicidal activity has already been described for EO's of plants of the genus *Lavandula* against other insects [33–35].

Similar to *L. hybrida* EO, *S. sclarea* EO exhibited activity against all the stages of *C. felis felis*. The expected result could be due to similar chemical composition which both have linalool and linalyl acetate as major constituents. The activity insecticide of this EO has already been described for EO of plants of the genus *Salvia* against lice [36], and larvicidal and adulticidal activity against mosquitoes of the genus *Anopheles* and *Aedes* [20, 37].

Comparing the EO results (Fig. 1), in the egg stage, it was possible to verify that the *L. hybrida* EO presented a lower LC_{50} value (171.4 $\mu g.cm^{-2}$) when compared to the *S. sclarea* EO (390.2 $\mu g.cm^{-2}$). When performing a calculation of relative potency, it was possible to notice that the *L. hybrida* EO was 2.27 times more potent when compared to *S. sclarea* EO.

Other EOs have demonstrated ovicidal activity against *C. felis felis* eggs. Conceição *et al*. [24] demonstrated activity for the OE of *Origanum vulgare* (LC_{50} = 94.5 $\mu g.cm^{-2}$), *Thymus vulgaris* (LC_{50} = 13.5 $\mu g.cm^{-2}$) and *Cinnamomum cassia* (LC_{50} = 3.0 $\mu g.cm^{-2}$). Freitas et al. [10] also demonstrated ovicidal activity for this same parasite for the EO of *Illicium verum* (LC_{50} = 36.9 $\mu g.cm^{-2}$) and *Pelargonium graveolans* (LC_{50} = 18.8 $\mu g.cm^{-2}$). When compared to the results obtained for the EOs evaluated in this work, it is possible to verify that all of them presented lower LC_{50} values.

For larvicidal activity, only *C. paradisi* EO did not presented it. Previous studies have shown EOs with larvicidal activity against *C. felis felis*, as EOs of *I. verum* (LC_{50} = 14.4 $\mu g.cm^{-2}$), *P. graveolans* (LC_{50} = 20.1 $\mu g.cm^{-2}$) [10], *C. cassia* (LC_{50} = 17.2 $\mu g.cm^{-2}$), *T. vulgaris* (LC_{50} = 35.4 $\mu g.cm^{-2}$) and *O. vulgare* (LC50 = 155.1 $\mu g/cm^2$) [24]. As for the three EOs, it is possible to infer that the *S. sclarea* EO presented the lowest LC_{50} value (103.7 $\mu g.cm^{-2}$), followed by EO from *L. hybrida* (188.4 $\mu g.cm^{-2}$) and *C. reticulata* (230.6 $\mu g.cm^{-2}$) against flea larvae. When comparing the EO from *L. hybrida* to the EO from *S. sclarea*, it was possible to notice that the ratio was less than 2.

Essential oils have already been demonstrated pupicidal activity against *C. felis felis*, as the EOs of *C. cassia* (LC_{50} = 34.6 $\mu g.cm^{-2}$), *O. vulgare* (LC^{50} = 98.4 $\mu g.cm^{-2}$), *T. vulgaris* (LC^{50} = 203.2 $\mu g.cm^{-2}$) [24], *I. verum* (LC_{50} = 35.4 $\mu g.cm^{-2}$) and *P. graveolans* (LC_{50} = 67.6 $\mu g.cm^{-2}$) [10]. *Lavandula hybrida, I. verum* and *C. reticulata* EOs presented activity against pupae of *C. felis felis*, being *C. reticulata* EO the lowest LC_{50} (233.1 $\mu g.cm^{-2}$), followed by *S. sclarea* EO (475 $\mu g.cm^{-2}$) and *L. hybrida* EO (672.9 $\mu g.cm^{-2}$). When compared *C. reticulata* EO to *S. sclarea* and *L. hybrida* EOs, the ratio was higher than 2, showing respectively 2.03 and 2.88 more potency for pupicidal activity.

The adulticidal activity of EO has already been described in the literature for the EOs of *I. verum* (LC_{50} = 121.7 $\mu g.cm^{-2}$), *P. graveolans* (LC_{50} = 173.0 $\mu g.cm^{-2}$) [10] *O. vulgare* (LC_{50} = 33.5 $\mu g.cm^{-2}$), *T. vulgaris* (LC_{50} = 64.5 $\mu g.cm^{-2}$) and *C. cassia* (LC_{50} = 74.7 $\mu g.cm^{-2}$) [24]. When compared to the LC_{50} results of EO for fleas, it was possible to verify in this study that the observed values of LC_{50} were higher than those found in the literature. In a comparative way, it is possible to infer that the EO of *C. paradisi* did show no activity, *C. reticulata* was the one that presented the best results against pupae and adult fleas, proving to be promising for the development of adulticidal formulations to control this ectoparasite. On the other hand, the EO of *S. sclarea* was the one that was able to eliminate the larvae of *C. felis felis* in lower concentrations, while the EO of *L. hybrida* demonstrated a significant characteristic for eliminating eggs, making them appropriate for formulations that may be applicable in the environment in order to promote the control of the immature forms of this insect.

Although most of the LC_{50} of the EOs evaluated in this study are greater than those described in the literature for most stages, it is important to emphasize that this is the first study evaluating the insecticidal activity of EOs from *C. reticulata, L. hybrida* and *S. sclarea* against fleas. Probably the insecticidal activity of EOs could be associated with their majority constituents. Still, further studies can be carried out evaluating the synergistic activity of these EOs for flea control.

Evaluating the BCI activity (Table 3), mortality was verified in the negative control and placebo of the tests carried out within the three EOs, and this percentage of mortality ranged from 5.5 to 25.3%. Despite being considered high for a negative/placebo control group, this percentage of mortality becomes acceptable within this type of test, since the effect is being evaluated from the

hatching of the larvae from eggs to adult emergence from the puparium. Within a healthy population of fleas there is a mortality considered normal that occurs in the ecdysis of each stage, in which the percentage of emergence of adults can vary from 70–100% [38]. Among the evaluated activities, BCI was the one that presented the lowest values of LC_{50} for the three EOs that showed such activity (*C. reticulata, L. hybrida* and *S. sclarea*). This lower concentration found to prevent the emergence of adult fleas, when compared with the immature stages in isolation, is most likely due to exposure of the parasite to a longer period to the EO, which may have resulted in chronic toxicity. Among the EOs analyzed, *C. reticulata* presented the highest LC_{50} values, probably caused by the fact that this EO has no ovicidal activity added to its final effect, resulting in higher concentrations to inhibit the emergence of adult fleas.

No other studies pointed the activity of the EOs evaluated in this study on the BCI of other insects. However, when compared to the LC_{50} of other EOs against *C. felis felis* as *S. aromaticum* (LC_{50} = 0.3 μg.cm^{-2}) [11], *C. cassia* (LC_{50} = 2.3 μg.cm^{-2}), *O. vulgare* (LC_{50} = 6.2 μg.cm^{-2}), *T. vulgaris* (LC_{50} = 4.7 μg.cm^{-2}) [24], *I. verum* (LC_{50} = 7.9 μg.cm^{-2}) and *P. graveolens* (LC_{50} = 12.5 μg.cm^{-2}) [10], it is possible realize that other EOs have better activity in the BCI when compared to those evaluated in this study.

Regarding the residual efficacy, EO of *C. reticulata* was the one that presented the longest period of residual efficacy (Fig. 2), during 21 days, followed by the *S. sclarea* EO (11 days) and the *L. hybrida* EO (6 days), however, *C. paradisi* has not presented residual efficacy. Such results show that EOs have a persistent insecticidal effect that decays over days, most likely, due to the fact that its constituents are volatile and are being lost over time. Although Ellse and Wall [9] question the application of EOs for the control of ectoparasites due to its low residual effect, the results demonstrated in this study show that some EOs have greater persistence for insect control than others, and perhaps the development of some pharmaceutical formulations that reduce the volatility of the constituents increases this persistence of effectiveness when used for the animal treatment. On the other hand, the fact that the effectiveness of these products is lower, shows that in theory are less harmful to the environment, since their residue does not remain persistent as a contaminant [39, 40]. The residual efficacy of fipronil was 99% after 21 days of impregnation, showing that synthetic products last longer, mainly because they do not have the characteristic of being volatile. However, in the same way, they remain for more time as environmental contaminants [41].

The repellent activity of EOs has already been reported against several insect species of importance in agriculture and health [42, 43]. Specifically for fleas, it has already been described for the species *C. felis felis* [25] and *Pulex irritans* [44]. For the EO of *C. reticulata* it was not described for any insect species in the literature. However, other plants that have β-caryophyllene as a major constituent have already demonstrated repellent activity [45, 46], then, the possible repellent activity of this EO is attributed to its major constituent.

The repellent activity of *C. paradisi* EO has already been described for *Sitophilus oryzae* (Coleoptera: Curculionidae) with RP of 86% for this insect considered a problem for the grain storage [47]. For fleas, the highest RP was obtained (Fig. 3) in the highest concentration (800 μg.cm^{-2}) in the first hour evaluation, decreasing linearly in the subsequent evaluations. The major constituent of *C. paradisi*, D-limonene, it has also already demonstrated insect repellent activity [48, 49]. However, as observed in this work and in comparison, to the other EOs evaluated, *C. paradisi* did not prove to be the most effective to repel adult fleas.

The repellent activity for the EO of *L. hybrida* has already been described for *Sitophilus zeamais*, *Cryptolestes ferrugineus* and *Tenebrio molitor* [50]. Other species within the genus *Lavandula*, as *L. angustifolia*, showed repellent activity against *Sitophilus granarius* [51, 52].

The repellent activity of EO from different species of the genus *Salvia* has already been described for hematophagous insects [53, 54] and insects of grain storage [55]. The major constituents of the EOs of *L. hybrida* and *S. sclarea*, linalool and linalyl acetate, have also been shown to have repellent activity isolated. Linalool demonstrated repellent activity against mosquitoes [56] and ticks [57]. Just as linalyl acetate has already demonstrated repellent activity against nymphs of *Rhodnius prolixus* [58].

In results of data presented, we can observe the effectiveness of the oils in the following order: *Salvia sclarea* EO > *Copaifera reticulata* EO > *Lavandula hybrida* EO > *Citrus paradisi* EO. The *C. reticulata* EO was also the essential oil that showed the most prolonged efficacy when compared to other EOs, showing residual efficacy of 21 days.

Comparing the four EOs evaluated in this work, it is possible to notice the *C. paradisi* EO was the one that presented the lowest percentages of repellency in the concentrations used,. The *C. reticulata* and *S. sclarea* EOs stood out as a repellent activity, and showed no statistical difference ($p > 0.05$) in any of the evaluations performed at any of the concentrations when compared with the control positive (DEET). The EO of *L. hybrida* showed similar RP results in the first evaluation (1h). However, from the second evaluation (3h) the RP was less than 50% differing statistically from the *C. reticulata* and *S. sclarea* EOs and the positive control.

This research it is the first to evaluate the EOs potential for flea cat control, contributing to the academic environment. However, further studies with the essential oils tested are needed for other parasites of animals of company, as well as carrying out in vivo studies.

Conclusion

It was observed that *C. paradisi* EO did not show any insecticidal or repellent activity. *Copaifera reticulata* EO presented the best results against pupae and adult fleas, proving to be promising for the development of adulticidal formulations to control this ectoparasite. *Salvia sclarea* EO proved the most potential effects for repellency.

The present research can contribute to the academic environment, stimulating more studies with the EOs tested for other animal parasites, as well as carrying out *in vivo* studies.

Declarations

Declaration of competing interesting

The authors declare they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval

All experiments were authorized by the Committee on Ethical Use of Animals (CEUA/IV) of Federal Rural University of Rio de Janeiro (UFRRJ) under protocol number 43131104193.

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Figures

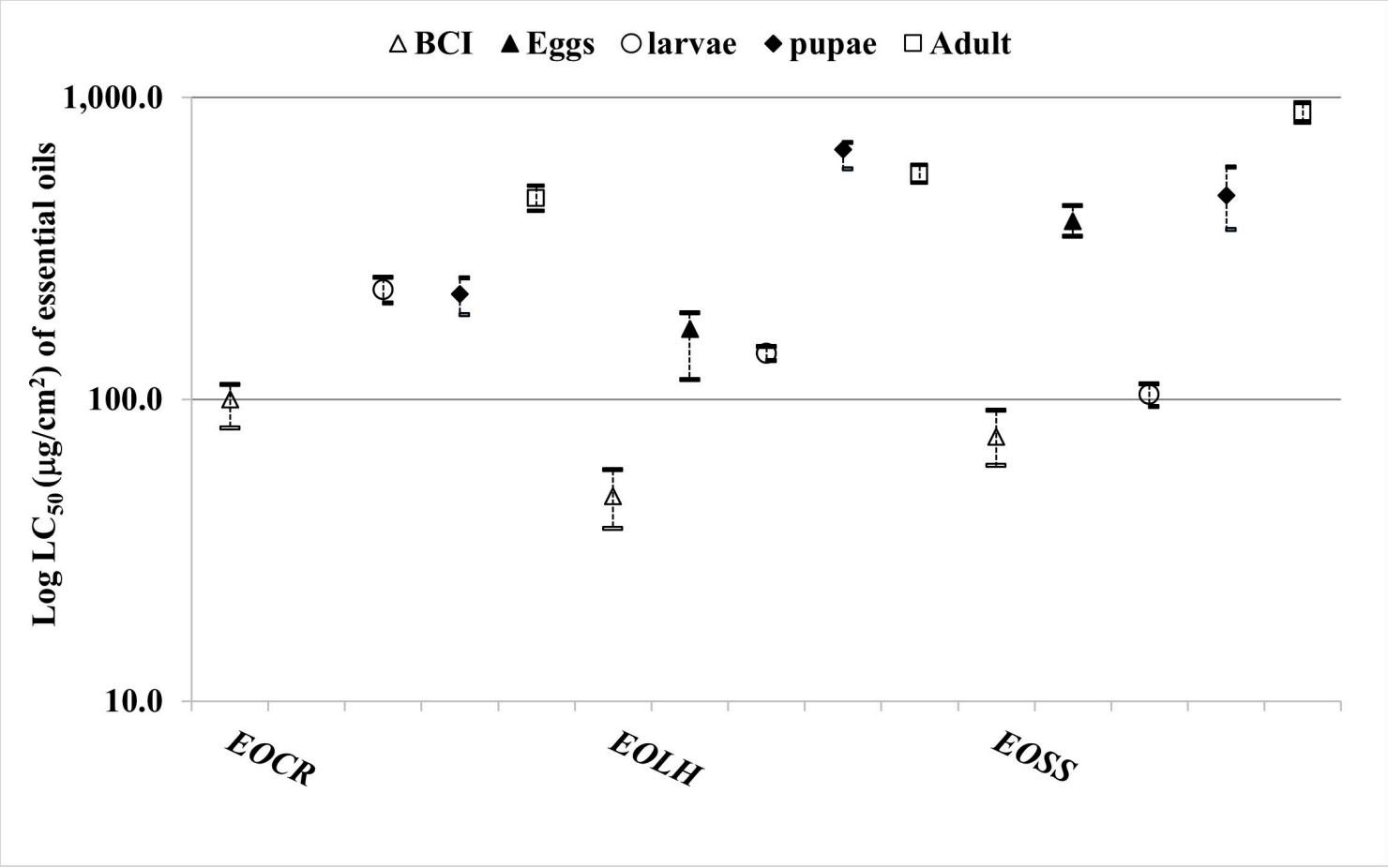

Figure 1

Comparison of LC_{50} between essential oils. BCI = Biological Cycle Inhibition; EOLH = essential oil of *Lavandula hybrida;* EOSS = essential oil of *Salvia sclarea*; EOCR = essential oil of *Copaifera reticulata*.

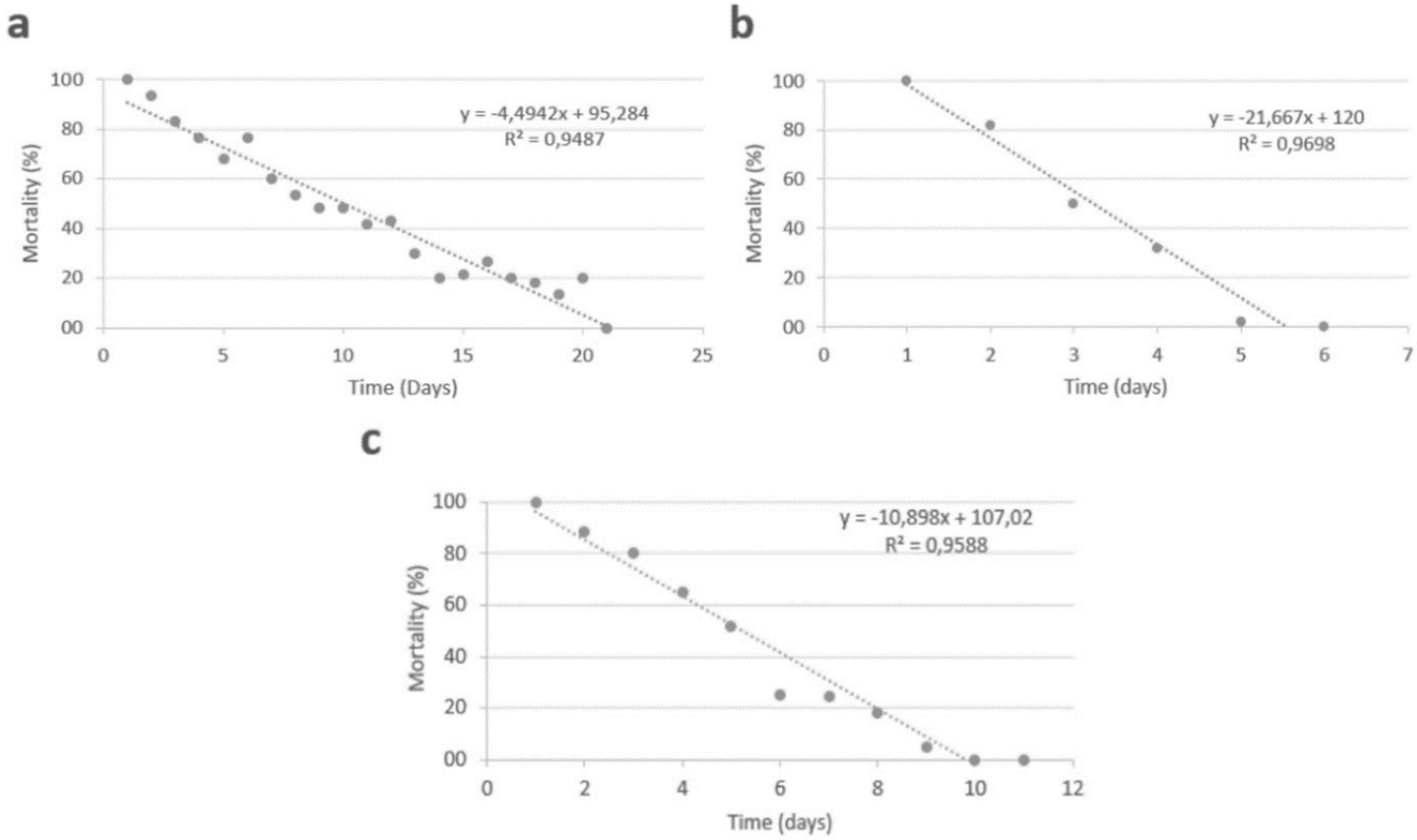

Figure 2

Comparison of residual effect of essential oils against *C. felis felis* through the days. a = *Copaifera reticulata* EO; b = *Lavandula hybrida* EO; c = *Salvia sclarea* EO.

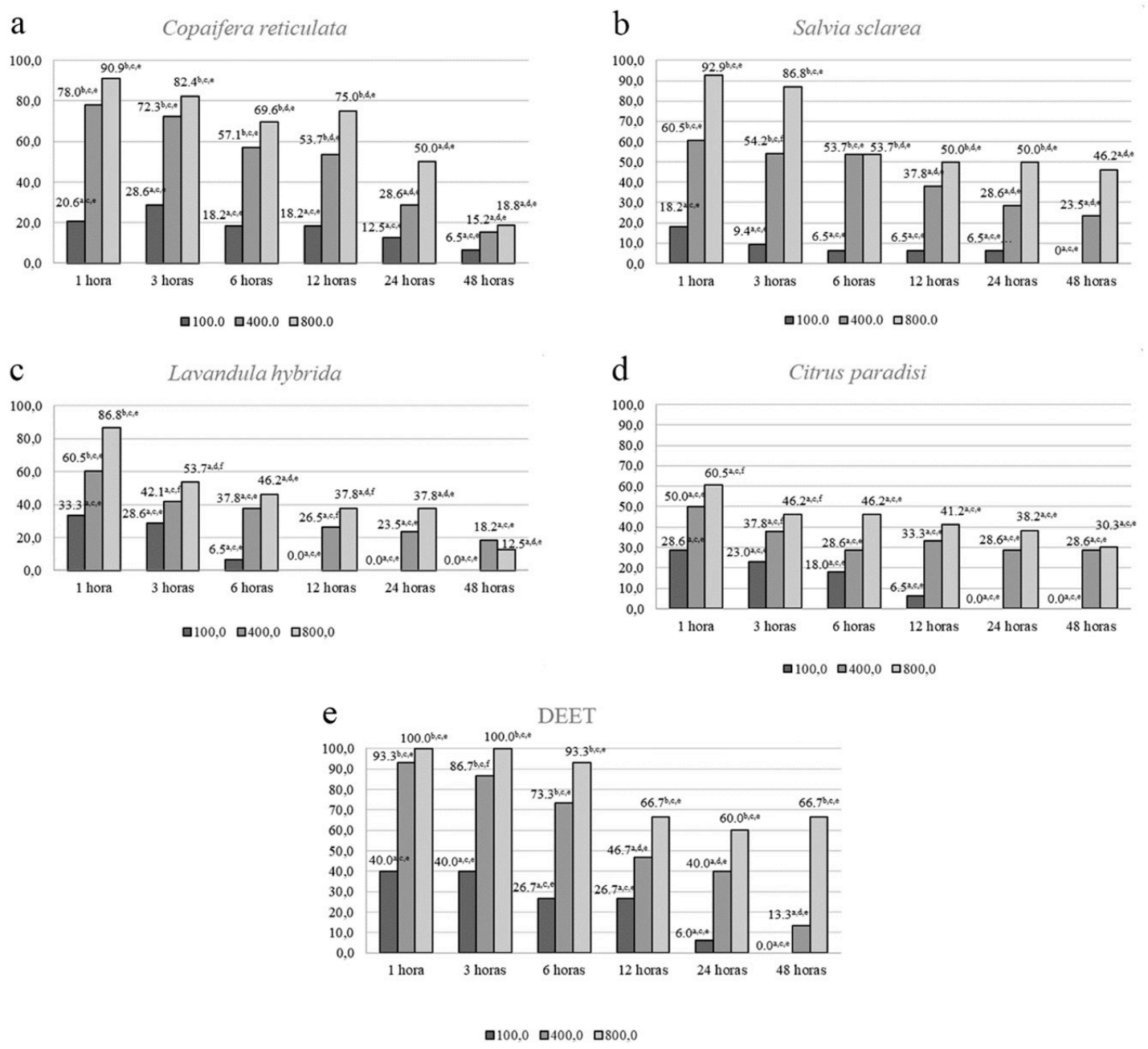

Figure 3

Repellency percentage of *Copaifera reticulata, Salvia sclarea, Lavandula hybrida* and *Citrus paradisi* essential oils against adult fleas of *Ctenocephalides felis felis*. Different letters [a b] = Statistical difference between concentrations of the same essential oil (or DEET) in different time frames; [c d] = Statistical difference of the same concentration in different measurements time; [e f] = statistical difference between different essential oils at the same concentration and same intake time.