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Short Communication

Insecticidal activity of essential oil of *Cannabis sativa* against the immature and adult stages of *Ctenocephalides felis felis*

Atividade inseticida do óleo essencial de *Cannabis sativa* frente aos estágios imaturos e adultos de *Ctenocephalides felis felis*

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Abstract

Essential oil (EO) of *Cannabis sativa* (*C. sativa*) was evaluated against the egg, larval, pupal, and adult stages of the flea *Ctenocephalides felis felis.* The chemical composition of EO was determined by gas chromatography with flame ionization and mass spectrometry. EO mainly comprised γ-elemene (16.2%) and caryophyllene oxide (14.2%) as major compounds. To evaluate the mortality of flea stages *in vitro*, filter paper tests were performed at different concentrations. EO of *C. sativa* showed insecticidal activity (100% mortality at the highest concentrations) for flea control at egg, larval, pupal, and adult stages, with lethal concentrations (LC_{50}) of 32.45; 91.61; 466.41 and 927.92 µg/cm^2, respectively. EO of *C. sativa* indicated the potential for the development of ectoparasiticide for veterinary use, especially for fleas in egg and larval stages.

Keywords: Flea, ectoparasite, mortality, pets, volatile oil, biocontrol.

Resumo

O óleo essencial (OE) de *Cannabis sativa* (*C. sativa*) foi avaliado contra os estágios de ovo, larva, pupa e adulto da pulga *Ctenocephalides felis felis*. A composição química do OE foi determinada por cromatografia gasosa com ionização de chama e espectrometria de massa. O OE foi composto principalmente de γ-elemeno (16,2%) e óxido de cariofileno (14,2%) como compostos majoritários. Para avaliar a mortalidade dos estágios de pulgas *in vitro*, foram realizados testes de papel filtro em diferentes concentrações. O OE de C. sativa apresentou atividade inseticida (100% de mortalidade nas maiores concentrações), para controle de pulgas nos estágios de ovo, larva, pupa e adulto, com concentrações letais (CL_{50}) de 32,45; 91,61; 466,41 e 927,92 µg/cm^2, respectivamente. O OE de C. sativa indicou potencial para o desenvolvimento de ectoparasiticida para uso veterinário, principalmente para pulgas em fase de ovo e larva.

Palavras-chave: Pulgas, ectoparasitas, mortalidade, animais domésticos, óleos voláteis, biocontrole.

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Ctenocephalides felis felis (Bouché) is a critical parasitic insect of dogs and cats and is important for public health as is a vector of several pathogenic agents to animals and humans. Of the cosmopolitan distribution, cat fleas are the most abundant ectoparasites in cats worldwide, causing discomfort to pets and their owners. They are associated with several diseases, such as flea allergy dermatitis (FAD) and feline leukemia (Rust, 2020; Vobis et al., 2003).

The cat flea *C. felis felis* is taxonomically grouped in the order Siphonaptera and family Pulicidae. It is classified as a holometabolic cycle insect, with four stages in its life cycle: egg, larva, pupa, and adult. The entire life cycle can be finalized in 12–14 days or prolonged up to 140 days, depending primarily on temperature and humidity (Blagburn & Dryden, 2009). In the egg, larvae, and pupae stages, most flea populations are present around the host animals' habitat, yet they live and feed on the host animal in the adult stage (Wright & Elsheikha, 2014).

The estimated annual worldwide spending to control fleas on pets is approximately US$ 15 billion (Zhang et al., 2021). Most treatments and methods for controlling cat fleas comprise chemical insecticide applications. Nevertheless, in recent years, multiple alternatives have been found for flea control, corresponding with the new wave of sustainable strategies and concerns about reducing the use of chemical pesticides due to the resistance that cat fleas have developed to some conventional treatments, which are mostly based on residual topical or oral medications (Rust, 2020).

Essential oils (EOs) have been studied as alternatives to control ectoparasites of veterinary importance with great relevance, including *C. felis felis* (Freitas et al., 2021; Lambert et. al., 2020; Batista et al., 2016).

Cannabis sativa L. is an important herbaceous species that belongs to the family Cannabaceae and is used in medicine and as a source of textile fiber. It was traditionally cultivated on a large scale in Austria until the 20th century. Essential oil of *C. sativa* has been used for multi-purpose applications in the pharmaceutical industry, especially because of its very low quantity of tetrahydrocannabinol, excluding its psychoactive effect (Novak et al., 2001). Essential oil of *C. sativa* comprises two main fractions: monoterpenes and sesquiterpenes (Fiorini et al., 2019). Studies have shown that EOs from industrial hemp are effective as a larvicide against flies (Benelli et al., 2018) and for their anti-tick activity (Nasreen et al., 2020). Despite the literature showing positive activity against some parasites, the number of studies on fleas is scarce. Hence, this study aimed to evaluate the insecticidal activity of EO of *C. sativa* against egg, larval, pupal, and adult stages of *C. felis felis*.

In this study, we used 24-h and 5-, 10-, and 14-day-old eggs, larvae, pupae, and adults respectively. All flea stages were obtained from a colony maintained in cats of the Laboratório de Quimioterapia Experimental em Parasitologia Veterinária (LQEPV), with all experiments authorized by the standards established by Comissão de Ética no Uso de Animais (CEUA/IV) under protocol number 4313110419, both situated on the Universidade Federal Rural do Rio de Janeiro (UFRRJ).

Cannabis sativa EO was obtained from Canapse® (Process number:23083002965/2020-11) and subjected to gas chromatography (GC) to establish its chemical composition. Gas chromatography was recorded in the Laboratório de Plantas Aromáticas e Medicinais (LABPAM) at UFRRJ using a flame ionization detector (FID) and a split/split-less injector used to detect and separate the constituents of *C. sativa* EO.

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. The injector, oven, and detector temperatures were determined according to Adams (2007). The percentage of EO compounds was calculated from the relative area of each peak analyzed by GC-FID. Moreover, the carrier gas temperature conditions, flow, and capillary column used for GC/MS analysis were the same as those described for GC/FID (Adams, 2007). EO was analyzed using GC-MS QP-2010 Plus (Shimadzu, Japan). Operating conditions of the mass spectrometer were as follows: 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 identified by comparing their mass spectra with the NIST-Mass Spectrometry Data Center library and data from Adams (2007).

The insecticidal activity was divided into two steps. The first was a screening test in which exposure of different flea stages to a range of 10 different concentrations of EO was performed. These tests were performed in duplicates for each concentration, with the positive control and placebo also in duplicates. We subsequently determined the lethal concentration (LC) determination (definitive test), where five concentrations were chosen among the mortality screening range from the first step to be included in a probit analysis. Moreover, the tests were performed in sextuplicate with a positive control and placebo group. To estimate the screening range for adult and immature stages when preparing the concentrations for the first step, a 1:2 serial dilution was performed to obtain concentrations of 40000; 20000; 10000; 5000; 2500; 1250; 625; 312.5; 156.25 and 78.12 µg/mL. The following

were concentrations in grams for the adult and immature stages: 800; 400; 200; 100; 50; 25; 12.5; 6.25; 3.12 and 1.56 µg/cm².

In vitro testing was performed using the filter paper impregnation method with a 10 cm² (1 × 10 cm) Whatman nº1 (80 g) for an area with a stock solution at a concentration of 0.200 mL of the EO for adults. For other appraisals, the same filter paper with 23.76 cm² of the area and impregnation concentration of 0.470 mL was used. After impregnation, the material remained on the bench for 30 min for complete evaporation of the acetone before commencement of the tests. Acetone alone was used as the negative control. To test insecticidal activity, the concentrations used were 12–200; 25–400; 200–1600 and 400–2000 µg/cm² in the eggs, larvae, pupae, and adults, respectively. As a positive control, fipronil (8 µg/cm²) was used for the larvae, pupae, and adults, and pyriproxyfen (8 µg/cm²) was used for eggs.

To evaluate the insecticidal activity of EO against adult fleas, 10 adult fleas (five males and five females), not fed, at 14 days of age were selected for each repetition. The fleas were placed in a 1 x 10 cm test tube with filter paper impregnated with different concentrations. To test insecticidal activity against immature stages, 10 eggs aged 24 h (1 day old), 10 larvae aged 5 days (third larval instar), and 10 pupae aged 10 days were selected by repetition. All groups were placed in 60×15-cm plastic Petri dishes, which were contained inside the filter paper disk impregnated with EO.

After the test, the material was incubated in climatized chambers with biochemical oxygen demand and controlled temperature and relative humidity (27±1°C; 75%±10%), where they were maintained for up to 15 days depending on the stage assessed. The evaluation period was 24 h for adult fleas and larvae, 72 h for eggs, and 15 days after incubation for pupae.

The criterion used to establish the motility of adult fleas and larvae was movement, where any kind of movement presented by the insects indicated that they were alive. Eggs were considered dead if they did not hatch to larvae. For pupae, those that did not emerge as adult fleas were considered dead. Following evaluations, data were collected, and the percentage of mortality was calculated.

The percentage of mortality was calculated for each concentration using the formula described by Abbott (1987): 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).

After determining the mortality rate, the LC_{50} value was 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 analysis of the constituents of EO of *C. sativa*, the two constituents that stood out in the chemical composition were γ-elemene (16.2%) and caryophyllene oxide (14.2%) (Table 1).

The chemical composition of EO (Table 1) is consistent with the findings of other studies, except for the percentage of γ-elemene, which was found in large quantities. Nevertheless, we did not detect a significant amount of monoterpenes. Bertoli et al. (2010) have shown a constant frequency of sesquiterpenes as a major component, especially caryophyllenes.

As shown in Table 2, the mortality rate was 100% at the highest concentrations (200; 400; 1600 µg/cm²) in the egg, larval, and pupal stages. However, for adults, the mortality rate reached 90% at a concentration of 2000 µg/cm², which is a relevant result, even if it is not an absolute mortality rate.

The mortality rate was 100% for positive controls and <5% for placebo sample at all stages, as expected. The estimated LC_{50} values were 32.45 (11.1–69.1); 91.6 (63.9–130.7); 466.4 (300.4–643.7) and 927.9 µg/cm² (653.9–1198.5) for the egg, larvae, pupae, and adult stages, respectively, indicating that EO of *C. sativa* showed insecticidal activity against the immature and adult stages of *C. felis felis*.

Moreover, when comparing the minimum and maximum ranges of LC_{50} among groups, immature stages eggs (11.07–69.13 µg/cm²) and larvae (63.89–130.70 µg/cm²) showed a better response when compared to adult (300.35–643.71 µg/cm²) and pupae (653.92–1198.53 µg/cm²).

These data allowed us to verify the difference in the susceptibility of each stage to EO of *C. sativa*, and when comparing the LC_{50} values of each stage, it was possible to notice that the larval stage was 10.12 times more susceptible than the adult stage, the egg stage was 28.59 times more susceptible than the adult stage, the larval stage was 5.09 times more susceptible than the pupal stage, the egg stage was 14.37 more susceptible than the pupal stage, and the egg stage was 2.82 times more susceptible than the larval stage, indicating that EO of *C. sativa* produced a better response and potency at a lower concentration in the egg and larval stages. This could be related to the fact that adults and pupae are more resistant to ectoparasiticides than larvae and eggs (Rust, 2020).

Table 1. Major substances obtained from the EO of *Cannabis sativa.*

Peak	Retention time	Linear Retention Index	%	Component
1	9.086	931	1.246	α-pinene
2	10.896	976	0.4536	β-pinene
3	11.221	985	5.5975	β-mircene
4	13.095	1027	2.3299	Limonene
5	13.334	1032	0.9226	Eucalyptol
6	13.792	1042	5.8167	1,3,6-Octatriene
7	16.397	1098	1.3577	Linalool
8	31.341	1412	0.4831	Bergamotene
9	31.621	1418	0.5546	Caryophyllene
10	31.812	1423	**16.2067**	γ-elmene
11	32.077	1429	0.9223	Bicyclo[3.1.1]hept-2-ene
12	32.217	1432	3.9773	1,6,10-Dodecatriene
13	32.978	1449	7.0382	α-humulene
14	33.387	1458	5.2006	Alloaromadendrene
15	34.611	1486	0.4608	γ-amorphene
16	34.865	1492	1.8945	Viridiflorene
17	35.152	1498	2.5317	α-farnesene
18	35.365	1503	0.3312	β-bisabolene
19	35.47	1506	0.8691	Germacrene A
20	35.923	1517	0.4725	δ-Cadinene
21	36.179	1523	2.1633	(Z)-nerolidol
22	36.683	1535	2.8117	α-cadinene
23	36.937	1541	7.8595	Selina-3,7(11)-diene
24	37.149	1546	10.0128	(E)-Dauca-4(11),7-diene
25	37.675	1559	1.3268	Germacrene B
26	37.932	1565	**14.1597**	Caryophyllene oxide
27	38.911	1588	0.669	Viridiflorol
28	40.569	1629	0.791	Unidentified
29	41.825	1661	0.7826	Intermedeol
30	43.57	1705		N-heptadecane

Table 2. Mortality rates and LC_{50} in different concentrations of EO of *Cannabis sativa* in different flea stages of life after treatment.

Eggs (72 hours)			Larvae (24 hours)			Pupae (15 days)			Adults (24 hours)		
µg/mL	**µg/cm²**	**Mortality (%)**	**µg/mL**	**µg/cm²**	**Mortality (%)**	**µg/mL**	**µg/cm²**	**Mortality (%)**	**µg/mL**	**µg/cm²**	**Mortality (%)**
Placebo	- - -	0	**Placebo**	- - -	0	**Placebo**	- - -	0	**Placebo**	- - -	0
625	12	17.6	1250	25	5	10000	200	15	20000	400	12
1250	25	23.5	2500	50	20	20000	400	40	40000	800	36
2500	50	41.2	5000	100	45	40000	800	75	60000	1200	60
5000	100	64.7	10000	200	90	60000	1200	85	80000	1600	77
10000	200	100	20000	400	100	80000	1600	100	100000	2000	90
LC_{50}		32.45 µg/cm²	**LC_{50}**		91.61 µg/cm²	**LC_{50}**		466.41 µg/cm²	**LC_{50}**		927.92 µg/cm²
Minimum - Maximum		(11.07 - 69.13 µg/cm²)	**Minimum - Maximum**		(63.89 - 130.70 µg/cm²)	**Minimum - Maximum**		(300.35 - 643.71 µg/cm²)	**Minimum - Maximum**		(653.92 - 1198.53 µg/cm²)
R^2		0.927	**R^2**		0.986	**R^2**		0.969	**R^2**		0.987

These activities could be associated with the composition of EOs. γ-elemene has been cited in the literature as a potential toxic factor for *Anopheles subpictus*, *Aedes albopictus*, and *Culex tritaeniorhynchus* (Govindarajan et al.,

2018). Furthermore, γ-elemene is included in formulations patented to control insects by targeting sundry receptors, including tyramine receptors (Enan, 2008); however, its mechanism of action has not been fully elucidated.

The insecticidal and antiparasitic activities of the compound caryophyllene oxide have already been demonstrated in arthropods in the literature (Bettarini et al., 1993), but not in some insects with medical importance, including mosquitoes from the genera *Culex* sp. and *Aedes aegypti* L. (Hung et al., 2019; Abé et al., 2018). Its mechanism acts as a nerve poison to pests via sodium channel modulators (Liu et al., 2012).

Caryophyllene oxide is commercially available and has low mammalian toxicity, which is a good factor for use in contact with pet animals, and it contains an easily modifiable functional group that could make it possible to change the synthesis of derivatives to study the effect of structural modifications on insecticidal activities (Bettarini et al., 1993).

The higher prevalence of sesquiterpenes in the examined EO could be due to the drying process, which might have induced some chemical modifications in the composition of the starting material, including the evaporation of the low boiling-point compounds and occurrence of oxidative reactions, as in the conversion of β-caryophyllene in caryophyllene oxide, a major component of EO of *C. sativa* (Fiorini et al., 2019).

These two compounds have been already associated with a repellent activity against *Lasioderma serricorne* Fabricius, indicating a potential synergy between compounds, showing that this can influence the insecticidal activity against *C. felis felis* (You et al., 2015).

These results allowed us to propose caryophyllene oxide and γ-elemene as the major active compounds that might be important for the development of newer parasiticides, especially for cat fleas.

The present study showed for the first time the potential of *C. sativa* EO as a botanical insecticide against *C. felis felis,* especially during the egg and larval stages. Future studies should be conducted to develop EO formulations and test their efficacy against other medically important parasites.

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Ethical declaration

All experiments authorized by the standards established by Comissão de Ética no Uso de Animais (CEUA/IV) under protocol number 4313110419 situated on Universidade Federal Rural do Rio de Janeiro (UFRRJ).

Conflict of interest

No potential competing interest was reported by the authors.

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