



Insecticidal activity of essential oils extracted from the leaves of three *Lippia* sp. against *Callosobruchus maculatus* (Fabricius, 1775)

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Received: 30 June 2025 / Accepted: 26 April 2026 / Published online: 23 May 2026
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Abstract

Callosobruchus maculatus (Fabricius, 1775) is considered the main pest that damages stored *Vigna unguiculata* (L.) Walp grains. Control of this insect is usually achieved through the use of synthetic insecticides, whose residual effects pose risks to human health and the environment. Given the important economic losses caused by the infestations of this pest and the need to develop less harmful control methods, this study aimed to evaluate the insecticidal potential of essential oils (EOs) from *Lippia thymoides* Martius & Schauer, *Lippia lasiocalycina* Schauer, and *Lippia insignis* Moldenke against *C. maculatus*. The chemical constituents of the EOs were analyzed, and an in vitro bioassay was conducted using a concentration of 10 $\mu\text{L mL}^{-1}$. Distilled water and a 1% Tween[®] 20 solution were used as negative controls, and Lambda-cyhalothrin was used as a positive control. Insect mortality was recorded at 24 and 48 h after treatment. The results showed that among the tested species, *L. insignis* was the most effective, causing 100% mortality after 48 h. Subsequently, the EO of *L. insignis* was tested at five different concentrations (0.625, 1.25, 2.5, 5 and 10 $\mu\text{L/mL}$) to determine the median lethal concentration (LC_{50}), which was found to be 3.11 $\mu\text{L/mL}$. Based on this result, a concentration of 5 $\mu\text{L/mL}$ was selected to assess the efficacy of two application methods: topical contact and exposure to contaminated surfaces. Both methods proved to be effective in controlling *C. maculatus*.

Keywords Verbenaceae · Weevil · Biopesticide · Stored grain pest · Topical contact · Treated surface

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Introduction

Callosobruchus maculatus (Fabricius 1775) popularly known as bean weevil, is the most important cause of damage to beans of the *Vigna* genus. *Vigna* beans constitute one of the most important foods in tropical and neotropical regions, especially in the Americas, where they account for 47% of world production. In Brazil, the bean trade significantly moves the national GDP, with an estimated production of 1,058.6 thousand tons produced in the period from 2020 to 2021 (CONAB 2020). However, despite the great importance of this type of bean, the use of inadequate practices of transport and storage of grains leads to the proliferation of pests and consequent economic loss (Lorini et al. 2015).

The chemical products most used today for the control of *C. maculatus* have proven to entail several problems for humans and the environment, such as residues in food, elimination of natural enemies, intoxication of applicators, selection of pest populations resistant to insecticides, among

other direct and indirect effects (Carneiro et al. 2015; Babarinde and Akinyemi 2024). Indeed, for a long time, the use of chemical insecticides was the most common approach to pest control. However, due to the numerous health problems resulting from its excessive use, for example: residual accumulation on food, soil and water contamination, in addition to the progressive harmful effects on human health, the use of synthetic pesticides (like organophosphates) is currently prohibited. Nonetheless, many compounds from the group of carbamates, dithiocarbamates, benzoyl ureas, diacylhydrazines, amidines, hydrazones, triazineamines and pyrethroids still have their permitted uses (Pinto-Zevallos and Zarbin 2013). In this context, the use of secondary metabolites by plants represents a relevant alternative for pest control, as it involves the production of chemical compounds with different biological activities against various organisms, making it essential to identify pest management strategies that are both effective and environmentally sustainable (Pino et al. 2013; Cherif et al. 2025; Gupta et al. 2025).

Among the great floristic wealth found in the Caatinga biome, the *Lippia* genus can be mentioned, which is one of the most important representatives of the *Verbenaceae* family. This genus has approximately 200 species of herbs, shrubs and small trees; its major centers of dispersion are found in countries in South and Central America, as well as in territories in tropical Africa (Salimena and Múlgura 2015). In Brazil, this genus can be found mainly in the States of Minas Gerais and Bahia, with most species distributed in the Cerrado and Caatinga biomes (Cardoso et al. 2018). Some *Lippia* species have great economic potential, mainly due to the use of their essential oils in the food, cosmetics, medicine and agricultural industries, especially in the fight against pathogens and pests, reported in several studies, such as Babarinde et al. (2016), R. Silva et al. (2018b) and Baldim et al. (2019). In the literature, the increasing number of studies on the insecticidal activity of species of the genus *Lippia*, such as the results reported by Torre et al. (2024) for *Lippia alba* (Mill.) N.E.Br. in the protection of cowpea seeds, the literature remains focused on a limited number of species, with emphasis, in addition to this species, on *Lippia gracilis* Schauer (Monteiro et al. 2020) and *Lippia sidoides* Cham. (Santos et al. 2018), highlighting the incipient nature of these studies in view of the diversity of the genus. Accordingly, this work sought to evaluate the insecticidal potential of essential oils of three different species of *Lippia* against *Callosobruchus maculatus*, in addition to testing their effectiveness in two different methods, topical application and exposure to contaminated surface.

Materials and methods

Collecting plant species

The plant species used in the experiments were collected from the collection of Medicinal and Aromatic Plants of the Forest Garden Experimental Unit, belonging to the State University of Feira de Santana (HORTO/UEFS), Feira de Santana, Bahia. The species were identified in the Herbarium of the institution (HUEFS) (Voucher: *Lippia thymoides*: 115371; *Lippia lasiocalycina*: 197676 and *Lippia insignis*: 197674). These genotypes were registered with the platform of the National System of Genetic Resource Management and Associated Traditional Knowledge (SISGEN) under reference code A1C1DEB.

Collecting and breeding insects

The specimens of *Callosobruchus maculatus* were collected from samples of cowpea grains, obtained from the supply center in the city of Feira de Santana, taken to the laboratory and then placed in closed plastic pots, where holes were made in the lid, in order to allow air circulation. The pots were kept in a room with environmental temperature and fluorescent lighting. The insects were constantly exchanged between the pots, allowing for the maintenance of multiple generations during the experiment. The identification of insects was carried out according to specialized literature by Athié and Paula (2002).

Extraction of essential oils

In order to perform the extraction of essential oils (EOs), 200 g of dry leaves of each *Lippia* species (Fig. 1. A) were crushed in a blender with distilled water, placed in a glass flask, where distilled water was added in sufficient volume to cover the plant material. The extraction method was carried out by hydrodistillation, using the Clevenger apparatus, coupled in glass flasks, which were heated by electric thermal blankets with thermostat (Fig. 1. B). The extraction lasted 3 h, starting from the appearance of the first condensed drop, and the volume of EO was verified in the graduated column of the Clevenger apparatus, and after extraction, anhydrous sodium sulfate was added to the oil to eliminate residual water, following the method described by A. Silva et al. (2018a). Subsequently, using a Pasteur-type pipette, the oil was collected and transferred to an amber glass vial, and stored in a freezer until chemical analysis and its insecticidal bioassay were carried out (Fig. 1. C).

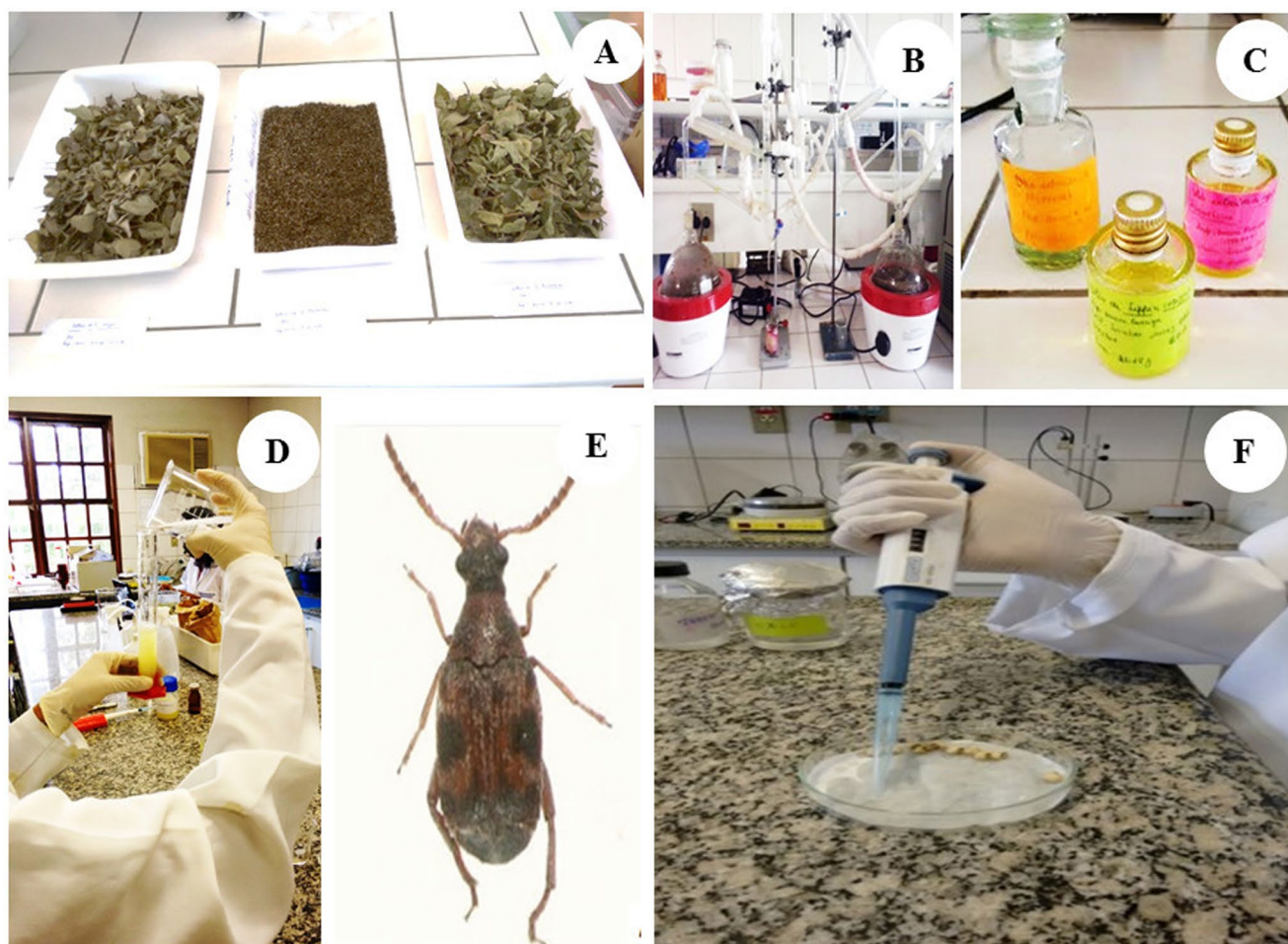


Fig. 1 Steps of the used method: **A:** Dried leaves of the three analyzed *Lippia* species; **B:** Hydrodistillation by steam distillation; **C:** essential oils; **D:** Dilution of essential oils in different concentrations to determine LC_{50} ; **E:** *Callosobruchus maculatus* specimen; **F:** Method of

treated surface used to evaluate the insecticidal potential of the species named *Lippia thymoides*, *Lippia lasiocalycina* and *Lippia insignis* against *Callosobruchus maculatus*

Chemical analysis of essential oils

For chemical composition analysis, 20 mg of essential oils were previously diluted in 1 mL of dichloromethane. Quantification was performed by Gas Phase Chromatography coupled to a Flame Ionization Detector (GC/FID). In order to identify the constituents, Gas Phase Chromatography coupled to Mass Spectrometry (GC/MS) was used. The identification of the constituents was carried out by calculating the Kovats indices, obtained by co-injection of the sample with a homologous series of n-alkanes (C_8 to C_{24}), by comparing the mass spectra with the equipment library and by consulting the specialized literature (Adams 1995). The quantification of the relative percentage of the identified constituents was obtained based on the areas of the corresponding chromatographic peaks, through the normalization method.

Preliminary tests of the insecticidal potential of the EOs of the tested *Lippia* species

The initial concentration tested was 1%, using 500 μ L of oil for 50 mL of distilled water ($v v^{-1}$) equivalent to 10 μ L of oil for each mL of distilled water. Dilution in water was conducted with the aid of Tween® 20 surfactant in a proportion of 1% (Fig. 1. D). In order to carry out the preliminary tests, the method of exposure to treated surface was used according to the methodology adapted from Santos et al. (2010). Petri dishes measuring 9.0 cm in diameter were lined with filter paper and ten uninfested cowpeas grains were placed in each dish. With the aid of a micropipette, each filter paper was soaked with 1.5 mL of the solution containing EO and then ten mixed-sex adult specimens of *Callosobruchus maculatus* were placed per plate (Fig. 1. E-F). The plates were covered with voile-type fabric held by an elastic band,

allowing air circulation. Distilled water and distilled water with Tween® 20 were used as negative controls, reflecting the solvents used to dilute the essential oil, in addition to the commercial agrochemical, Lambda-cyhalothrin (positive control). In order to evaluate mortality, all insects that moved any part of the body when stimulated, were considered alive (Santos et al. 2007). A cumulative dead insect count was performed 24 and 48 h After Treatments (HAT).

The experiments was conducted in a completely randomized design with four replications, each replication consisting of a plate with ten insects. Data were analyzed using the R statistical program (R Core Team 2015), version 3.2.2. As they did not comply with the assumptions of normality and homogeneity, the data were analyzed by means of the non-parametric Kruskal-Wallis test for the 5% probability level and compared by rank.

Determination of median lethal concentration (LC₅₀)

After establishing the initial range in the preliminary tests, the oil obtained from *Lippia insignis* (the species that evoked greater insecticidal activity) was diluted in five different concentrations in geometric progression, 0.625, 1.25, 2.5, 5, 10 $\mu\text{L mL}^{-1}$, in order to determine the concentration capable to achieved at least 50% of bruchids mortality of the individuals, and, as an experimental control, a distilled water solution and Tween® 20 (1%) was used. Four replications per concentration were used, and mortality was assessed at 48 HAT. Tests were performed to verify the statistical assumptions of normality and homogeneity through the Shapiro-Wilk and Barlett tests, respectively. Once the assumptions were met, analysis of variance and data regression were conducted using the simple linear regression model through the SISVAR program, version 5.7.

The concentration of 5 $\mu\text{L mL}^{-1}$ of the essential oil of *L. insignis* was selected and evaluated for toxicity in two different application methods (exposure to treated surface and topical application), according to the methodology adapted of Santos et al. (2010).

Preliminary insecticidal activity tests of the species and products

Exposure by topical application

Ten specimens of *C. maculatus* were placed in petri dishes measuring 9.0 cm in diameter, lined with filter paper, and containing ten uninfested cowpea grainss and covered with elastic voile-type fabric. Subsequently, 1.0 mL of solutions containing essential oil of *L. insignis* were sprayed at a concentration of 5 $\mu\text{L mL}^{-1}$ per plate. The experiment was carried out in a completely randomized design

with four replications, including controls. As negative and postive controls, distilled water and distilled water solution+Tween® 20 were used respectively. Mortality was evaluated at 48 HAT. Mortality evaluation was performed as established by Santos et al. (2007), where all insects that moved any part of the body were considered alive, even those that only moved when stimulated.

Exposure to treated surface

The same bioassay solution was used as for topical application experiment above. Petri dishes lined with filter paper containing ten uninfested cowpea grains were soaked with 1.5 mL of the solution containing essential oil of *L. insignis* (5 $\mu\text{L mL}^{-1}$) using a micropipette. After that, ten insects were introduced and the opening closed with a voile-type fabric, held by an elastic band. Insect mortality was evaluated according to Santos et al. (2007). For the individual analyses, each method consisted of ten repetitions per treatment, in a completely randomized design. As they did not meet the assumptions of normality and homogeneity, the data were analyzed and compared using the non-parametric Kruskal-Wallis test. For purposes of comparison between the groups, the non-parametric method designed by Mann Whitney was used for independent samples through the statistical program R (R Core Team 2015), version 3.2.2.

Results and discussion

Chemical characterization of essential oils

The chromatogram analysis of EOs from *L. thymoides*, *L. lasiocalycina* and *L. insignis* resulted in the identification of 83.80, 96.30 and 95.40% of the chemical constituents, respectively. The major compounds found in the EO of *L. thymoides* were: E-caryophyllene (29.55%), caryophyllene oxide (8.17%), germacrene D (6.59%) and cis-calamenene (5.59%). For *L. lasiocalycina*, the predominant compounds were: β -myrcene (31.17%), E-ocymenone (24.10%) and p-cymene (7.17%). Finally, for *L. insignis*, the constituents with the highest occurrence in the samples were: E-ocymenone (26.11%), limonene (14.73%), β -myrcene (12.43%) and p-cymene (7.24%) (Table 1).

Analysis of the insecticidal potential of the species

The evaluation of the insecticidal activity of essential oils from *Lippia* species against *Callosobruchus maculatus* 24 h after treatment showed that the essential oils of *Lippia thymoides* (15.0%), *Lippia lasiocalycina* (60.0%), and *Lippia insignis* (67.5%) did not differ statistically from each

Table 1 Major compounds present in essential oils extracted from leaves of the species named *Lippia thymoides* Martius & Schauer, *Lippia lasiocalycina* Cham and *Lippia insignis* Moldenke

Compostos	Kovats index of literature	Calculated Kovats index	Lippia thymoides (%)	Lippia lasiocalycina (%)	Lippia insignis (%)
β -myrcene	990	989–91	-	31.17 $\pm$ 1.16	12.48 $\pm$ 0.1
p-cymene	1026	1024	-	7.17 $\pm$ 0.62	7.24 $\pm$ 0.83
Limonene	1029	1029	-	-	14.73 $\pm$ 0.60
Z-ocymenone	1226	1241	-	6.51 $\pm$ 0.13	-
E-caryophyllene	1226	1426	29.55 $\pm$ 0.47	-	-
E-ocymenone	1238	1240–41	-	24.10 $\pm$ 0.80	26.11 $\pm$ 1.05
germacrene D	1485	1588	6.59 $\pm$ 2.98	-	-
cis-calamenene	1485	1485	5.59 $\pm$ 0.99	-	-
caryophyllene oxide	1529	1034	8.17 $\pm$ 3.40	-	-

other. However, the essential oil of *L. insignis* caused the highest mortality, followed by *L. lasiocalycina*, and did not differ statistically from the commercial insecticide lambda-cyhalothrin. In contrast, *L. thymoides* showed low mortality, similar to the negative controls, whereas *L. lasiocalycina* did not differ from the DWS+Tween® 20 (1%) control. The distilled water control resulted in the lowest mortality (5.0%), confirming the reliability of the bioassay and indicating *L. insignis* as the species with the highest insecticidal potential among those evaluated.

After 48 h, *L. thymoides*, which showed the lowest mortality (40%), differed significantly from *Lippia insignis*, whereas the remaining treatments exhibited the same pattern observed during the first 24 h. The essential oil of *L. insignis* stood out by promoting the highest cumulative insect mortality (100%), differing from both the distilled water control (10%) and the DWS+Tween® 20 (45%) control, and showing statistically equivalent results to those observed for the commercial agrochemical lambda-cyhalothrin. Of the three analyzed species, *L. insignis* was the only one that indicated the presence of Limonene in its constitution, which is a constituent with insecticidal action established in the literature and present in some current agrochemicals (Brito et al. 2021) (Table 2).

The insecticidal activity of essential oils of *L. insignis* after 48 h of application is in line with the satisfactory results of other studies that evaluated the insecticidal potential of

essential oils of *Lippia sp* species (Santos et al. 2018, 2020). Terpenes comprise a number of plant substances whose activity as plant defensives is well established. These exert a proven function of protecting the plants that produce them. A hypothesis for the outstanding biological activity of *L. insignis* against *C. maculatus*, verified in this work, is due to the majority presence of the compounds like E-ocymenone, β -myrcene and, mainly, limonene, verified in isolation only in samples of this species. The highest concentration of monoterpenes in samples of EOs from *Lippia* genus has already been reported by Alves et al. (2015) when verifying the predominance of geranial (43.3%) and neral (32.5%) in the samples of EO extracted from *Lippia alba* also against *C. maculatus*. According to the authors, EOs have greater lipophilicity and, therefore, greater easiness for penetration into the integument of insects.

Essential oils compromise the physiology of *C. maculatus* due to the inhibition of acetylcholinesterase in insects, which can cause growth inhibition, maturation damage, reduced reproductive capacity, feeding suppression and death by starvation or direct toxicity (Viegas Júnior 2003). Another effect of EOs already demonstrated is the inhibition of the neuromodulator named octopamine, leading to hyperpolarization of calcium channels modulated by GABA (gamma-aminobutyric acid). Octopamine acts as a neurohormone and neurotransmitter, responsible for regulating the heartbeat, behavior and metabolism of insects (Coitinho et al. 2011). The EOs of some botanical species also have the ability to inhibit the endocrine system of insects, leading to a deficiency in the production of the hormones named ecdysone and 20-hydroxy-ecdysone, responsible for the ecdysis stage in insects, causing delays in the maturation and reproduction of these pests (Marangoni et al. 2013).

Determination of median lethal concentration (LC₅₀)

Regression analysis to estimate LC₅₀ from different concentrations of the essential oil of *L. insignis* on the mortality of *C. maculatus* showed a linear ascending behavior on the percentage of dead insects, with a coefficient of determination

Table 2 Median mortality of *Callosobruchus maculatus* (Fabricius, 1775) in tests with the essential oils of the species named *Lippia thymoides* Martius & Schauer, *Lippia lasiocalycina* Cham and *Lippia insignis* Moldenke after 24 and 48 h of exposure

Treatments	Mortality	
	24 h	48 h
<i>Lippia thymoides</i>	15.0 bcd	40.0 bc
<i>Lippia lasiocalycina</i>	60.0 abc	75.0 ab
<i>Lippia insignis</i>	67.5 ab	100.0 a
DWS+ Tween® 20 (1%)	25.0 cd	45.0 bc
DWS	5.00 d	10.0 c
Lambda-cyhalothrin	100.0 a	100.0 a

* Data were submitted to non-parametric Kruskal-Wallis analysis and, later, compared by rank. *Medians followed by the same lower-case letter in the column do not differ from each other

(R^2) equal to 0.90, thus indicating an adequate fit of the data to the simple linear regression model (Fig. 2).

From the straight-line equation, it was possible to estimate, within the tested interval, that the concentration of $3.11 \mu\text{L ml}^{-1}$ was the lowest concentration responsible for the mortality of 50% of the individuals (LC_{50}). Nevertheless, it is possible to infer that better responses in relation to the mortality in *C. maculatus* can be verified in higher concentrations, reaching 72.5% of mortality in the concentration, taking into account the tested interval.

Insecticidal evaluation of *Lippia* sp species using the methods of contaminated surface and topical application

The toxicity of EOs of *L. insignis* applied by the methods of treated surface and topical application individually were significantly higher than that observed in controls, therefore being effective. The method of topical application caused mortality rate of 70% in relation to the controls with distilled water (0%) and DWS+Tween[®] (20%) (Fig. 3A), while the method of exposure to contaminated surface caused 65% mortality compared to DWS (0%) and DWS and Tween[®] (30%) at 48 HAT (Fig. 3B).

When comparing the mortality of *C. maculatus*, using the two methods of insecticide evaluation, treated surface and topical application, there was no significant difference between the methods, with 65% mortality of *C. maculatus*, using the treated surface, and 70% in topical application, using a concentration of 0.5% of the EO of *L. insignis*, in

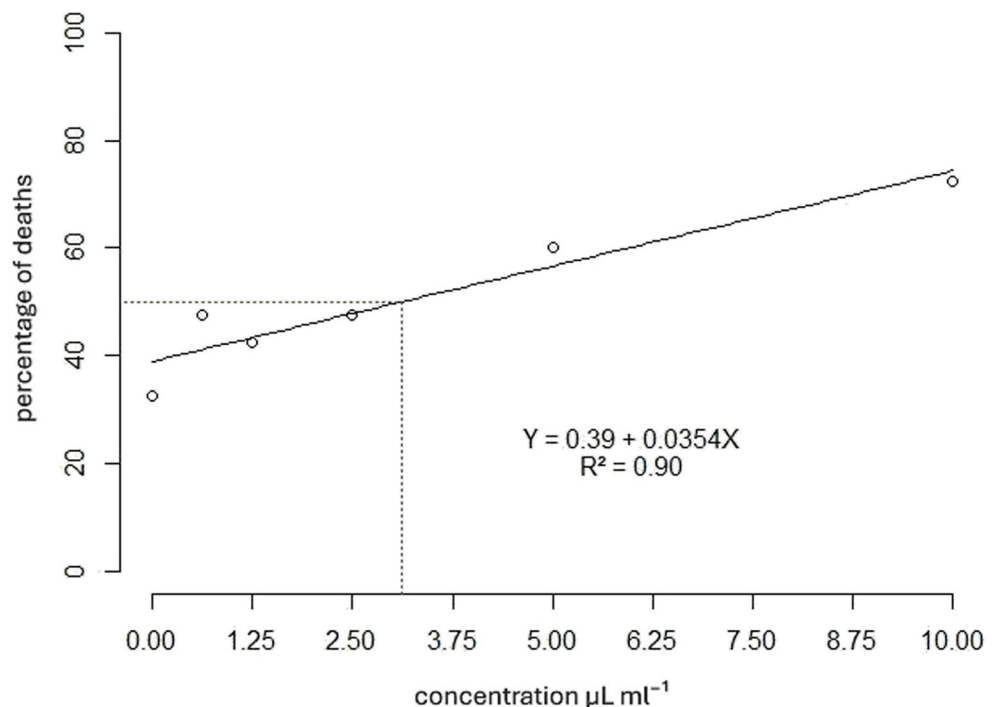
both methods. The results of this study were obtained using a concentration ten times lower than those used by Estrela et al. (2006) in the control of *Sitophilus zeamais*, who obtained 100% insect mortality from *Piper hispidinervum* and *Piper aduncum* EOs at a concentration of 35%.

The *C. maculatus* mortality through the topical application of EO of *L. insignis*, in vitro, corroborates the findings of Peixoto et al. (2015) and suggests the possible efficiency of the product on populations of *C. maculatus* in vivo, by highlighting the relevance of the speed of the method of topical application for the control of insect-pests, especially in field conditions, since the contact of insects with the EOs can be relatively quick in these conditions. On the other hand, treated surface tests indicate the need to develop more stable structures, such as nanocapsules and nanoemulsions (Singh et al. 2021), in addition to tests in storage places, consisting of a future alternative for the control of *C. maculatus* under these conditions.

Conclusion

The results obtained in vitro demonstrated that the three assayed EOs had insecticidal potential against *Callosobruchus maculatus*, but *L. insignis* displayed superior efficacy. Further studies are needed to elucidate the mode of action of the EOs and the major compounds identified in them, as well as for the development of stable formulations from the EOs.

Fig. 2 Median Lethal Concentration (LC_{50}) of the essential oil of *Lippia insignis* Moldenke on the mortality rate of *Callosobruchus maculatus* (Fabricius, 1775)



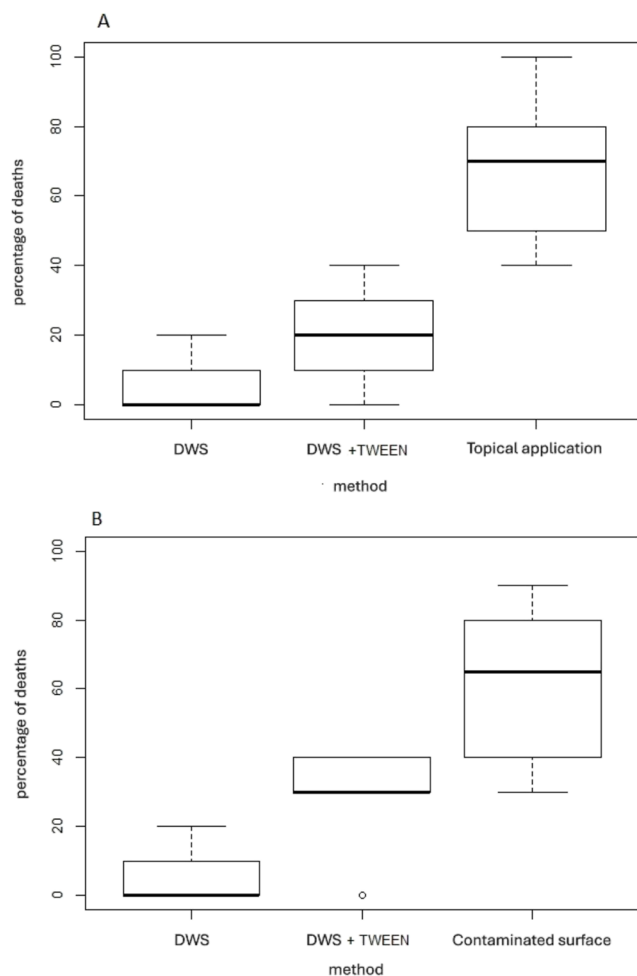


Fig. 3 Toxicity of the essential oil of *Lippia insignis* Moldenke (5 μ L mL⁻¹) against *Callosobruchus maculatus* (Fabricius, 1775) in two different application methods (**A**- Topical application and **B**- Contaminated surface) *DWS: distilled water; Tween®: surfactant (1%)

Acknowledgements The authors are grateful to the Research Support Foundation of the State of Bahia (FAPESB) and the National Council for Scientific and Technological Development (CNPq) for the funding provided to carry out this project. We would also like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support, through Financing Code 001, State University of Feira de Santana (UEFS) and the Graduate Program in Plant Genetic Resources (PPGRGV/UEFS).

Author contributions Project, methodology – D.R.S., L.M.O., J.D.C. and A.M.L.; Research – D.R.S., L.M.O., J.D.C., M.S.L. and A.M.L.; Writing – original draft, D.R.S.; Proofreading and editing, L.M.O., J.D.C., M.S.L., M.C.A.L.R. and A.M.L.; Acquisition of funding – L.M.O. and A.M.L.; supervision – L.M.O., A.M.L. and J.D.C. All authors contributed to the conception and design of the study. The preparation of material, data collection, analysis and the first draft of the manuscript were held by D.R.S. and all authors commented on versions prior to the final manuscript.

Funding The Article Processing Charge (APC) for the publication of this research was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (ROR identifier:

00x0ma614). This study was funded by the Research Support Foundation of the State of Bahia (FAPESB) through the project “Research network in bioprospecting and sustainable use of native plant species in the semiarid region for the production of bioactive compounds”, approved in public notice 12/2012 and registered with the State University of Feira de Santana (UEFS). And was supported by a grant from the National Council for Scientific and Technological Development (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support, through Financing Code 001.

Data availability The datasets generated and analyzed during the current study are not publicly available as they are on personal file at the time, but are available from the corresponding author upon request.

Declarations

Competing interests The authors declare no competing interests.

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