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Toxicity of botanical insecticides on the stingless bee *Tetragonisca angustula*

Toxicidade de inseticidas botânicos na abelha nativa sem ferrão *Tetragonisca angustula*

Toxicidad de insecticidas botánicos sobre la abeja nativa sin aguijón *Tetragonisca angustula*

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ABSTRACT
This study aimed to investigate the toxicity of botanical insecticides on the stingless bee *Tetragonisca angustula* (Apidae: Meliponini). Bioassays evaluated the effects of essential oils (EO) of *Allium sativum* (Amaryllidaceae), *Eucalyptus citriodora* (Myrtaceae), and commercial oil (CO) of *Azadirachta indica* (Meliaceae), in addition to ethanolic extracts (EE) of *Piper nigrum* (Piperaceae), and *Eugenia caryophyllata* (Myrtaceae). It was also assessed the effect of Surfactant addition to essential oils. The probit analysis was used to determine LC_{50} and LC_{90}. Results showed essential oil of *E. citriodora* at 1% v/v was toxic to *T. angustula*, with mean mortalities of 42.00 and 46.00% at 96 hours and survival times of 107.52 and 105.60 hours for contact and ingestion, respectively. Ethanolic extracts of *P. nigrum* and *E. caryophyllata* caused 100% mortality within 24 hours through ingestion but were less toxic through contact. Surfactants increased mortality caused by the essential oil of *E. citriodora* from 47.67 to 93.33% and the commercial oil of *A. indica* from 38.00 to 100%. Essential oil of *E. citriodora* had the lowest LC_{50} and LC_{90} (0.075 and 1.45% v/v), indicating high toxicity to *T. angustula*. Ethanolic extracts of *P. nigrum* and *E. caryophyllata* had higher LC_{50} and LC_{90} values, showing lower toxicity. In conclusion, the oils of *A. indica* and *E. citriodora* and ethanolic extracts of *P. nigrum* and *E. caryophyllata* are toxic to stingless bee *T. angustula*.

Keywords: Bio-Insecticides, Plant Extracts, Stingless Bees, Meliponini, Apidae.

RESUMO
Este estudo teve como objetivo investigar a toxicidade de inseticidas botânicos sobre a abelha sem ferrão *Tetragonisca angustula* (Apidae: Meliponini). Foi avaliada a toxicidade de óleos essenciais (OE) de *Allium sativum* (Amaryllidaceae), *Eucalyptus citriodora* (Myrtaceae) e óleo comercial (OC) de *Azadirachta indica* (Meliaceae), além de extratos etanólicos (EE) de *Piper nigrum* (Piperaceae) e *Eugenia caryophyllata* (Myrtaceae). Também foi avaliado o efeito da adição de surfactante aos óleos essenciais. A análise probit foi usada para determinar CL_{50} e CL_{90}. Os resultados mostraram que o óleo essencial de *E. citriodora* a 1% v/v foi tóxico para *T. angustula*, com mortalidades médias de 42,00 e 46,00% em 96 horas e tempos de sobrevivência de 107,52 e 105,60 horas para contato e ingestão, respectivamente. Extratos etanólicos de *P. nigrum* e E. *caryophyllata* causaram 100% de mortalidade em 24 horas por ingestão, mas foram menos tóxicos por contato. Os surfactantes aumentaram a mortalidade causada pelo óleo essencial de *E. citriodora* de 47,67 para 93,33% e do óleo comercial de *A. indica* de 38,00 para 100%. O óleo essencial de *E. citriodora* apresentou as menores CL_{50} e CL_{90} (0,075 e 1,45% v/v), indicando alta toxicidade para *T. angustula*. Extratos etanólicos de *P. nigrum* e *E. caryophyllata* apresentaram maiores valores de CL_{50} e CL_{90}, mostrando menor toxicidade. Em conclusão, os óleos de *A. indica* e *E. citriodora* e os extratos

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etanólicos de *P. nigrum* e *E. caryophyllata* são tóxicos para a abelha sem ferrão *T. angustula.*

Palavras-chave: Bio-Inseticidas, Extrato de Plantas, Abelhas sem Ferrão, Meliponini, Apidae.

RESUMEN
Este estudio tuvo como objetivo investigar la toxicidad de insecticidas botánicos en la abeja sin aguijón *Tetragonisca angustula* (Apidae: Meliponini). Se evaluó la toxicidad de aceites esenciales (AE) de *Allium sativum* (Amaryllidaceae), *Eucalyptus citriodora* (Myrtaceae) y *Azadirachta indica* (Meliaceae), además de extractos etanólicos (EE) de *Piper nigrum* (Piperaceae) y *Eugenia caryophyllata* (Myrtaceae). También se evaluó el efecto de agregar surfactante a los aceites esenciales. Se utilizó el análisis Probit para determinar LC_{50} y LC_{90}. Los resultados mostraron que el aceite esenciale de *E. citriodora* al 1% v/v fue tóxico para *T. angustula*, con mortalidades promedio de 42,00 y 46,00% en 96 horas y tiempos de supervivencia de 107,52 y 105,60 horas por contacto e ingestión, respectivamente. Los extractos etanólicos de *P. nigrum* y *E. caryophyllata* causaron un 100% de mortalidad dentro de las 24 horas posteriores a la ingestión, pero fueron menos tóxicos al contacto. Los surfactantes aumentaron la mortalidad causada por el aceite esencial de *E. citriodora* de 47,67 a 93,33% y el aceite esencial de *A. indica* de 38,00 a 100%. El aceite esencial de *E. citriodora* presentó las LC_{50} y LC_{90} más bajas (0,075 y 1,45% v/v), lo que indica una alta toxicidad para *T. angustula*. Los extractos etanólicos de *P. nigrum* y *E. caryophyllata* presentaron valores más altos de CL_{50} y CL_{90}, mostrando menor toxicidad. En conclusión, los aceites de *A. indica* y *E. citriodora* y los extractos etanólicos de *P. nigrum* y *E. caryophyllata* son tóxicos para la abeja sin aguijón *T. angustula*.

Palabras clave: Bioinsecticidas, Extracto de Plantas, Abejas sin Aguijón, Meliponini, Apidae.

1 INTRODUCTION

The stingless bees, regarded as the most efficient pollinators worldwide (Castilhos *et al*., 2019), are a crucial part of our ecosystem. The meliponine, or social stingless (Apidae: Meliponini), are the most diverse group of eusocial tropical bees and may be the most abundant clade of bees on earth. Among the species, the bee *Tetragonisca angustula* (Apidae: Meliponini) is one of the most abundant stingless bees in the Neotropical region (Xavier *et al*., 2010). This species thrives in urban environments, requiring minimal space for breeding, and plays a crucial role in pollinating native species essential for ecosystem

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maintenance (Vieira *et al*., 2016; Giannini *et al*., 2020).

Using substances of natural origin has been widely encouraged to ensure more sustainable agricultural practices that can minimize the impact of pesticides on bees, assuming that such compounds are less harmful to beneficial insects. Such compounds, labeled as biopesticides, have received considerable attention in organic production systems and have expanded to conventional cultivation systems. They also aim for more sustainable production (Tomé *et al*., 2015). In this sense, botanical insecticides present some advantages, such as fast degradation, lower environmental persistence, and reduced impact on beneficial organisms, humans, and the environment (Xavier *et al*., 2010).

However, despite the importance of studying the toxicity of botanical insecticides on essential pollinators, mainly native stingless bees, very few studies have been reported. This lack of research underscores the need for more comprehensive studies to ensure the safety of these essential pollinators.

This work aimed to investigate and evaluate the toxicity of commercial oil of *A. indica*, essential oil of *E. citriodora*, and *A. sativum* and ethanolic extracts of *P. nigrum* and *E. caryophyllata* on the native stingless bee *T. angustula* through ingestion and contact exposure.

2 THEORETICAL FRAMEWORK

The stingless bees, regarded as the most efficient pollinators worldwide (Castilhos *et al*., 2019), are a crucial part of our ecosystem. The meliponine, or social stingless (Apidae: Meliponini), are the most diverse group of eusocial tropical bees and may be the most abundant clade of bees on earth. Worldwide, an estimated 516 species of stingless bees belong to 60 genera (Rasmussen; Cameron, 2010). The Neotropics harbor the most extraordinary stingless bee diversity, with 417 known species, accounting for 80% of the total diversity (Bueno *et al*., 2023; Camargo *et al*., 2012). They are essential to the environment as they pollinate more than 60 tropical crops, and the pollination services likely contribute billions of dollars to tropical economies every year (Brosi, 2009). Among the species, the bee *Tetragonisca angustula* (Apidae: Meliponini) is one of the most abundant

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stingless bees in the Neotropical region (Xavier *et al*., 2010). This species thrives in urban environments, requiring minimal space for breeding, and plays a crucial role in pollinating native species essential for ecosystem maintenance (Vieira *et al*., 2016; Giannini *et al*., 2020).

The loss of bees, a current large-scale problem, is not just a threat to beekeeping and natural ecosystems but also to our agricultural production and biodiversity. This negative trend in pollinator populations is catastrophic, affecting plant fertility and decreasing world populations of many bee species (Castilho *et al*., 2019). The current consensus is that a decline in bee species and other pollinators does not have a single cause but from several factors primarily associated with human activities, including habitat loss and degradation, pesticides, parasites, pathogens, invasive species, and climate change (Castilhos *et al*., 2019; Brosi, 2009; Giannini *et al*., 2020). Pesticides are suspected to be the leading cause of Brazil's high bee loss rates, and they are proposed as the leading cause of pollinator decline (Castilho *et al*., 2019).

The indiscriminate use of synthetic insecticides to control insect pests that are not selective to bees likely contributes to the harm and threat to the existence of these essential pollinators, potentially causing the decline of their population (Lima; Rocha, 2012). In addition to killing non-target insects, insecticides can influence the behavior, structures, and functions of natural communities (Rosa *et al*., 2019). Furthermore, they can exert sublethal effects on individual bees and colonies, including immune system impairment and neural and locomotor disorders (Barbosa *et al*., 2015; Desneux *et al*., 2007).

Using substances of natural origin has been widely encouraged to ensure more sustainable agricultural practices that can minimize the impact of pesticides on bees, assuming that such compounds are less harmful to beneficial insects. Such compounds, labeled as biopesticides, have received considerable attention in organic production systems and have expanded to conventional cultivation systems. They also aim for more sustainable production (Tomé *et al*., 2015). In this sense, botanical insecticides present some advantages, such as fast degradation, lower environmental persistence, and reduced impact on beneficial organisms, humans, and the environment (Xavier *et al*., 2010).

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Previous studies have shown that botanical insecticides such as citronella oil (*Cymbopogon citratus*, Poaceae), eucalyptus oil (*Eucalyptus* sp., Myrtaceae), and neem oil (*Azadirachta indica*, Meliaceae) are toxic to native stingless bee *T. angustula* (Xavier *et al*., 2010). In addition, *A. indica* oil was also harmful to the stingless bee, *Melipona quadrifasciata* (Barbosa *et al*., 2015). Furthermore, *A. indica* oil has other sublethal effects on native stingless bees, such as growth regulating, antifeeding effect, and impairment of individual flight take-off of worker bees (Xavier *et al*., 2010; Barbosa *et al*., 2015; Tomé *et al*., 2015; Bernardes *et al*., 2015). The main active ingredient of *A. indica* oil is azadirachtin, a complex tetranortriterpenoid limonoid (Mordue; Nisbet, 2000), which is toxic to insects and exhibits a repellent effect, inhibits their feeding and growth, and is widely used as an insecticide globally (Xavier *et al*., 2010).

However, despite the importance of studying the toxicity of botanical insecticides on essential pollinators, mainly native stingless bees, very few studies have been reported. This lack of research underscores the need for more comprehensive studies to ensure the safety of these essential pollinators. In addition to *A. indica* oil, several botanical insecticides such as *Eucalyptus* sp. (Myrtaceae), *Piper nigrum* (Piperaceae), *Eugenia caryophyllata* Syn. *Syzygium aromaticum* (Myrtaceae) and *Allium sativum* (Amaryllidaceae) are widely used worldwide. However, there is little information about the negative effect of these botanical insecticides in benefical insects such as the essential pollinators native stingless bess.

3 METHODOLOGY

3.1 *TETRAGONISCA ANGUSTULA* BEES

The *T. angustula* bees were obtained in the Experimental Farm of the Federal University of Mato Grosso (UFMT), located in the "Cerrado" biome of Brazil at Latitude 15° 51' 01.7" S and Longitude 56° 04' 14.9" W. The swarm of bees was captured using bait nests made of two-liter PET bottles covered with newspaper sheets and black plastic to control temperature and light levels (adaptation from Oliveira *et al*., 2013). A hole was

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made on the side of each trap, where the upper part of a two-liter plastic bottle, shaped like a funnel, was attached to allow the swarms to enter while making it difficult for the ants to enter. A *T. angustula* bee propolis solution was used as an attractant.

After the consolidation of the captured swarms, which took 45 to 60 days after capture, they were transferred to model wooden boxes designed at the Instituto Nacional da Amazonia - INPA, containing a nest module with 12 x 12 x 7 cm^3 of internal space and a honey storage module with 12 x 12 x 5 cm^3 of internal space. The bees were kept in the meliponary of the Experimental Farm of UFMT until the swarm was established. Once established, the bees were taken to the Meliponiculture and Ecological Pest Management laboratory.

3.2 BOTANICAL INSECTICIDES

The main active ingredient of *P. nigrum* is Piperine, an alkaloid insoluble in water (Matena *et al*., 2021; Chopra *et al*., 2017). As ethanolic extract yields the highest amount of active components (Toussirot *et al*., 2023), the extraction is usually performed using 20 - 40 g of dried mature fruits for 400 mL of ethanol (Matena *et al*., 2021; Toussirot *et al*., 2023; Yaseen, 2020). Extraction of 10 g of *P. nigrum* fruit powder in 100 mL of ethanol can yield 1 g/15 mL of Piperine (Matena *et al*., 2021). In this study, for the preparation of the concentrated solution, 10 g of powdered dried mature fruit *P. nigrum* and 100 mL of 70° alcohol (ethanol) were placed in a container and left for seven days (Matena *et al*., 2021). Then, the extract was diluted in pure water at the desired concentration.

The main active ingredient of *E. caryophyllata* is eugenol (Gohary *et al*., 2021). Eugenol is an aromatic compound of the phenol group, moderately soluble in water and organic solvents such as alcohol (Ulanowska; Olas, 2021). The extraction is usually performed using 30 g of *E. caryophyllata* flower buds for 200 mL of ethanol. This method is more effective in terms of extraction efficiency (Gohary *et al*., 2021; Jung; Yang, 2014). Extraction with 70° alcohol can yield 58% extract, with eugenol comprising 88.7% (Jung; Yang, 2014). Therefore, to prepare the concentrated solution, 15 g of crushed dried *E. caryophyllata* flower buds and 100 mL of 70° alcohol were placed in a container and

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left for 24 hours (Jung; Yang, 2014). Then, the extract was diluted in pure water at the desired concentration.

The primary active molecule of *A. sativum* is diallyl-disulfide, an oil-soluble organosulfur compound (Sundaram; Milner, 1996), constituting 18.62% of *A. sativum* essential oil (Plata-Rueda *et al*., 2017). For this study, the commercial essential oil of *A. sativum* Distriol® was used. For survival analysis, a concentration of 10% v/v was used (Jess *et al*., 2017; Hussain *et al*., 2023; Oparaeke *et al*., 2007).

The main active ingredient of *E. citriodora* is citronellal, a monoterpenoid that makes up 60 to 90% of Eucalyptus essential oil (Gusmão *et al*., 2013; Insuan; Chahomchuen, 2020). The commercial essential oil of *E. citriodora* (Saunalim©) in a concentration of 1% v/v was used for this study.

The main active ingredient of *A. indica* is azadirachtin, a complex tetranortriterpenoid limonoid (Mordue; Nisbet, 2000). This study used the commercial *A. indica* oil Original Nim® (Azadirachtin 0.12%) in a concentration of 1% v/v was used.

All the products to prepare the botanical insecticides were acquired from the local marketplace. Furthermore, the products used were Alcohol 70° (Itajá®, Jalles©) and dishwashing detergent (Vida®, Grupo Altolim©).

3.3 TOXICITY OF BOTANICAL INSECTICIDES TO *T. ANGUSTULA* BEES

Two bioassays were conducted to assess the toxicity of botanical insecticides on adult *T. angustula* bees' survival through contact and ingestion exposure. The insecticides were prepared as previously described according to methods detailed earlier. The treatments included ethanolic extracts (EE) of *P. nigrum* 10% v/v and *E. caryophyllata* 10% v/v, essential oils (EO) of *A. sativum* 10% v/v and *E. citriodora* 1% v/v, commercial oil (CO) of *A. indica* and pure water as a control.

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3.4 CONTACT EXPOSURE

To assess the toxicity of botanical insecticides to *T. angustula* bees through contact exposure, one mL of the botanical insecticide was applied to a filter paper disc (8.5 cm diameter) using a syringe (10 mL). After drying the discs, each disc was placed in a Petri dish (9 cm diameter x 2 cm height). A cotton ball (1 cm diameter) moistened with 2 mL of a 10% v/v sugar water solution was added to each Petri dish to provide food for the bees. Subsequently, ten adult *T. angustula* bees were transferred to each Petri dish using a manual sucker. The Petri dishes were covered with plastic film to prevent the bees from escaping. The manual sucker, composed of two hoses of different diameters separated by a piece of organza fabric, facilitated the transfer of bees from the INPA box to the Petri dishes. Each experimental unit consisted of one Petri dish with ten bees. The experiment followed a completely randomized design with six treatments and five replications. The number of alive and dead insects was recorded at 24, 48, 72, 96, 120, 144, and 168 hours after the beginning of the experiment.

3.5 INGESTION EXPOSURE

To assess the effect of botanical insecticides on *T. angustula* bees through ingestion exposure, one mL of the botanical insecticide and 1 mL of a 10% v/v sugar water solution were applied to a cotton ball (1 cm diameter) using a 10 mL syringe. The sugar water solution is used to stimulate bee feeding, facilitating the ingestion of the insecticides. Then, the treated cotton ball was placed into a 500 mL glass container with small holes in the lid for insect breathing. Ten adult *T. angustula* bees were transferred to each glass container using a manual sucker. The experiment followed a completely randomized design with ten treatments and five replications. A glass with ten bees formed each experimental plot. The number of alive and dead insects was recorded at 24, 48, 72, 96, 120, 144, and 168 hours after the beginning of the experiment.

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3.6 EFFECT OF SURFACTANT ON ESSENTIAL OILS TOXICITY

This bioassay was conducted to assess the effect of detergent addition on essential oils through ingestion exposure. The treatments evaluated were *A. indica* CO 1% v/v, *A. indica* CO 1% v/v + detergent 5% v/v, *E. citriodora* EO 1% v/v, *E. citriodora* EO 1% v/v + detergent 5% v/v, detergent 5% v/v, and pure water as a control.

The insecticides were prepared as previously described. The bioassay was carried out using the same method previously described for contamination of the bees through ingestion exposure. The experiment was conducted in a completely randomized design with six treatments and five replications. The number of alive and dead insects was recorded at 24 and 96 hours after the beginning of the experiment.

3.7 LETHAL CONCENTRATIONS OF ESSENTIAL OIL OF *E. CITRIODORA* AND ETHANOLIC EXTRACTS OF *E. CARYOPHYLLATA* AND *P. NIGRUM*

This part of the experiment assessed the lethal concentration to kill 50 and 90% of the *T. angustula* bees' population (LC_{50} and LC_{90}) of botanical insecticides through ingestion exposure. For the *E. citriodora* EO and *A. indica* CO and EE of *E. caryophyllata* and *P. nigrum*, the concentrations of 0, 0.01, 0.1, 1, 5, and 10% were assessed.

The insecticides were prepared as previously described. The bioassay was carried out using the same method previously described for contamination of the bees through ingestion exposure. Five Petri dishes with ten *T. angustula* bees each were used for each concentration, totaling 50 bees. The number of alive and dead insects was recorded at 96 hours after the beginning of the experiment.

3.8 DATA ANALYSIS

In assessing the toxicity of botanical insecticides to *T. angustula* bees through contact and ingestion exposure, the percentage of bees' survival was submitted to Analysis of Variance (ANOVA). The data were transformed in an arc.sin$\sqrt{(x/100)}$ when

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necessary to meet the ANOVA assumptions. Then, the Tukey's test ($P < 0.05$) was used to compare the means. As low differences along time were observed, only the mortality data at 96 and 168 hours were used for ANOVA.

The data of all assessment times (24, 48, 72, 96, 120, 144, and 168 hours) were used to perform a survival analysis to assess the mean survival time of *T. angustula* bees exposed to each treatment through contact and ingestion exposure. The survival analysis was performed using the non-parametric procedure PROC LIFETEST (SAS Institute Inc., 2013), which uses Kaplan–Meyer estimators and provides χ^2 tests, as well as mean and median survival times for insects of each group and Tukey's adjustment for multiple comparisons (Etikan *et al.*, 2017). The bees that did not die up to 168 h were considered censored observations. A total (n) of 50 *T. angustula* bees were used for all treatments.

In assessing the effect of emulsifiers on oil toxicity, the percentage of bees' survival at 24 and 96 hours was submitted to Analysis of Variance (ANOVA) and transformed in an arc.sin$\sqrt{(x/100)}$ when necessary to meet the ANOVA assumptions. Then, the Tukey's test ($P < 0.05$) was used to compare the means.

The concentration-mortality data were subjected to probit analyses with 95% confidence intervals of toxicity, which were used to estimate the LC_{50} and LC_{90}. The chi-square test (χ^2) assessed the curve's adjustment.

4 RESULTS AND DISCUSSIONS

4.1 CONTACT EXPOSURE

There was a significant difference in mortality of *T. angustula* bees among treatments through contact exposure at 96 and 168 hours after the beginning of the experiment (Table 1), where the *E. citriodora* EO 1% v/v caused mortality of *T. angustula* bees (46.00 and 48.67%, respectively) higher than the control ($P < 0.05$). However, the *A. sativum* EO 10% v/v, the *A. indica* CO 1% v/v, and *E. caryophyllata* EE and *P. nigrum* EE 10% v/v caused mortalities below 30.00% and did not differ from control.

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Table 1. Effect of botanical insecticides on mortality (mean ± standard error) on *T. angustula* bees through contact and ingestion exposure in two assessment times 96 and 168 hours. The means followed by the same letters are not different, according to Tukey's test supported by Anova. The ethanolic extracts of *E. caryophullata* and *P. nigrum* 10% v/v in the ingestion exposure test were not used for Anova.

Treatment	Mean ± standard error							
	96 h				168 h			
	Contact exposure							
Water	0.00	±	0.00	b	4.00	±	2.45	b
A. sativum EO 10% v/v	5.64	±	3.69	ab	30.00	±	15.16	ab
E. citriodora EO 1% v/v	46.00	±	22.27	a	48.67	±	21.06	a
A. indica CO 1% v/v	10.67	±	6.86	ab	10.67	±	6.86	ab
E. caryophyllata EE 10% v/v	4.00	±	4.00	ab	7.33	±	4.52	ab
P. nigrum EE 10% v/v	23.43	±	12.96	ab	28.89	±	10.01	ab
ANOVA	$F_{(5,24)}$ =2.68; *P*=0.046				$F_{(5,24)}$=3.02; *P*= 0.03			
	Ingestion exposure							
Water	2.00	±	2.00		4.00	±	2.45	b
A. sativum EO 10% v/v	2.00	±	2.00		8.00	±	3.74	b
E. citriodora EO 1% v/v	42.00	±	23.75		62.00	±	23.32	a
A. indica Co 1% v/v	3.82	±	2.34		27.64	±	18.59	b
E. caryophyllata EE 10% v/v	100.00	±	0.00	N	100.00	±	0.00	N
P. nigrum EE 10% v/v	100.00	±	0.00	N	100.00	±	0.00	N
ANOVA	$F_{(3, 16)}$=2.55; *P*=0.09				$F_{(3, 16)}$= 3.38; *P*=0.043			

Source: Authors

The survival analysis showed differences in mean survival time among treatments through contact exposure (Table 2). Besides causing the highest mortality, the *E. citriodora* EO 1% v/v resulted in the lowest survival time of *T. angustula* bees (107.52 hours). Although EE of *P. nigrum* 10% v/v did not significantly affect the mortality of *T. angustula* bees, it reduced their mean survival time (142.56 hours) according to the Holm-sidak method ($P < 0.05$). The *A. sativa* EO 10% v/v, *A. indica* CO 1% v/v, and *E. caryophyllata* EE 10% v/v did not affect survival time of *T. angustula* bees through contact exposure, with means survival times above 157.44 hours.

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Table 2. Mean survival time, standard error, and confidence interval (95%) (CI) for *T. angustula* bees exposed to different treatments through contact and ingestion exposure. Means of survival time followed by the same letter are not different according to the Holm-sidak method ($P < 0.05$).

Treatments	Contact exposure				Ingestion exposure			
	Survival Time (h)	Std. error		CI (95%)	Survival Time (h)	Std. error		CI (95%)
Water	167.04	1.34	a	164.41 - 169.67	165.60	2.40	a	160.90 - 170.30
A. sativum EO 10% v/v	163.20	2.81	a	157.69 - 168.71	166.56	1.65	a	163.33 - 169.79
E. caryophyllata EE 10% v/v	162.24	4.61	a	153.21 - 171.27	24.00	-	b	-
E. citriodora EE 1% v/v	107.52	10.09	b	87.75 - 127.29	105.60	9.64	b	86.71 - 124.49
A. indica CO 1% v/v	157.44	5.93	a	145.83 - 169.06	159.36	4.96	a	149.63 - 169.09
P. nigrum EE 10% v/v	142.56	7.02	b	128.79 - 156.33	24.00	-	b	-
ANOVA	Log-Rank = 47.79; df = 5; P <0.001				Log-Rank = 269.79; df = 5; P <0.001			

Source: Authors

4.2 INGESTION EXPOSURE

The ethanolic extracts of *P. nigrum* and *E. caryophyllata* 10% v/v killed all *T. angustula* bees within 24 hours. Consequently, both treatments were removed from the ANOVA analysis. Significant differences between treatments were observed only 168 hours after the beginning of the bioassay (Table 1). Similar to the results for contact exposure, the *E. citriodora* EO caused mortality to *T. angustula* bees (62.00%) higher than control ($P < 0.05$). The *A. sativum* EO 10% v/v and the *A. indica* CO 1% v/v caused mortalities below 30.00% and did not differ from the control.

The survival analysis also indicated differences in mean survival time among treatments for the ingestion exposure (Table 2). The *E. caryophyllata* EE and *P. nigrum* EE 10% v/v, as well as the *E. citriodora* EO, resulted in lower mean survival time compared to the control according to the Holm-sidak method ($P < 0.05$). The lowest mean survival time was caused by EE of *E. caryophyllata* and *P. nigrum* 10% v/v (24 hours).

In general, the effect *E. citriodora* EO 1% v/v in *T. angustula* through contact and ingestion exposure were similar, with mortality rates at 168 hours of 48.67 and 62.00% and mean survival times of 107.52 and 105.60 hours, respectively. The findings of our study underscore the significant toxicity *E. citriodora* EO 1% v/v to *T. angustula* bees through contact and ingestion exposure. In the species *E. citriodora*, the monoterpenoid citronellal constitutes 60 to 90% essential oil (Gusmão *et al.*, 2013; Insuan; Chahomchuen, 2020). The *E. citriodora* EO has shown insecticidal activity against several insects

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through contact exposure and fumigation (Gusmão *et al*., 2013; Rehman *et al*., 2024). It may explain its toxicity to *T. angustula* through ingestion and contact exposure.

The *A. sativum* EO 10% v/v, and *A. indica* CO 1% v/v, showed no toxicity to *T. angustula* through either contact or ingestion exposure. However, for *P. nigrum* EE and *E. caryophyllata* EE 10% v/v, the mortalities through ingestion exposure (100%) were much higher than those through contact exposure (10.67 and 28.89%, respectively). Therefore, the following bioassays were performed through ingestion.

Essential oil of *A. sativum* has shown toxicity to several pests such as Red flour beetle, *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) (Chaubey, 2013), Meal-worm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) (Plata-Rueda *et al*., 2017). The main active ingredient of *A. sativum* is diallyl disulfide, an oil-soluble organosulfur compound (Sundaram; Milner, 1996). This compound can act in several ways, such as repellent and feeding deterrent, reducing oviposition potential, reducing the transformation of larvae into pupae and adult emergence, inhibiting acetylcholinesterase enzyme activity (Chaubey, 2013), and interfering with the cellular and nervous functions of insect (Plata-Rueda *et al*., 2017). When bees come into direct contact with the oil, it penetrates through the cuticle and reaches the nervous system, leading to death (Stejskal *et al*., 2021). However, the results of our study showed that the *A. sativum* EO was not toxic to *T. angustula* bees (Xavier *et al*., 2010).

4.3 EFFECT OF SURFACTANT ON ESSENTIAL OILS TOXICITY

Significant differences among treatments were observed at 24 and 96 hours (Table 3). Anova showed that adding detergent increased the mortality caused by the *A. indica* CO from 2.00 to 44.00% after 24 hours and from 38 to 100% after 96 hours. For the *E. citriodora* EO, adding detergent increased the mortality of *T. angustula* bees from 34.00 to 80.00% after 24 hours and from 47.69 to 93.33 % after 96 hours. The results also showed that pure detergent was not toxic to *T. angustula* bees, confirming that the increased toxicity resulted from a combination of detergent and essential oil synergistic effect.

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Table 3. Effect of botanical insecticides with or without detergent addition on *T. angustula* bees through ingestion exposure at 24 and 72 hours. The means followed by * did not differ from the control (water) according to Tukey's test supported by Anova.

Treatments	24 hours				96 hours			
	Mean		Std. erro		Mean		Std. erro	
Water	**1.82**	**±**	**1.82**	b	1.82	±	1.82	b
Detergent (5% w/w)	1.82	±	1.82	b	21.82	±	19.62	b
A. indica CO 1% v/v	2.00	±	2.00	ab	38.00	±	5.83	b
E. citriodora EO 1% v/v	34.00	±	21.35	ab	47.69	±	20.38	ab
A. indica CO 1% v/v + detergent 5% v/v	44.00	±	16.00	b	100.00	±	-	a
E. citriodora EO 1% v/v + detergent 5% v/v	80.00	±	20.00	a	93.33	±	6.67	a
ANOVA	$F_{(5,24)} = 5.51$, $P = 0.02$				$F_{(5,24)} = 10.39$, $P < 0.001$			

Source: Authors

The commercial oil of *A. indica* caused low mortality to *T. angustula* bees through contact and ingestion exposure and did not reduce the mean survival time of *T. angustula* bees. However, after detergent addition, the mortality increases dramatically, reaching 100.00%. It highlights the potent synergistic effect of detergent addition to essential oils, as observed for *E. citriodora*. The detergent acts as a surfactant and is often used as an adjuvant for pesticide application to enhance the effectiveness of the pesticide formulation by enhancing the solubility or the compatibility of the active ingredients or to enhance adsorption, penetration and translocation of the active ingredients into the target (Krogh *et al*., 2003). Although detergent was not toxic to *T. angustula* bees, it likely acted synergistically with insecticide oils, increasing the absorption of the insecticides to *T. angustula* bees and, therefore, its toxicity.

As previously observed, *A. indica* CO was toxic to *T. angustula* bees (Xavier *et al*., 2010). In addition, sublethal effects of *A. indica* oil on bees have been reported, such as hindering individual flight take-off of worker bees, potentially compromising foraging activity and colony survival (Tomé *et al*., 2015), and inducing antifeeding effects (Bernardes *et al*., 2017). *A. indica* oil contains several biologically active compounds, particularly azadirachtin, which is toxic to insects. Azadirachtin exhibits a repellent effect, inhibits feeding and growth, and is widely used as an insecticide globally (Xavier *et al*., 2010).

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4.4 LETHAL CONCENTRATIONS

The essential oil of *E. citriodora* had the lowest LC_{50} and LC_{90} (0.075 and 1.45% v/v, respectively) (Table 4), which correspond to around 0.45 - 0.674 mL/ 1 mL of citronellal (Gusmão *et al*., 2013; Insuan; Chahomchuen, 2020), proving highly toxic to *T. angustula* bees.

Table 4. Concentration-mortality of four botanical insecticides to *T. angustula* bees through ingestion exposure.

Botanical insecticide	No. Insects	Slope ± SE	LC_{50} (95%)	CI	LC_{90} (95%)	CI	$\chi 2$	*P*
E. citriodora EO	250	0.998 ± 0.093	0.075	0.009 - 0.29	1.45	0.35 - 10.63	4.81	0.1
P. nigrum EE	300	12.28 ± 1.39	7.21	6.63 - 7.53	9.17	8.54 - 9.97	2.03	0.5
E. caryophyllata EE	300	5.49 ± 0.74	7.35	5.96 - 8.94	12.58	10.02 - 22.48	4.21	0.1

Source: Authors

Previous studies determined an LC_{95} of 449.50 ppm for *E. citriodora* EO against *C. maculatus* (Gusmão *et al*., 2013) and an LC_{50} of 32.25 µL/mL against *S. oryzae* (Rehman *et al*., 2024). It was also shown that *E. citriodora* EO contains only 0.75% of eucalyptol and 58.42% of citronellal (Rehman *et al*., 2024). Despite a low amount in *E. citriodora*, the main component of the eucalyptus genus is the monoterpene 1.8-cineole, known as eucalyptol, which can reach up to 94.4% of the EO of *E. globulus* (Pineda *et al*., 2023) and 27.91% of *E. camaldulensis*. The eucalyptol has insecticidal activity on several insects (Pineda *et al*., 2023; Sukontason *et al*., 2004), including *T. angustula* (Xavier *et al*., 2010).

The probit analysis showed an LC_{50} and LC_{90} for *P. nigrum* EE of 7.21 and 9.17 % v/v, respectively, which corresponds to around 721 mg/15 mL and 917 mg/15 mL of piperine (Matena *et al*., 2021). The LC_{50} and LC_{90} for *E. caryophyllata* EE were 7.35 and 12.58 % v/v, respectively, corresponding to around 56.74 mg/mL and 97.12 mg/ml of eugenol (Jung; Yang, 2014).

The ethanolic extract of *P. nigrum* 10% v/v was highly toxic to *T. angustula* bees, primarily through ingestion exposure. The mortality observed in the *P. nigrum* EE could be attributed to piperine, an alkaloid that acts on the insect's nervous system, causing

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disturbances, intoxication, and death (Ikechi-Nwogu; Omeke., 2020; Yaseen, 2020). A previous study showed that oil extract of *P. nigrum* at a concentration of 5% w/v can kill up to 100% of *S. zeamais* at 96 hours of exposure (Choden *et al*., 2021). In addition, the *P. nigrum* EO showed 100% mortality of *A. gambiae* larvae (Kemabonta *et al*., 2018). The *P. nigrum* EE dried fruits (50 mg/mL) exhibited 100% larvicidal activity against the cattle farming parasite *Rhipicephalus australis* larvae (Toussirot *et al*., 2023). Simas *et al*. (2007) found the LC_{50} of the *P. nigrum* EE and piperine for *Aedes aegypti* larvae to be 0.98 ppm and 1.53 ppm, respectively. Antifeeding activity has also been reported (Choden *et al*., 2021; Fan *et al*., 2011; Khani *et al*., 2012).

The ethanolic extract of *E. caryophyllata* 10% v/v was highly toxic to *T. angustula* bees through ingestion exposure. These results indicated that the primary toxicity of *E. caryophyllata* is caused by the bees' ingestion of this botanical insecticide. Previous studies showed that *E. caryophyllata* EE is more toxic through inhalation than contact exposure (Aimad *et al*., 2021). Eugenol has feeding deterrent activity and can affect the respiratory system (Badgujar *et al*., 2017), inhibit acetylcholinesterase and adenosine triphosphatase activities, and cause histological alterations (Gohary *et al*., 2021). Previous studies showed that *E. caryophyllata* EO at a concentration of 20 µl/l can kill 100% of *C. maculatus* within 72 hours (Aimad *et al*., 2021). In addition, Gohary *et al*. (2021) found the LC_{50} and LC_{95} for *E. caryophyllata* EE against *Culex pipiens* larvae of 47 ppm and 357.8 ppm, respectively.

5 CONCLUSION

The essential oil of *E. citriodora* 1% v/v was toxic to *T. angustula* bees both trough contact and ingestion exposure and addition of detergent 5% v/v increases mortality drastically. The commercial oil of *A. indica* with addition of detergent 5% v/v was highly toxic to *T. angustula* bee trough ingestion exposure. The probit analysis showed de LC_{50} and LC_{90} for *E. citriodora* EO of 0.075 and 1.45% v/v, respectively. The probit analysis showed an LC_{50} and LC_{90} for *P. nigrum* EE of 7.21 and 9.17 % v/v, respectively, and LC_{50} and LC_{90} for *E. caryophyllata* EE of 7.35 and 12.58 % v/v, respectively.

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