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Ensayo entomológico / Entomological Esssay

Insecticidal and antifeedant bioactivities of *Melaleuca alternifolia* essential oil on *Ascia monuste orseis*

Bioactividades insecticidas y antialimentarias del aceite esencial de *Melaleuca alternifolia* en *Ascia monuste orseis*

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Abstract: The bioactive effects of *Melaleuca alternifolia* essential oil on the behavior and mortality of *Ascia monuste orseis* caterpillars were studied. The experiment was conducted under controlled laboratory conditions using a completely randomized design with six treatments, represented by *A. monuste orseis* caterpillars fed on cabbage leaves treated with 0 (control), 5, 10, 20, 30, or 40 mg mL^{-1} essential oil. Treatments were applied to discs of collard leaves by immersion for 1 min. The leaves were offered to the insects *ad libitum*. Each treatment consisted of five replicates of four third-instar caterpillars. Mean daily intake of collard leaves, faeces production, mortality, and behavioral changes were assessed for 15 days after the beginning of the experiment. Data on leaf intake and faeces production were subjected to analysis of variance by the *F*-test; when significant differences were found, data were subjected to regression analysis. Corrected mortality rate was calculated and subjected to analysis of variance by the *F*-test followed by Tukey's test at the 5 % significance level for comparison of means. The median lethal concentration (LC_{50}) was also evaluated. Two very clear feeding behaviors were observed, one in caterpillars exposed to essential oil concentrations of 5, 10, and 20 mg mL^{-1}, which showed little difference in leaf intake from the control, and the other in caterpillars exposed to the highest concentrations (30 and 40 mg mL^{-1}), which showed a decrease of 76 % to 93 % in feed intake compared with the control. Lower leaf intake resulted in a decrease in faeces production, and the negative effects increased linearly with essential oil concentration. The mortality curve showed a linear and positive response to essential oil concentration, reaching 100% in insects exposed to the highest concentrations. The LC_{50} was 13.93 mg mL^{-1}.

Keywords: Bioinsecticide, cabbage, caterpillar, feeding deterrence, mortality, tea tree.

Resumen: Este estudio evaluó los efectos bioactivos del aceite esencial de *Melaleuca alternifolia* sobre el comportamiento y la mortalidad de las orugas *Ascia monuste orseis*. El experimento se llevó a cabo en el laboratorio bajo condiciones controladas utilizando un diseño completamente al azar con seis tratamientos, representados por orugas de *A. monuste orseis* alimentadas con hojas de berza tratadas con 0 (control), 5, 10, 20, 30 o 40 mg mL^{-1} aceite esencial, los tratamientos se aplicaron a discos de hojas de col por inmersión durante 1 min. Las hojas se ofrecieron a los insectos *ad libitum*. Cada tratamiento consistió en cinco repeticiones de cuatro orugas de tercer estadio. Se evaluó la ingesta diaria media de hojas de berza, la producción de heces, la mortalidad y los cambios de comportamiento durante 15 días después del comienzo del experimento. Los datos sobre el consumo de hojas y la producción de heces se sometieron a análisis de varianza mediante la prueba F; cuando se encontraron diferencias significativas, los datos se sometieron a análisis de regresión. Se calculó la tasa de mortalidad corregida y se sometió a análisis de varianza mediante la prueba F seguida de la prueba de Tukey al nivel de significancia del 5 % para la comparación de medias. También se evaluó la concentración letal mediana (CL_{50}). Se observaron dos comportamientos de alimentación muy claros, uno en orugas expuestas a concentraciones de aceite esencial de 5, 10 y 20 mg mL^{-1}, que mostraron poca diferencia en el consumo de alimento del control, y el otro en orugas expuestas a las concentraciones más altas (30 y 40 mg mL^{-1}), que mostró una disminución de 76 % a 93 % en el consumo de alimento en comparación con el control. Una menor ingesta de hojas resultó en una disminución en la producción de heces y los efectos negativos aumentaron linealmente con la concentración de aceite esencial. La curva de mortalidad mostró una respuesta lineal y positiva a la concentración de aceite esencial, alcanzando el 100 % en los insectos expuestos a las concentraciones más altas. La CL_{50} fue de 13,93 mg mL^{-1}.

Palabras clave: Árbol de té, berza, bioinsecticida, disuasión de alimentación, mortalidad, oruga.

Introduction

Collard (*Brassica oleracea* var. *acephala*), belonging to the family Brassicaceae, is a leafy green native to the Mediterranean area. It can be consumed fresh, cooked, or processed (Viana et al., 2021). From an economic perspective, the crop is particularly attractive because it has a short return-on-investment period, even for small-scale systems (Melo et al., 2019). In addition to having high economic importance for farmers, vegetables such as collard greens are also valued by consumers for their nutritional properties, given their high antioxidant activity and ability to scavenge reactive oxygen species, which are linked to the development of chronic diseases in humans (Chawner & Hetherington, 2021; Fayet-Moore et al., 2020) frequency of intake, proportion meeting ADG recommendations, most popular food groups, intake at each reported eating occasion, and the profile of high and low vegetable consumers (based on the median servings). With the increasing demand for vegetables, consumers have attributed greater importance to nutrient composition, palatability, and product appearance (Makhal et al., 2021). These factors are considered a limitation in collard production, as the crop is highly susceptible to pests and affected plants have reduced marketability.

Ascia monuste orseis (Godart, 1819) (Lepidoptera: Pieridae), known in Brazil as cabbage caterpillar and curuquerê-da-couve, is a major pest of Brassicaceae in the Neotropical region, especially in collard fields. Being voracious feeders, *A. monuste orseis* larvae can cause great damage to crops, leading to total loss of production in some cases (Baldin et al., 2015). Current strategies for the prevention and control of *A. monuste orseis* are mostly based on synthetic insecticides. However, long-term intensive use of pesticides can disrupt the ecological balance, damage the environment, induce insect pest resistance, and eliminate non-target organisms (Mapeli et al., 2010). In order to effectively prevent and control damage caused by *A. monuste orseis*, it is crucial to develop environmentally safe control methods, such as the use of biological agents and botanical insecticides.

Several plants have been found to contain bioactive secondary metabolites that can be used as natural insecticides (e.g., terpenoids, flavonoids, and alkaloids) (Liao et al., 2017). It is noteworthy that the development of effective plant products based on plant extracts and secondary metabolites is already a reality in cutting-edge agricultural research, and novel pesticide formulations often contain ingredients of plant origin. Products based on secondary metabolites represent a more sustainable control method, as they are environmentally safe and have high biodegradability (Kedia et al., 2015).

An interesting source of compounds with insecticidal potential is the native Australian species *Melaleuca alternifolia* (Myrtales: Myrtaceae), commonly known as tea tree. The leaf glands of this small tree produce and store valuable essential oil rich in monoterpenes (Baldissera et al., 2014). Tea tree has several biological properties: insecticidal, insect repellent, antibacterial, antifungal, anti-inflammatory, and analgesic, among others (Callander & James, 2012).

In general, lipophilic essential oils interfere with metabolic, biochemical, physiological, and behavioral processes of insects (Arun et al., 2009). Plant essential oils have been widely researched for their fumigation potential. However, these agents are rarely applied for the control of lepidopterans (Liao et al., 2017). There is limited information on the use of tea tree essential oil as a feeding deterrent, repellent, or insecticide against *A. monuste orseis*. In view of the above, this study aimed to assess the bioactive potential of tea tree essential oil in inducing behavioral changes and mortality in *A. monuste orseis* caterpillars.

Material and methods

Adults and caterpillars of *A. monuste orseis* were collected at the experimental field of the State University of Maringá (23°47′25″S 53°15′31.0″W, 412 m a.s.l.), Umuarama campus, Paraná, Brazil, and taken to the Agricultural Entomology Laboratory for mass rearing. Insects were reared according to a method adapted from Liu (2005). Mass rearing and experimental analyses were carried out under controlled laboratory conditions (25 ± 2 °C, 75 ± 5% relative humidity, 12 h photoperiod). The insects were fed a natural diet consisting of *B. oleracea* var. *acephala* leaves.

Tea tree essential oil was extracted from plants obtained from the Physic Garden of the State University of Maringá. Extraction was performed by hydrodistillation of dried shoots for 4.5 h using a graduated Clevenger-type apparatus, according to the method of Wahba (2020).

Essential oil constituents were detected by gas chromatography/mass spectrometry (GC/MS) as reported by Daniel et al. (2020). Chemical compounds were identified based on library and GC-MS Postrun Analysis software and by comparison of their linear retention, calculated based on a homologous series of n-alkanes (C7–C26), against literature data (Adams, 2007).

For assessment of antifeeding and toxic effects, tea tree essential oil was serially diluted to concentrations of 5, 10, 20, 30, and 40 mg mL^{-1}. Each concentration represented a treatment. Tween 80 (0.01%) was added to each dilution as an emulsifier. The control consisted of water and emulsifier only, according to the method adapted from Liao et al. (2017). Five replications were performed per treatment, and each replicate consisted of four third-instar caterpillars in a 150 mL polypropylene arena, consisting of a flask with a diameter of 10 cm, with lids adapted with holes and sealed with voile fabric to enable gas exchange and prevent insects from leaving. Treatments were applied to discs of collard leaves (5.5 cm in diameter) by immersion for 1 min. After drying, leaves were placed on wet filter paper and offered to the insects *ad libitum.*

The mean daily intake of collard leaves was calculated as the difference in weight between the amount of feed provided and the amount remaining in the arena. Daily feed intakes were corrected for moisture loss during the period, estimated by determining the initial and final weight of two replicates per treatment not provided to the insects. During each feed assessment period, all faeces produced by caterpillars were collected and weighed daily. These data were used to evaluate the possible anti-nutritional effects of tea tree essential oil. Behavioral parameters and insect mortality were recorded daily until all individuals died or reached the pupal stage.

Mean values of leaf intake and faeces production were subjected to analysis of variance by the *F*-test ($p < 0.05$). When differences between treatments were significant, the data were subjected to regression analysis. In the cases of inflection points that could not be explained by a single linear or exponential model, the data were analyzed by segmented regression analysis.

Corrected mortality was calculated using Abbott's equation (Abbott, 1925). Mortality data were then subjected to analysis of variance by the *F*-test, followed by Tukey's test at the 5% level for comparison of means. Mortality results were also used to estimate the median lethal concentration (LC_{50}) and to develop a mathematical model to represent the effect of essential oil concentration on insect mortality. Statistical analyses were performed using R software (R Core Team, 2016) and RStudio software (RStudio Team, 2015).

Results and discussion

The essential oil constituent's analysis identified the following major compounds: terpinen-4-ol (44.48 %), γ-terpinene (20.69 %), γ-elemene (5.11 %), and α-terpineol (4.56 %).

The feeding behavior of third-instar larvae of *A. monuste orseis* fed on collard leaves treated with tea tree essential oil is depicted in Figure 1. The results indicate two feeding behaviors, as evidenced by the breakpoint (20 mg L^{-1} essential oil) of the regression curve. Leaf consumption varied little between treatments containing the three lowest oil concentrations (5, 10, and 20 mg mL^{-1}), as shown by the small slope of the first segment of the regression curve (Figure 1A). This finding suggests a small difference in leaf consumption between caterpillars subjected to these treatments and those subjected to the control treatment. In the first segment, collard intake ranged from 5.37 g day^{-1} (control) to 4.39 g day^{-1} (5 mg mL^{-1} essential oil), representing a variation of about 18 %.

The breakpoint or change of trend occurred at the oil concentration of 20 ± 6.8 mg mL^{-1} (Figure 1A). From this estimated point onward, the model exhibited a different behavior, as depicted by the steep slope of the curve. Thus, insects exposed to leaves treated with 30 or 40 mg mL^{-1} oil exhibited a marked decrease in feed intake, which was 76 % and 93 % lower than that of control caterpillars, respectively (Figure 1).

The antifeeding action of tea tree oil and the decrease in leaf consumption were confirmed indirectly by data shown in Figure 1B. The amount of faeces produced during the experimental period reduced significantly with increasing oil concentrations. Such a negative relationship between faeces production and oil concentration corroborates the effect of tea tree oil on the feeding behavior of *A. monuste orseis* larvae. Essential oil treatment resulted in reduced feed intake, possibly exerting deleterious or antibiosis effects on larvae. Caterpillars exposed to 30 or 40 mg mL^{-1} oil were expected to excrete faeces, given that they consumed leaves during the experimental period, albeit at a low rate; however, because of the reduction in metabolism, which culminated in death, this physiological process was not observed.

Mortality rates caused by the intake of leaves treated with tea tree oil are shown in Table 1. Total mortality was observed in treatments in which insects were fed collard leaves containing 30 or 40 mg mL^{-1} oil. The other oil concentrations caused mortality rates from 27.37 to 42% (Table 1).

Table 1. Corrected mortality (Abbott, 1925) of *Ascia monuste orseis* caterpillars exposed to different concentrations of *Melaleuca alternifolia* essential oil.

Essential oil concentration (mg mL^{-1})	Corrected mortality (%)
5	27.37 ± 12.28 a
10	28.42 ± 14.28 a
20	42.11 ± 15.34 ab
30	100.00 ± 0.00 b
40	100.00 ± 0.00 b
CV = 32.57%	

CV, coefficient of variation. Values are presented as mean ± standard error. Means followed by the same letter are not significantly different at p ≤ 0.05 by Tukey's test.

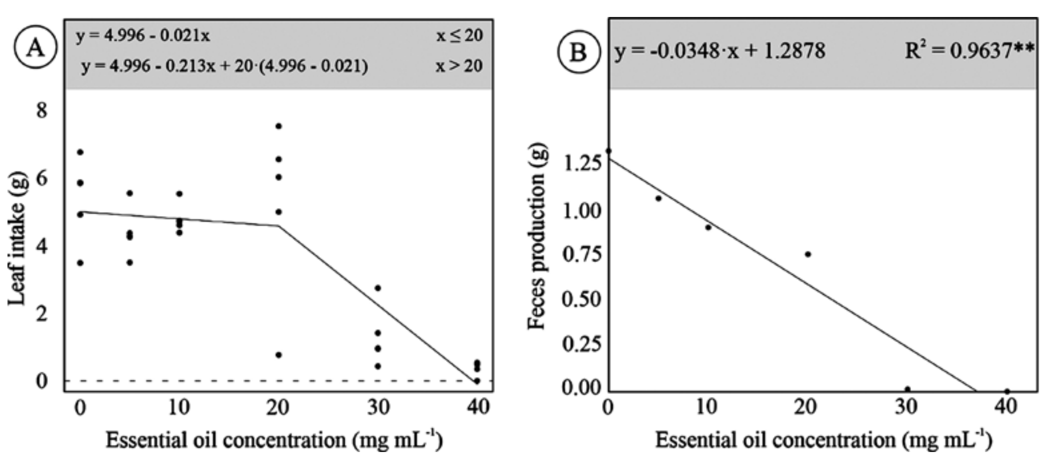

Figure 1. (A) Segmented regression model for daily intake of *Brassica oleracea* var. *acephala* leaves by *Ascia monuste orseis* caterpillars as a function of *Melaleuca alternifolia* essential oil concentration added to the diet. (B) Linear regression model for feces production during the larval period as a function of *M. alternifolia* essential oil concentration. **(p value<0.01).

The mathematical model that best represented the mortality of *A. monuste orseis* larvae as a function of tea tree oil concentration was linear, with a coefficient of determination greater than 0.90, demonstrating a good fit to experimental data, as reinforced by the significant *F*-value at $p < 0.05$ (Figure 2A). The LC_{50}, that is, the concentration necessary to kill 50% of larvae within 48 h of exposure to tea tree oil, was found to be 13.9322 mg mL^{-1} (Figure 2B). Thus, even at low concentrations, tea tree oil may be an efficient agent to control *A. monuste orseis* (Figure 2).

It is common for essential oils to exert negative effects on insects. Studies on the actions of essential oils have indicated not only repellent activity but also insecticidal activity through contact and fumigation and behavioral modifications that lead to reduced feeding (Arasu et al., 2013; Abdullah et al., 2015; El-Wakeil, 2013). In this study, we investigated the residual action of different concentrations of tea tree essential oil on the behavior and mortality of *A. monuste orseis.* We found that the oil had negative residual effects on feed consumption and larval mortality.

Major variations in *B. oleracea* var. *acephala* leaf consumption by caterpillars were observed when using the highest oil concentrations, namely 30 and 40 mg mL^{-1}. Under these conditions, larvae initially rejected food, possibly indicating the beginning of repellency, and later foraged treated leaves but consumed smaller quantities of the plant material. Leaf intake decreased by 76 % and 93% with exposure to 30 and 40 mg mL^{-1} tea tree oil, respectively, compared with the control. Callander and James (2012) observed that feeding was reduced in *Lucilia cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae) larvae exposed to tea tree essential oil through an artificial diet. In *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera: Noctuidae) condiments essential oils, such as garlic, cinnamon, cloves, ginger, mint and thyme tested at sublethal concentrations, caused a considerable decrease in feeding and oviposition (Ribeiro et al., 2015). The reduced intake of treated collard leaves might be associated with aversion or irritability caused by high tea tree oil concentrations, attributed to the presence of terpinen-4-ol, γ-terpinene, γ-elemene and α-terpineol, which are toxic to insects (Noosidum et al., 2014).

According to Hammer et al. (2006) the major compound of tea tree essential oil is terpinen-4-ol. This compound accounted for 45 % of tea tree oil in the present study. Thus, it is suggested that the antifeeding action of tea tree oil observed here is associated with the presence of this major compound, which is known to cause deterrent effects on lepidopteran species. Liao et al. (2016) reported that terpinen-4-ol had the most distinct and important deterrent action on *Helicoverpa armigera* (Hübner, 1805) (Lepidoptera: Noctuidae). According to the authors, it can be said that terpinen-4-ol is the oil component that has the greatest insecticidal action on caterpillars; the compound affects mainly the central nervous system, showing high specificity for the enzyme acetylcholinesterase.

According to Parra et al. (2009) feces production is directly related to feed intake in insects. *A. monuste orseis* larvae showed a decrease in leaf consumption with increasing oil concentrations, leading to a direct effect on faeces production, as demonstrated in Figure 1B. The amount of feces produced by insects may serve as an indication of food use for the calculation of approximate digestibility. Low feces production may, therefore, be associated with high food digestibility (Coudron et al., 2006). However, in the current study, the drastic decrease in faeces production was related to reduced consumption and interruption of the life cycle due to intoxication and death (as observed in most treatments), not too high digestibility (Figure 1A). Exposure of *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae) larvae to the essential oil of *Curcuma longa* (Zingiberaceae), rhizomes led to a significant reduction in faeces production throughout insect development. Essential oil not only reduced fecal production but also triggered several responses that compromised nutrient absorption capacity, ultimately affecting immature development (Veeran et al., 2017).

In the present study, mortality was found to increase with increasing tea tree oil concentrations. The two highest concentrations resulted in total larval control, demonstrating the important insecticidal potential of tea tree oil against *A. monuste orseis* (Figure 2A). Larvae fed with discs of collard leaves exposed to 40.0 mg mL^{-1} oil died in less than 48 h, whereas larvae exposed to 30.0 mg mL^{-1} oil exhibited reduced food consumption, which evolved to mortality. Thus, the essential oil caused toxic effects due to the ingestion of portions of treated leaves, as also described by Benelli et al. (2013), Klauck et al. (2014), and Wahba (2020) for other insect species.

The LC_{50} of tea tree oil for *A. monuste orseis* larvae was found to be 13.9322 mg mL^{-1} (Figure 2B), allowing to classify the botanical insecticide as having moderate toxic action, according to Hammer et al. (2006). This is the first study reporting the mortality effects of tea tree essential oil on *A. monuste orseis*, although the oil has been shown to control

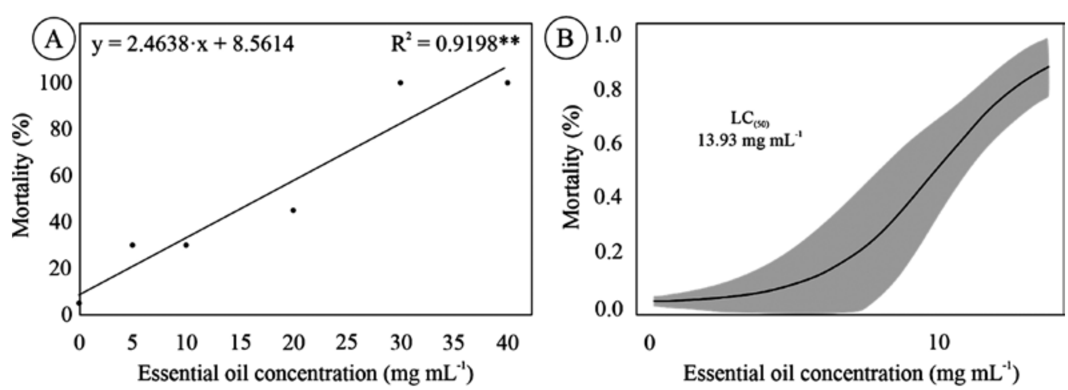

Figure 2. (A) Linear regression model for larval mortality as a function of *Melaleuca alternifolia* essential oil concentration added to the diet. **(p value<0.01). (B) Estimation of the median lethal concentration (LC_{50}) of *M. alternifolia* essential oil for *Ascia monuste orseis* caterpillars.

other pest species. Halbert et al. (2009) demonstrated that tea tree oil repelled the corn leaf aphid, *Rhopalosiphum maidis* (Fitch, 1856) (Hemiptera: Aphididae) and Liao et al. (2018) reported the potent toxic effects of the oil on *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) adults via fumigation, which extended over time. Liao et al. (2016), in assessing the insecticidal effects of tea tree on *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) observed that mortality occurred at 24 h after exposure. Fumigation effects increased with increasing essential oil concentrations and became more intense after 24 h, affording mortality rates of up to 92 %. In a previous study, tea tree achieved satisfactory control of sheep lice, *Bovicola ovis* (Schrank) (Phthiraptera: Trichodectidae) (Callander & James, 2012).

Although studies have demonstrated the important action of tea tree essential oil for the control and repellency of insects and mites, there is a need for more in-depth investigations to elucidate the mechanism of action of major oil components. The oil possibly acts on neurotoxic pathways (Tripathi et al., 2009) and might induce losses in the octopaminergic system of insects, resulting in the blockage of octopamine receptors (Kostyukovsky et al., 2002).

Conclusions

Collard leaves treated with tea tree essential oil in underdosed conditions induces a decrease in foliar consumption of *A. monuste orseis* larvae, and concentrations above 30 mg mL^{-1} oil caused leaf intake to decrease more than 76 %. *A. monuste orseis* larvae total mortality was observed in treatments in which insects were fed collard leaves treated 30 and 40 mg mL^{-1} tea tree essential oil.

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Author Contribution

Authors Paulo Henrique Martins da Silva e Julio César Guerreiro, conceived research. Paulo Henrique Martins da Silva, Camila da Silva e Erci Marcos Del Quiquiconducted experiments. Evandro Pereira Prado, Pedro José Ferreira-Filho, João Paulo Francisco, analysed data and conducted statistical analyses. Paulo Henrique Martins da Silva and Julio César Guerreiro wrote the manuscript.

Conflict interests

The authors declare no conflict interests.