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Original Article

Efficacy and residual effect of *Illicium verum* (star anise) and *Pelargonium graveolens* (rose geranium) essential oil on cat fleas *Ctenocephalides felis felis*

Eficácia e efeito residual dos óleos essenciais de *Illicium verum* (anis-estrelado) e *Pelargonium graveolens* (gerânio rosa) em pulgas de gato *Ctenocephalides felis felis*

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Abstract

The essential oils (EOs) of *Illicium verum* and *Pelargonium graveolens* were evaluated for lethality, inhibition of development and residual efficacy against the flea *Ctenocephalides felis felis*. Their chemical composition was characterized by means of gas chromatography with a flame ionization and mass spectrometry detection. Mortality at different immature stages and among adult fleas was measured through *in vitro* filter paper tests at different concentrations of EOs. The chemical characterization of *I. verum* volatile oil showed that *E*-anethole (79.96%) was the major constituent, while the major compounds in *P. graveolens* were citronellol (29.67%) and geraniol (14.85%). Insecticidal activity against both immature and adult flea stages were observed. The EO of *I. verum* had insecticidal activity for approximately 18 days, while the EO activity of *P. graveolens* lasted for 13 days. The pulicidal activity of *I. verum* remained above 70% for up to 9 days, while the activity of *P. graveolens* was 41.7% for up to 2 days. Essential oils, especially that of *I. verum*, showed insecticidal activity for flea control at different life cycle stages and have potential for the development of ectoparasiticides (biopesticides) for veterinary use.

Keywords: Flea, volatile oil, phenylpropanoid, terpenes, geranium, gas chromatography.

Resumo

Os óleos essenciais (OE) de *Illicium verum* e *Pelargonium graveolens* foram avaliados quanto à letalidade, inibição do desenvolvimento e eficácia residual contra a pulga *Ctenocephalides felis felis*. Sua composição química foi caracterizada por meio de cromatografia gasosa com detector de ionização de chama e espectrometria de massas. A mortalidade entre os diferentes estágios imaturos e pulgas adultas foi avaliada por meio de testes *in vitro* em papel filtro, contendo diferentes concentrações de OEs. A caracterização química do óleo volátil de *I. verum* mostrou que o E-anetol (79,96%) foi o constituinte majoritário, enquanto os principais compostos de *P. graveolens* foram citronelol (29,67%) e geraniol (14,85%). Foi observada atividade inseticida contra os estágios imaturos e adulto da pulga. O OE de *I. verum* teve atividade inseticida por aproximadamente 18 dias, enquanto o de *P. graveolens* durou 13 dias. A atividade pulicida de *I. verum* permaneceu acima de 70% até o 9º dia, enquanto a atividade de *P. graveolens* foi de 41,7% até o 2º dia. Os óleos essenciais, principalmente de *I. verum*, apresentam atividade inseticida para o controle de pulgas em diferentes estágios do ciclo de vida e têm potencial para o desenvolvimento de ectoparasiticidas (biopesticidas) de uso veterinário.

Palavras-chave: Pulga, óleo volátil, fenilpropanóide, terpenos, gerânio, cromatografia gasosa.

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Introduction

Ownership of pets such as dogs and cats is increasing, causing rising concern over methods to control infestations caused by ectoparasites such as fleas. *Ctenocephalides felis felis* is the most prevalent flea subspecies parasitizing companion animals worldwide. They are responsible for causing blood loss and irritation of parasitized animals (Rust, 2020). Saliva inoculation can lead to the development of flea allergy dermatitis, which represents a large part of dermatological care in dogs and cats (Streicher, 2019). Furthermore, they are responsible for the transmission of several pathogens such as: *Bartonella* species, *Coxiella burnetii*, Hemoplasmas, *Rickettsia* species and *Yersinia pestis* (Lappin et al., 2020).

Essential oil are complex mixtures originated from the secondary metabolism, produced by the glandular trichomes, and in different secretory structures. Then can be compose of terpenes (mainly mono and sesqui) and/or phenylpropanoids. These can be applied by industry, food and pharmaceuticals (Dawood et al., 2021). Activity of essential oils against fleas has been reported (Lambert et al., 2020; Dos Santos et al., 2020; Conceição et al., 2020). It is believed that these oils can act by means of several mechanisms, since the chemical composition of essential oils is complex, possibly including mono and sesquiterpenes and/or phenylpropanoids.

Illicium verum, known as star anise, has shown activity against several insects of agricultural and veterinary importance (Matos et al., 2020). Its major compound is the phenylpropanoid *E*-anethole. *Pelargonium graveolens*, known as rose geranium, is also toxic to various insects (Saraiva et al., 2020). Its oil is rich in the monoterpenes citronellol and geraniol (Saraiva et al., 2020).

The aim of the present study was to conduct an *in vitro* evaluation on the insecticidal activity of the essential oils of star anise and rose geranium against the different immature stages and residual efficacy against the adult form of the flea *Ctenocephalides felis felis*.

Material and Methods

Essential oils

Essential oils of *Illicium verum* L. (Schisandraceae) (star anise) - and *Pelargonium graveolens* L'Hér. ex Aiton (Geraniaceae) (rose geranium) were purchased commercially (Via Aroma©). The EOs were kept protected from the light and were stored at -20 °C until the time of the chromatographic and biological analyses.

Dereplication of chemical constituents

The analysis was performed by means of a gas chromatography (GC) device equipped with a flame ionization detector (FID) and a split/split-less injector to separate and detect the constituents of the volatile oils of *I. verum* and *P. graveolens*. The compounds were separated using a fused silica capillary column (5% phenyl; 95% dimethylpolysiloxane), of dimensions 30 m × 0.25 mm (i.d.) × 0.25 µm (film thickness). Helium was used as the carrier gas at a flow rate of 1 mL/min. The column temperature was programmed as follows: 60 °C for 2 min followed by heating at 5 °C/min to 110 °C, followed by heating at 3 °C/min to 150 °C and, lastly, followed by heating at 15 °C/min up to 290 °C and holding this constant for 15 min. The injector temperature was 220 °C and the detector temperature was 290 °C. To separate and identify the substances, 1 µL of volatile oils samples diluted in dichloromethane (10 µl/ml) was injected at the times defined, into the gas chromatograph coupled to a mass spectrometer (GC-MS) QP-2010 Plus (Shimadzu, Japan). The flow of the helium carrier gas, the capillary column and the temperature conditions for the GC-MS analysis were the same as described for the GC. The temperature of the injector was 220 °C and the interface temperature was 250 °C. Mass spectra were obtained with a quadrupole detector operating at 70 eV, with a mass range of 40-400 *m/z* and a scanning rate of 0.5 scan/s.

The chemical identification of the essential oil was based on linear retention indices (LRI) and mass spectra of the samples. These were compared with authentic standards injected under the same conditions, and using the NIST database (2008) and the Kovats index (KI) (Adams, 2007). The LRI was calculated based on co-injection of alkane series (van Den Dool & Kratz, 1963).

Flea origin

Different stages (adults, eggs, larvae and pupae) of *Ctenocephalides felis felis* (Bouché, 1935), obtained from a laboratory colony maintained in cats without introduction of external specimens and without exposure to

insecticides since 1998, were used in this study. This species is registered in the National System of Genetic Heritage Management and Associated Traditional Knowledge (SisGen) under number A710DC4 and the flea colony was approved by the Ethics Committee for Animal Use of the Veterinary Institute, Federal Rural University of Rio de Janeiro, under protocol number 4313110419.

Essential oil activity against *Ctenocephalides felis felis in vitro*

The technique using filter paper was used for the bioassays. Dilutions of the essential oils of *I. verum* and *P. graveolens* were made using acetone as diluent and as a negative control. For positive control, a solution of fipronil (400 $\mu g.mL^{-1}$) was used. Direct dilutions of the essential oil were made, to obtain 10 solutions at concentrations ranging from 40,000 $\mu g.mL^{-1}$ to 100 $\mu g.mL^{-1}$ for *I. verum* and from 12,000 $\mu g.mL^{-1}$ to 50 $\mu g.mL^{-1}$ for *P. graveolens*, according to the flea stage under analysis. The bioassays were performed in sextuplicate, with filter paper strips of 10 cm^2 and filter paper discs of 23.76 cm^2, for adult fleas and immature phases, respectively. The strips were impregnated with 200 µL and the discs with 475 µL of their respective dilutions, so that the final concentrations for *I. verum* and *P. graveolens* were in the range of 800.00 to 100.00 $\mu g.cm^{-2}$ and 240.00 to 120.00 $\mu g.cm^{-2}$ respectively. After impregnation, the tapes and discs were dried for 1 h before the bioassay procedure.

The *in vitro* activity of the essential oils against immature stages of *C. felis felis* was evaluated using filter paper tests against (Conceição et al., 2020) eggs, larvae and pupae. The inhibition of flea development was also analyzed. The dried impregnated discs were inserted in petri dishes containing 10 eggs, larvae or pupae of *C. felis felis*. After sealing, the petri dishes were kept in a climatized chamber (Eletrolab®, Model: 102FC, Serie: 970603) at 28 ± 1 °C and relative humidity of 75 ± 10%.

The evaluation criteria for ovicidal, larvicidal and pupicidal tests were the hatching, motility and emergence of adult fleas, respectively. In the evaluation of developmental inhibition, 10 newly collected eggs were incubated in a petri dish with the impregnated disc, and the emergence of adult fleas was evaluated. The results from the ovicidal test were evaluated after 72 h, as was also the development inhibition test, the larvicidal test after 24 and 48 h, while the pupicidal test was evaluated after 15 days. All tests were performed in sextuplicate.

In vitro insecticidal activity against *C. felis felis* adults was evaluated using the impregnated filter paper technique (Conceição et al., 2020) 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). After sealing with non-woven tissue and rubber bands, the tubes were kept in a climatized chamber at 28 ± 1 °C and relative humidity of 75 ± 10%. Insecticidal activity was evaluated according to the motility of the fleas, which were deemed to be alive at the slightest movement observed. The tests were performed in sextuplicate for each concentration. The mean number of live adult fleas per concentration was evaluated after 24 and 48 h using a stereo microscope (Wild Heerbrugg, M5-52796).

Efficacy evaluation and establishment of LC_{50} and LC_{90} and Statistical analysis

To calculate the efficacy, Abbott's formula (Abbott, 1987) was used: percentage efficacy = [(mean number of fleas (corresponding flea stage) of the control group – mean number of fleas (corresponding flea stage) of the treated group)/ (mean number of fleas (corresponding flea stage) of the control group)] × 100. LC_{50} and LC_{90} were established through Probit analysis using the RStudio Team software (2020, RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA, USA). Statistical significance was set at 5% ($P < 0.05$).

To compare the mortality means between the different concentrations, the Shapiro Wilks test was performed to determine the normality of the data. The comparison of means was performed using the ANOVA test followed by the T-test (LSD). The BioEstat version 5.3 program was used for the statistical analysis of the data, considering a significance level (α) 5%.

Residual efficacy

The residual efficacy of the essential oils against adults of *C. felis felis* was assessed by means of the filter paper impregnation technique (Conceição et al., 2020) against unfed fleas. The impregnated and dried strips were inserted into glass tubes containing 10 unfed adult cat fleas (five males and five females). After sealing with non-woven tissue and rubber bands, the tubes were kept in a climatized chamber at 28 ± 1 °C and relative humidity of 75 ± 10%. Every 24 h, live fleas were counted and the fleas in the tubes were replaced with 10 new fleas. The impregnated

tape was maintained to assess the durability of the insecticidal activity of the essential oils in a single application. The tests were performed in sextuplicate, using the concentration responsible for 100% mortality: 800 μg.cm^{-2} and 240 μg.cm^{-2}, for *I. verum* and *P. graveolens*, respectively. Insecticidal activity was evaluated according to the motility of the fleas, which were deemed to be alive at the slightest movement observed.

Results

Chemical analysis

The gas chromatography analysis (Table 1) led to identification of 15 compounds in *I. verum* EO, among which the phenylpropanoid (*E*)-anethole was the major compound (79.96%) and the other constituents were in a concentration lower than 10% (Figure 1a). In turn, the *P. graveolens* EO was found to contain 27 compounds, among which the major ones were the class of monoterpene oxygenated as citronellol (29.67%) and geraniol (14.85%) (Figure 1b).

Efficacy and LC_{50} and LC_{90}

The essential oil of *I. verum* caused 100% mortality of adult fleas at a concentration of 400.0 μg.cm^{-2} after 48 hours. This same total mortality was observed for concentrations of 80.0 μg.cm^{-2}, 20.0 μg.cm^{-2} and 140.0 μg.cm^{-2} against the egg, larval and pupal stages, respectively. The concentration of 40.0 μg.cm^{-2} totally inhibited development from egg to adult. In turn, the essential oil of *P. graveolens* caused 100% mortality at lower concentrations after exposure for 48 hours: 240.0 μg.cm^{-2} against adult fleas; 100.0 μg.cm^{-2} against pupae; and 35.0 μg.cm^{-2} for inhibition of the biological cycle. On the other hand, the concentrations causing 100% mortality were greater for ovicidal activity (60.0 μg.cm^{-2}) and larvicidal activity (40.0 μg.cm^{-2}) (Table 2).

Comparison of the estimated lethal concentration values revealed that the immature stages were more susceptible when measured using the LC_{50} values, ranging from 7.9 to 12.4 μg.cm^{-2} for inhibition of the biological cycle, 18.8 to 36.9 μg.cm^{-2} against eggs, 12.1 to 16.3 μg.cm^{-2} versus larvae, and 35.4 to 67.6 μg.cm^{-2} against pupae. The same pattern was observed for the estimated LC_{90} values, ranging from 20.4 to 30.1 μg.cm^{-2} for inhibition of the biological cycle, 54.5 to 55.6 μg.cm^{-2} against eggs, 16.8 to 32.3 μg.cm^{-2} against larvae and 85.4 to 87.9 μg.cm^{-2} versus pupae. Both essential oils presented similar LC_{50} and LC_{90} values for inhibition of the biological cycle and egg stage, while for larvicidal activity, the *I. verum* essential oil presented relative potency (LC_{90}) 2-fold higher. For adult fleas, the insecticidal activities measured by LC_{50} and LC_{90} ranged from 119.1 to 164.6 μg.cm^{-2} and from 209.5 to 258.1 μg.cm^{-2}, respectively (Table 3).

Residual efficacy

The residual efficacy of the essential oils was measured to assess the longevity of insecticidal activity (Figure 2a and 2b). The EO of *I. verum* retained its insecticidal activity for approximately 18 days, while the OE activity of *P. graveolens* lasted for 13 days. The EO of *I. verum* caused 80% mortality on the 7th day of exposure, decreasing to approximately 50% on the 10th day of analysis. On the 13th day, it was still able to kill 36.7% of the fleas and this went down to 1.7% activity on the 18th day. Although the EO of *Pelargonium graveolens* had the lowest concentration capable of causing 100% mortality, its effectiveness did not last long. On the 3rd day of exposure, the pulicidal activity of the *P. graveolens* EO declined to approximately 50% mortality, falling to just under 30% on the 5th day, 9.5% on the 12th day and no activity on the 13th day of exposure.

Discussion

This is the first study to show the insecticidal activity of essential oils from *I. verum* and *P. graveolens* against adult fleas and their immature stages, as well as their residual activity. The chromatographic analysis showed a total of 16 substances in *I. verum* and 27 in *P. graveolens*. GC/MS identified that monoterpenes were the major class in *P. graveolens* (63.01%) and phenylpropanoids formed the majority in *I. verum* (86.34%). As described by other authors, *E*-anethole (1) was the major compound of star anise essential oil, but other phenylpropanoids, such as (*Z*)-anethole (2) and estragole (3), were also abundant (Yu et al., 2021). E-anethole has already been described as the major constituent of *I. verum* essential oil in some studies that report insecticidal activity (Nilprapat et al.,

Table 1. Chemical characterization of *Illicium verum* and *Pelargonium graveolens* essential oils.

Compounds	AIT	IVEO	PGEO
Monoterpenes hydrocarbons			
α-pinene	932	0.97	-
α-phellandrene	1002	0.57	-
Carene	1008	0.36	-
Limonene	1024	2.05	0.80
β-phellandrene	1025	0.43	-
Monoterpenes oxygenated			
1,8-cineol	1026	0.42	-
Linalool	1095	-	5.00
(*E*)-rose oxide	1122	-	2.58
β-terpineol	1140	3.58	-
(+)-menthone	1148	-	2.50
(-)-menthone	1148	-	5.72
4-terpineol	1174	0.29	-
α-terpineol	1186	-	1.29
Citronellol	1223	-	29.67
Neral	1235	-	0.77
Geraniol	1249	-	14.85
Geranial	1264	-	0.45
Citronellyl formate	1271	-	9.41
Neryl formate	1280	-	4.13
Sesquiterpenes hydrocarbons			
α-cubebene	1348	-	0.40
Ylangene	1373	0.31	-
α-ylangene	1375	-	0.96
β-elemene	1389	-	0.34
(*Z*)-farnesene	1440	-	3.37
6,9-guaiadiene	1442	-	1.62
(*E*)-Muurola-3.5-diene	1451	-	0.71
α-clovene	1452	-	0.55
α-humulene	1452	-	0.35
Sesquiterpenes oxygenated			
β-thujaplicin	1475	0.39	-
Geranyl propanoate	1476	-	2.02
γ-himachalene	1481	1.20	-
Citronellyl butanoate	1532	-	0.84
2-phenyl ethyl tiglate	1584	-	1.86
γ-eudesmol	1630	-	2.32
α-eudesmol	1637	-	0.48
Citronellyl angelate	1656	-	0.66
Geranyl tiglate	1696	-	1.65
Phenylpropanoid			
Estragole	1195	5.75	-
Anisaldehyde	1247	2.49	-
(*Z*)-anethole	1249	0.63	-
(*E*)-anethole	1282	79.96	-
Monoterpenes hydrocarbons		4.38	1.98
Monoterpenes oxygenated		4.29	61.83
Sesquiterpenes hydrocarbons		1.51	8.30
Sesquiterpenes oxygenated		-	4.45
Phenylpropanoid		86.34	-
Total		95.92	95.75

The chemical composition was analyzed 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 (AIT). Not detected (–). IVEO – *Illicium verum* essential oil. PGEO – *Pelargonium graveolens* essential oil.

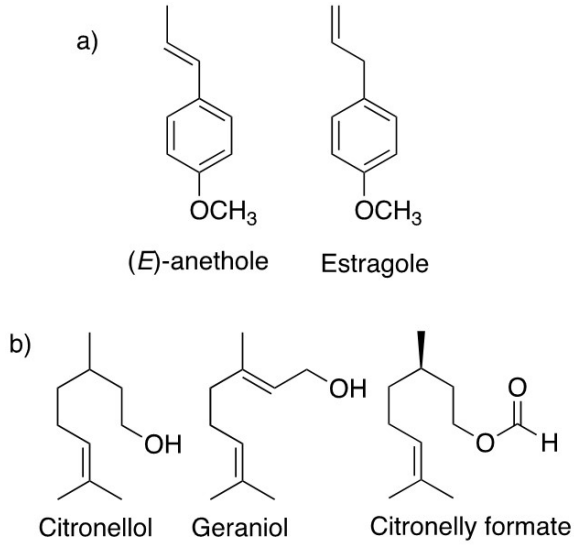

Figure 1. Major compounds identified in the (a) *Illicium verum* and (b) *Pelargonium graveolens* essential oil.

Table 2. Mortality (%) and life cycle inhibition of *Illicium verum* and *Pelargonium graveolens* volatile oils against different stage of the flea.

Illicium verum										
Conc. (µg.cm^{-2})	**Egg***	**Conc. (µg.cm^{-2})**	**Larva (24h)**	**Larva (48h)**	**Conc. (µg.cm^{-2})**	**Pupae***	**Conc. (µg.cm^{-2})**	**ID***	**Conc. (µg.cm^{-2})**	**Adult (24h)**	**Adult (48h)**
	Mortality (%)		**Mortality (%)**			**Mortality (%)**		**Mortality (%)**			
Cont. neg.	11.7^{a}±36.7	**Cont. neg.**	0^{a}±0	0^{a}±0	**Cont. neg.**	13.3^{a}±12.1	**Cont. neg.**	21.7^{a}±11.7	**Cont. neg.**	0^{a}±0	0^{a}±0
Placebo	15.0^{a}±10.5	**Placebo**	0^{a}±0	0^{a}±0	**Placebo**	13.3^{a}±11.6^{a}	**Placebo**	21.7^{a}±10.6	**Placebo**	0^{a}±0	0^{a}±0
30.0	26.7^{a}±8.2	**10.0**	21.7^{b}±11.7	26.7^{b}±10.3	**20.0**	25.0^{b}±8.4	**2.0**	4.3^{a}±7.5	**100.00**	40.0^{b}±25.0	41.7^{b}±19.4
40.0	61.7^{b}±14.7	**12.0**	38.3^{b}±11.7	45.0^{b}±5.5	**40.0**	58.3^{b}±24.0	**5.0**	41.7^{b}±14.7	**200.00**	75.0^{b}±21.8	75.0^{b}±18.7
50.0	78.3^{b}±29.3	**14.0**	53.3^{b}±17.5	75.0^{b}±22.4	**60.0**	66.7^{b}±25.8	**10.0**	78.3^{b}±14.7	**240.00**	85.0^{c}±17.6	85.0^{c}±17.6
60.0	93.3^{c}±8.2	**16.0**	71.7^{b}±21.4	80.0^{b}±46.4	**80.0**	85.0^{c}±10.5	**20.0**	85.0^{c}±16.4	**300.00**	90.0^{c}±11.0	93.3^{c}±5.2
70.0	98.3^{c}±4.1	**18.0**	86.7^{c}±13.7	95.0^{c}±5.5	**100.0**	93.3^{c}±8.2	**30.0**	95.0^{c}±8.4	**400.00**	96.7^{c}±5.2	100.0^{c}±5.5
80.0	100.0^{c}±0.0	**20.0**	98.3^{c}±4.1	100.0^{c}±0	**140.0**	100.0^{c}±0	**40.0**	100.0^{c}±0	**800.00**	100.0^{c}±0	100.0^{c}±0
Pelargonium graveolens											
Conc. (µg.cm^{-2})	**Egg***	**Conc. (µg.cm^{-2})**	**Larva (24h)**	**Larva (48h)**	**Conc. (µg.cm^{-2})**	**Pupae***	**Conc. (µg.cm^{-2})**	**ID***	**Conc. (µg.cm^{-2})**	**Adult (24h)**	**Adult (48h)**
	Mortality (%)		**Mortality (%)**			**Mortality (%)**		**Mortality (%)**		**Mortality (%)**	
Cont. neg.	11.7^{a} ±10.6	**Cont. neg.**	0^{a}±0	0^{a}±0	**Cont. neg.**	11.7^{a}±12.1	**Cont. neg.**	11.7±7.5	**Cont. neg.**	0^{a}±0	0^{a}±0
Placebo	13.3^{a}±10.3	**Placebo**	0^{a}±0	0^{a}±0	**Placebo**	13.3^{a}±8.6	**Placebo**	11.6±8.2	**Placebo**	0^{a}±0	0^{a}±0
10.00	26.7^{b}±11.7	**11.6**	15.0^{b}±10.5	21.7^{b}±11.7	**50.0**	36.7^{a}±12.1	**1.0**	4.8±7.5	**120.00**	10.2^{b}±21.9	13.2^{b}±22.2
20.00	55.0^{b}±12.1	**20.0**	23.3^{b}±37.8	33.3^{b}±13.7	**60.0**	55.0^{b}±15.2	**10.0**	28.7±9.8	**140.00**	18.3^{b}±13.3	23.3^{b}±16.3
30.00	60.0^{b}±10.5	**25.0**	53.3^{b}±10.3	70.0^{b}±6.3	**70.0**	70.0^{b}±12.6	**20.0**	63.7±16.7	**160.00**	38.3^{b}±11.7	41.7^{b}±14.7
40.00	75.0^{b}±11.0	**30.0**	70.0^{b}±11.0	86.7^{c}±12.1	**80.0**	83.3^{b}±16.3	**25.0**	75.1±8.9	**180.00**	53.3^{b}±17.5	65.0^{b}±25.1
50.00	90.0^{c}±5.5	**35.0**	93.3^{c}±8.2	98.3^{c}±4.1	**90.0**	91.7^{c}±7.1	**30.0**	85.1±11.7	**200.00**	70.0^{b}±15.5	83.3^{b}±10.3
60.00	100.0^{c}±0	**40.0**	100.0^{c}±0	100.0^{c}±0	**100.0**	100.0^{c}±0	**35.0**	100.0±0	**240.00**	96.7^{c}±8.2	100.0^{c}0

*Corrected mortality. ID = inhibition of development; h hours; Conc. = concentration; Neg. Cont. = negative control. Note: ANOVA test followed by the T-test (LSD). Different letters a b c indicate statistical difference.

Table 3. Lethal concentrations [LC_{50} and LC_{90} (μg.cm^{-2})] and slopes for *Illicium verum* and *Pelargonium graveolens* essential oils against different immature stages and adults of *Ctenocephalides felis felis.*

Essential oil	Flea stage	(95% CI)		Slope (SE)	R^2	χ^2
		LC_{50} (μg.cm^{-2})	LC_{90} (μg.cm^2)			
IVEO	Egg	36.9 (33.4-39.9)	55.6 (51.1-62.8)	7.20 (0.4)	0.953	0.900
	Larvae (24 h)	14.4 (13.5-15.3	20.8 (19.2-23.4)	8.1 (0.8)	0.951	0.568
	Larvae (48 h)	12.1 (11.21-12.7)	16.8 (15.7-18.5)	8.1 (0.9)	0.960	0.562
	Pupae	35.4 (29.1-41.1)	87.9 (75.2-108.4)	3.2 (0.3)	0.958	0.707
	ID	7.9 (3.7-9.3)	20.4 (15.3-30.5)	2.1 (0.2)	0.904	0.733
	Adult (24 h)	121.7 (82.4-152.1)	290.6 (230.5-434.1)	3.8 (0.4)	0.757	0.067
	Adult (48 h)	119.1 (65.4-157.4)	258.1 (194.4-494.1)	3.8 (0.6)	0.955	0.750
PGEO	Egg	18.8 (14.9-22.4)	54.5 (44.1-75.6)	2.8 (0.5)	0.989	0.964
	Larvae (24 h)	20.1 (18.7-23.1)	36.7 (31.7-44.7)	4.2 (1.1)	0.970	1
	Larvae (48 h)	16.3 (14.1-18.4)	32.3 (26.8-51.8)	3.6 (0.8)	0.978	1
	Pupae	67.6 (62.0-71.4)	85.4 (78.1-96.5)	7.4 (0.7)	0.970	0.384
	ID	12.5 (8.9-15.1)	30.1 (24.9-41.3)	3.4 (0.6)	0.997	0.851
	Adult (24 h)	173.0 (163.8-192.0)	229.0 (212.3-261.1)	10.5 (2.4)	0.904	0.337
	Adult (48 h)	164.6 (156.1-172.1)	209.5 (196.9-232.2)	12.2 (1.1)	0.917	0.357

ID = inhibition of development; h = hours; SE = standard error; CI = confidence interval. IVEO – *Illicium verum* essential oil. PGEO – *Pelargonium graveolens* essential oil.

2017; Gomes da Rocha Voris et al., 2018). Li et al. (2013). Wang et al. (2021) suggest that its mechanism of action is through the inhibition of acetylcholinesterase.

The major compounds in *P. graveolens* were citronellol and geraniol, according to the results of Verma et al. (2013). According to Gallardo et al. (2015), the main constituents of geranium essential oil showed insecticidal activity against *Musca domestica* alone. However, the synergistic effect was not observed when evaluated in combination. It is not known whether the same profile applies to the pulicidal effect. Therefore, studies with the constituents isolated and in association against *C. felis felis* should be encouraged.

The insecticidal activity of crude extract of *I. verum* fruits has already been reported (Sripongpun, 2008; Szczepanik & Szumny, 2011; Wei et al., 2014). Its essential oil has insecticidal activity for larvae (LC_{50} = 39.8 μg.mL^{-1}) and adults (LC_{50} = 10.3 μg.mg.female^{-1}) of *Aedes aegypti* (Gomes da Rocha Voris et al., 2018) and against the insect of importance for grain storage *Tribolium confusum* (LC_{50} = 552 μl.L^{-1}) (Popović et al., 2019). Acaricide activity has also been described for environmental mites *Dermatophagoides pteronyssinus* (LC_{50} = 0.032 μg.cm^{-2}) (Nilprapat et al., 2017) and tick nymph *Ixodes ricinus* (LC_{50} = 0.2 μl.cm^{-2}) (Elmhalli et al., 2018). Added to the results obtained in this study, star anise essential oil demonstrates to be able to act against different classes of arthropods, hematophagous or not, making it promising for the development of an ectoparasiticidal formulation.

Other essential oils have already demonstrated in vitro insecticidal activity against the *C. felis felis* flea. Essential oils that contain eugenol as their major constituent such as *Syzygium aromaticum* (Lambert et al., 2020) and *Ocimum gratissimum* (dos Santos et al., 2020) have low LC_{50} values for adults of *C. felis felis*. regarding the activity against the immature stages, the essential oils of *Cinnamomum* spp. (Conceição et al., 2020), *S. aromaticum* (Lambert et al., 2020) and *O. gratissimum* (dos Santos et al., 2020) demonstrated to be superior to those found in this study. However, it is important to highlight that both essential oils evaluated in this study presented satisfactory insecticidal activity,

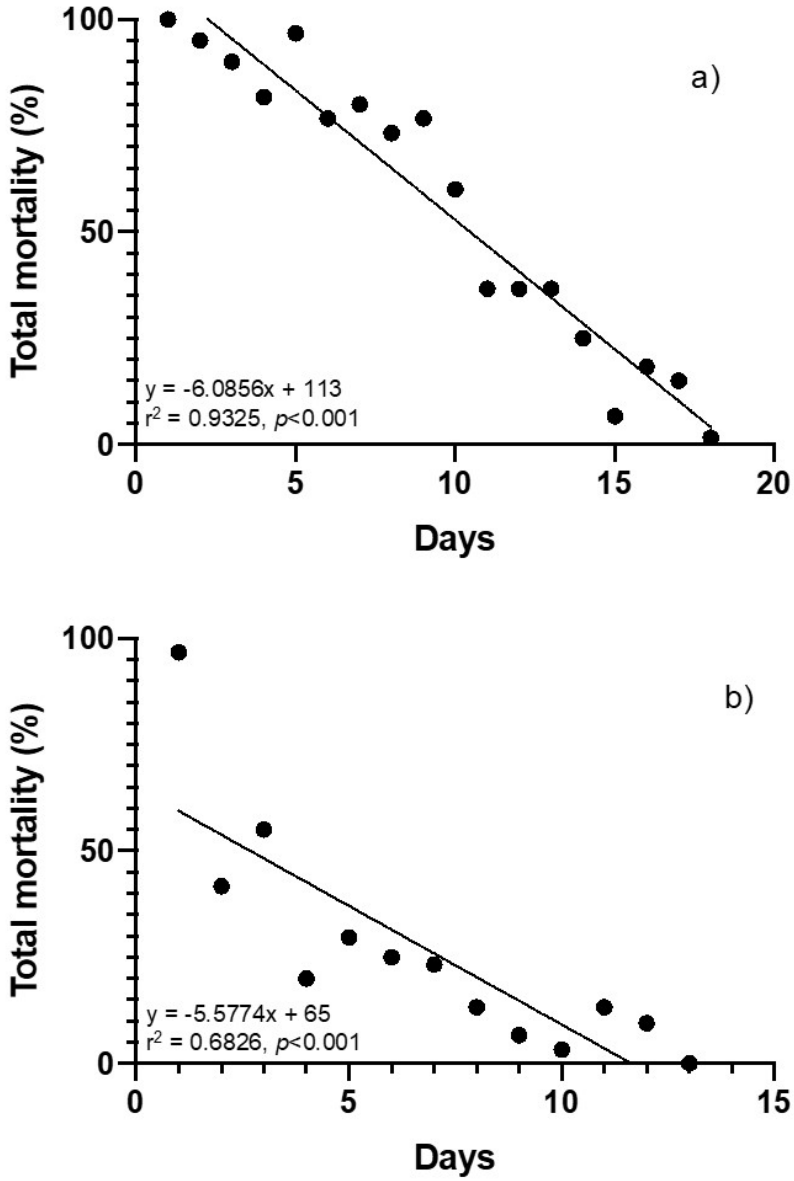

Figure 2. Residual efficacy of essential oils of (a) *Illicium verum* (800 µg·cm^{-2}) and (b) *Pelargonium graveolens* (240 µg·cm^{-2}).

which encourages future studies aiming at their applicability for the control of infestation in animals and in the environment.

We observed mortality of eggs and pupae in the negative control and placebo groups, as well as inhibition of development. According to Rust & Dryden (1997) and Rust (2005), the total emergence of adults, in *C. felis felis,* during the biological cycle can vary between 70-100%.

Both essential oils showed the same characteristic of susceptibility against different stages of the cat flea, which is, comparing the LC_{90} the order of susceptibility was ID<Larva<Egg<Pupae<Adult. The most susceptible was ID because of the essential oil's chronic exposure to all stages. Adults and pupae are the least susceptible and the larval stage most susceptible to ectoparasiticides going as described by Rust (2020).

Star anise is traditionally used as a mosquito repellent and can be used for vector control (Tisgratog et al., 2016). According to the Bio-Pesticides Database (BPDB), use of star anise (70-90% anethole) is authorized in several countries of the European Union (Lewis et al., 2016), although the threshold of toxicological concern is high (class III). Anethole and estragole, which are the major compounds, are effective against stored grain pests (Bedini et al., 2016) and mites (Shin et al., 2013).

The major compounds of *Pelargonium graveolens* were citronellol (29.67%) and geraniol (14.85%), which are both monoterpenes. Their chemical structures are similar, so both compounds can contribute to the effect against fleas, especially against eggs (LC_{50} = 20.97 μg.cm^{-2}) and for inhibition of development (12.45 μg.cm^{-2}). Commercial use of monoterpenes as repellents and insecticides is promising, such as in the product Fulltec®, a tick repellent containing 1% geraniol (Khallaayoune et al., 2009).

The residual effect should be highlighted in relation to development of new biopesticides. In the literature, there are few studies showing the residual effect of essential oils against fleas (Conceição et al., 2020). It is important for EOs to remain active in the environment or on the host after use, to prevent flea reinfestations. Our results showed that the pulicidal activity of the essential oils evaluated remained above 70% for up to 9 days at a concentration of 800 μg·cm^{-2} of *I. verum*. On the other hand, *P. graveolens* at 240 μg·cm^{-2} (100% mortality of adult stage) only had a residual effect of 41.7% after 2 days.

The future of biopesticides using essential oils is promising (Pavela & Benelli, 2016; Mossa, 2016; Raveau et al., 2020). Essential oils have high effectiveness, several mechanisms of action (good for use against resistant insects) and low toxicity, including towards humans. Furthermore, to obtain essential oils is generally relatively simple and cheap, and there is low health risk of intoxication (Pavela & Benelli, 2016).

As shown, both essential oils were able to eliminate both adult fleas and immature stages, in addition to inhibiting the development of the biological cycle of *C. felis felis*, showing potential for the development of formulations for the control of infestations in animals and also for the environmental control of this insect.

Conclusion

Use and application of essential oils requires attention to standardization of the product in order to avoid loss of activity. There are several websites that claim effectiveness of the essential oils of these species for tick control, either applied directly on the animal or in collars or in pharmaceutical medicines. The pulicidal activity and residual efficacy of the essential oils of star anise and rose geranium were demonstrated in the present study for the first time. This efficacy is attractive to the pharmaceutical and agronomy industry for development of biopesticides for veterinary medicine, because the essential oils are safe and environmentally friendly.

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