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Bioactivity of essential oils from *Artemisia* against *Diaphania hyalinata* and its selectivity to beneficial insects

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ABSTRACT: The demand for effective insecticides in pest control with low toxicity to the non-target organisms, such as natural enemies and pollinators, is increasing steadily. A good alternative for synthetic insecticides is natural compounds, including essential oils (EO). This work assessed toxicity of essential oils extracted from *Artemisia annua*, *A. absinthium*, *A. camphorata*, *A. dracunculus* and *A. vulgaris* against the melonworm *Diaphania hyalinata* (Linnaeus, 1758) (Lepidoptera: Crambidae) larvae, a pest of Cucurbitaceae, and their selectivity for fire ant *Solenopsis saevissima* (Smith) (Hymenoptera: Formicidae) and jataí bee *Tetragonisca angustula* (Latreille) (Meliponinae). The plants were grown in a greenhouse with mineral fertilization and were used for EO extraction. The insects in the bioassay belonged to the second instar of *D. hyalinata* and adult forms of *S. saevissima* and *T. angustula*. Essential oil from *A. annua* induced a high mortality rate in *D. hyalinata* (96 %) over a 48 h period. The same essential oil was selective for predator *S. saevissima* (42 % mortality) and pollinator *T. angustula* (74 % mortality), while causing high mortality in *D. hyalinata*. The insecticidal activity of *A. annua* oil was attributed to the synergism of its constituents viz., camphor and 1,8-cineole. Therefore, this essential oil contains constituents that are promising for effective use as insecticide due to its high toxicity and rapid action against *D. hyalinata* as well as low toxicity for predator and pollinator.
Keywords: *Solenopsis saevissima*, *Tetragonisca angustula*, melonworm, insecticide

Introduction

Diaphania hyalinata (Linnaeus, 1758) (Lepidoptera: Crambidae) is a melon caterpillar (melonworm) found in South and Central America and is considered the major agricultural pest of Cucurbitaceae.

Larvae of *D. hyalinata* attack leaves, berries, and stalks reducing the photosynthetic area of the plants, leading to fruit rot and making it useless for consumption. Fire ant *Solenopsis saevissima* (Smith) (Hymenoptera: Formicidae) is the natural enemy of *D. hyalinata* and feeds on larvae and pupae of *D. hyalinata*; thus, it plays a vital role as a biological pest control for these populations in these ecosystems (Pitts et al., 2005; Resende et al., 2016). Another important beneficial insect group associated with ecosystems are bees (Hymenoptera: Apidae), as they contribute positively to the ecosystem as pollinators (Aguiar et al., 2016; Brittain et al., 2010). *Tetragonisca angustula* (Latreille) (Meliponinae), popularly called *jataí*, is one bee species. Today, approximately 75 % of the species of agricultural crops worldwide benefit from insect pollination (Hladik et al., 2016).

Essential oils are very attractive products for insect control, because they have low environmental persistence and mammalian toxicity and are normally available at large amounts, justifying their use as fragrance and food flavors (Isman, 2006). Essential oils have several activities and applications such as insecticides, repellents, cosmetics, fragrances, herbicides, among others. Repellency of 17 essential oils against pestiferous yellowjackets wasps of human food sources was recently reported in the literature (Zhang et al., 2013). Research on natural products with insecticidal activity needs to determine the compounds responsible for this activity. This allows to standardize concentration of these compounds in the oil to maximize toxicity to the target organism. In addition, it enables the direct use of these compounds as insecticides (Passos et al., 2012).

This study investigated the insecticidal activity of the essential oils extracted from five *Artemisia* species, artificially fertilized with nitrogen, phosphorus, and potassium (NPK) and grown in a greenhouse. Insecticidal activities of essentials oils were evaluated against *D. hyalinata*, predator *S. saevissima* and pollinator *T. angustula* (bee). Toxicity of these natural terpenes against *D. hyalinata* and selectivity for predator and pollinator was then estimated. In addition, the compounds responsible for the insecticidal activity of the selected essential oil were determined.

Materials and Methods

Cultivation of *Artemisia* spp. and extraction of essential oils

The plants were cultivated in a greenhouse in Viçosa, Minas Gerais State (20°45′59.3″ S, 42°52′09.1″ W, altitude 663 m), from Mar 10 to Nov 15, 2013.

The species selected were *Artemisia annua*, *Artemisia absinthium*, *Artemisia camphorata*, *Artemisia dracunculus* and *Artemisia vulgaris*. Seeding was performed using 300 mL plastic cups containing a commercial substrate to accelerate rooting for subsequent transplantation to the pots.

A voucher of the species was deposited at the herbarium of the Federal University of Viçosa under the numbers: VIC 15614, VIC 42224, VIC 15592, VIC 42223, and VIC 42222.

For post soil analysis, the plants in each pot were fertilized with 6.5 g of ammonium sulfate (nitrogen source), 40 g of phosphorus (phosphorus source), and 3.0 g of potassium chloride (potassium source).

Crushed fresh leaves (100 g) of each plant species were hydrodistilled in a Clevenger apparatus for 2 h and the hydrolate was extracted with pentane (3 × 40 mL). The combined organic phases were dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated under reduced pressure in a rotary evaporator and the residue stored in glass vials at -4 °C.

Qualitative and quantitative analysis of essential oils

Quantitative analysis was performed in a Shimadzu GCMS5050 apparatus fitted with a capillary DB-5 column (30 m × 0.25 mm, with 0.25 µm film thickness) coupled with the flame ionization detector. Helium was the carrier gas used at a flow rate of 1.8 mL min^{-1} and the injector temperature was set at 220 °C. The initial column temperature was 40 °C. Isotherm was kept for 2 min, followed by heating from 3 °C min^{-1} up to 240 °C. Isotherm was kept for 15 min before injecting 1.0 µL of the sample [1 % of the sample in CH_2Cl_2 (m/v)], in a split ratio of 1:20 and a column pressure of 100 kPa.

The qualitative analysis was performed in a Shimadzu GC17A apparatus fitted with a capillary DB-5 column (30 m × 0.25 mm, with 0.25 µm film thickness) using electron impact ionization (70 eV). Injector temperature, isotherm, column pressure and heating rate were similar to the GC/FID analysis.

The analysis was performed in triplicate and concentration of each constituent was calculated as percentage of the corresponding peak area to the total area of all peaks. Components were identified by comparing retention times, in relation to series of alkanes (C_9 - C_{27}) and by comparing the mass spectra with library database of Wiley and Nist (05, 08 and 11).

Insecticidal bioassays

Second-instar *D. hyalinata* larvae were obtained from a population kept in the laboratory. Adults of *S. saevissima* and *T. angustula* were collected from nests located around the University campus. The average weight of each insect was estimated by measuring the mass of ten insects on an analytical balance. Bioassays were conducted by topical application. Each solution was prepared in acetone and applied (0.5 µL) on the abdominal tergum of each individual insect utilizing a Hamilton microsyringe (10 µL). For negative control, the insects were treated with an equal volume of acetone. As the positive control, the commercial insecticide neem oil (12 g L^{-1} of azadiractin, Parry India Limited, India) was used. Neem oil was used as a standard of efficiency because azadiractin is the most frequently natural constituent used for worm control (Isman, 2006). Besides, neem oil is an insecticide from a natural source, similar to the products evaluated in this work.

In this work, five bioassays were conducted. In the first, essential oil with the highest insecticidal activity against *D. hyalinata* was selected. In the second and third bioassays, dose-mortality curve and speed of action of the selected oil on *D. hyalinata* were determined. In the fourth bioassay, compounds responsible for the insecticidal activity of selected essential oil were determined. In the fifth bioassay, selectivity of essential oil for predator and bee was determined.

Selection of essential oil with insecticidal activity against *D. hyalinata*

The experimental design was completely randomized with six replicates. Each replicate consisted of a Petri dish (9 cm in diameter) containing ten second-instar caterpillars of *D. hyalinata*. The dose used in this bioassay was 20 µg of the compound per mg of larvae. The treatments comprised neem oil (positive control), negative control (acetone) and essential oils of *A. annua*, *A. absinthium*, *A. camphorata*, *A. dracunculus*. After application, a disc of chayote leaf was placed inside the Petri dish to feed the caterpillars. The Petri dishes were placed in an incubator at 25 ± 0.5 °C and 75 ± 5 % relative humidity with a 12-h photoperiod. Insect mortality was evaluated 48 h after application of the treatments. Larval mortality data were submitted to analysis of variance and the means were compared by the Tukey test at $p < 0.05$ (PROC GLM, SAS 9.2, SAS Institute Inc, Cary, USA).

Essential oils that induced more than 80 % of mortality in *D. hyalinata* were selected for the next bioassay. These criteria are used in Brazil to assess a product for its effectiveness in pest control (Bacci et al., 2007).

Dose-mortality curve for essential oil against *D. hyalinata*

The experimental design, conditions, and evaluations were similar to those of the previous bioassay. The treatments included doses of essentials oils selected in the previous bioassay and controls. Six doses were used (2.5; 5.0; 12.5; 15.0; 17.5; 20 mg g^{-1} of insect). Insect mortality was evaluated 48 h post treatment application, because this time allows to determine the dose to kill 50 % of the insects (LD_{50}) by the probit analysis.

The most active compounds were subjected to toxicity bioassays against *D. hyalinata* following the randomized experimental design, with six replicates. Each experimental unit included ten insects in a Petri dish (9 cm in diameter) covered with organza. The dose-mortality curves for *A. annua* and neem oil were constructed using four and six doses, respectively. These doses were established through preliminary bio-

assays with four concentrations for each compound to identify the concentration range inducing mortality greater than zero and less than 100 %.

The dose-mortality data were subjected to probit analysis (PROC PROBIT, SAS 9.2) to estimate the dose-mortality curves. The curves that presented probabilities greater than 0.05 by the χ^2 test were accepted. The lethal doses that induced 50 and 95 % mortality (LD_{50} and LD_{95}) were also estimated.

Action speed for selected essential oil against *D. hyalinata*

D. hyalinata was subjected to time-mortality bioassays when exposed to the LD_{90} of the most active oil (essential oil of *A. annua*) and control. One hundred insects were subjected to each treatment. Each experimental unit involved ten insects in a Petri dish (9 cm diameter) covered with organza. Larval mortality was recorded after 48 h. The intervals between the assessments for each treatment (ranging from 30 min to 3 h) were determined earlier.

Survival curves were estimated by the Kaplan-Meier product-limit method and compared by the log-rank test at $p < 0.05$ (PROC LIFETEST, SAS 9.2). Median lethal time (LT_{50}) of the insects were also estimated.

Determining compounds responsible for the insecticidal activity of selected essential oil against *D. hyalinata*

In this bioassay, the design was completely randomized with six replicates. This bioassay was conducted similarly to that for essential oil selection. The treatments were essential oil selected (*A. annua*), two compounds of this oil (camphor and 1,8-cineole) and control (acetone). These two oil compounds were used separately and in combination. The dose used of the essential oil selected was 20 µg mg^{-1} of insect. The doses of the two compounds were: 7.56 µg of camphor mg^{-1} of insect and 0.57 µg of 1,8-cineole mg^{-1} of insect. Forty-four hours after applications, insect mortality was evaluated. Mortality data were submitted to analysis of variance at $p < 0.05$ and the means of treatments were compared by the Tukey test (PROC GLM, SAS 9.2).

Selectivity of selected essential oil for predator and bee

S. saevissima and *T. angustula* were exposed to LD_{95} of the most active compounds against *D. hyalinata* in order to evaluate their selectivity. The experimental design was completely randomized, with six replications. Each experimental unit comprised ten *S. saevissima* and *T. angustula* insects placed in a Petri dish (9 cm in diameter) covered with organza. Insect mortality was recorded 48 h post treatment.

To evaluate selectivity, mortality of the non-target species was compared with *D. hyalinata* mortality by the student t-test for independent samples (PROC TTEST, SAS 9.2) at $p < 0.05$.

Results

Identification of chemical compounds

The Asteraceae family includes numerous flowering plants, involving around 1600 genera, comprising more than 23,000 species, namely mugwort (*Artemisia annua*), tarragon (*A. dracunculus*), wormwood (*A. absinthium*), camphor (*A. camphorata*) and wormwood (*A. vulgaris*). The species are highly aromatic and reported to have medicinal applications (Belhattab et al., 2014; Bessada et al., 2015; Brisibe et al., 2012).

Among the five species of *Artemisia*, the highest essential oil content was obtained for *A. camphorata* (2 %) followed by *A. dracunculus* (less than 2 %), *A. annua* (less than 1 %), *A. absinthium* (trace amount), and *A. vulgaris* (trace amount).

The chemical composition of the essential oils from the leaves of *A. annua*, *A. absinthium*, *A. camphorata*, *A. dracunculus* and *A. vulgaris* were determined and 96 compounds were identified.

In *A. annua* essential oil, 35 compounds were identified, accounting for 94 % of the essential oil, with the predominance of two compounds, camphor (32 %) and germacrene D (21 %). The main compounds of the essential oil of *A. absinthium* were *Z*-isocitral (22 %), myrcene (18 %) and β-pinene (15 %), from 30 compounds. *A. camphorata* showed the following major compounds: germacrene D-4-ol (22 %), 1,8-cineole (12 %), ascaridole (10 %), and borneol (10 %) from 28 compounds in the sample, representing 94 % identified. The chemical composition of *A. dracunculus* included 16 compounds with 99 % of the essential oils identified. The four major constituents methyleugenol (57 %), β-thujone (31 %), β-pinene (26 %), and 1,8-cineole (19 %) were identified in the species of *A. vulgaris*.

Selection of essential oil with higher insecticidal activity against *D. hyalinata*

Data on insect mortality caused in *D. hyalinata* allow to divide treatments of this bioassay into two groups. The first group comprised essential oil of *A. annua* (86 % mortality) and neem oil (68 % mortality), which were the treatments that caused the highest mortality to *D. hyalinata*. The second group comprised the treatments that caused mortality of *D. hyalinata* similar to the control and it was formed by essential oils of *A. camphorata*, *A. dracunculus*, *A. vulgaris* and *A. absinthium* (Figure 1). Therefore, *A. annua* essential oil was selected for the other bioassays.

Dose-mortality curves of selected essential oil and neem oil against *D. hyalinata*

Lethal doses of *A. annua* essential oil (LD_{50} = 2.27 µg mg^{-1} of insect and LD_{95} = 51.54 µg mg^{-1} of insect) were lower than those of neem oil (LD_{50} = 8.35 µg mg^{-1} of insect and LD_{95} = 101.35 µg mg^{-1} of insect). LD_{50} of these two oils showed that *A. annua* oil was 2.7 times more potent for *D. hyalinata* control than the efficiency standard was (Table 1).

Table 1 – Results of the probit regression analysis on *Diaphania hyalinata* larvae mortality, 48 h post application.

Oil	Equation*	χ^2	df	*p*	N	LD_{50}	LD_{95}
						μg of oil mg^{-1} of insect**	
Artemisia annua	Y = -0.43 + 1.21x	1.46	2	0.48	240	2.27 (1.26-3.47)	51.54 (30.09-118.49)
Neem oil	Y = -1.40 + 1.52x	3.86	4	0.43	360	8.35 (6.46-10.54)	101.35 (64.93-195.09)

df = degrees of freedom; *p* = probability; N = number of insect evaluated; LD_{50} = lethal dose for 50 % of the insect population; LD_{95} = lethal dose for 95 % of the insect population; *Y = probit mortality; x = dose in μg of oil mg^{-1} of insect; **The numbers outside the parenthesis are averages of the lethal dose. The values in parentheses are the fiducial intervals of the lethal doses.

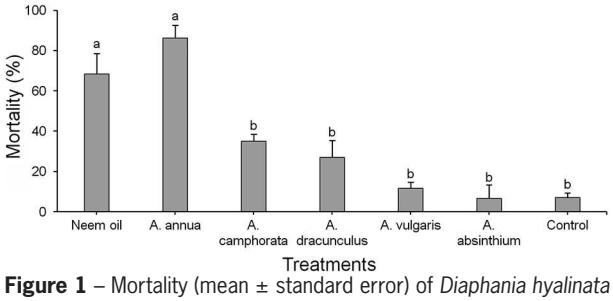

Figure 1 – Mortality (mean ± standard error) of *Diaphania hyalinata* larvae 48 h after application of the neem oil (positive control), five essential oils and control (acetone) at a dose of 20 μg of oil mg^{-1} per insect. Histograms followed by the same letter have averages that do not differ by the Tukey's test at $p < 0.05$.

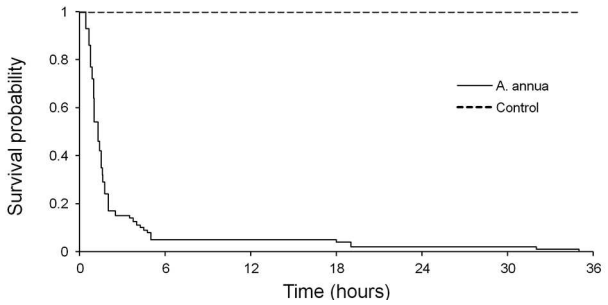

Figure 2 – Survival curves for the second instar *Diaphania hyalinata* larvae subjected to LD95 of the essential oil of *Artemisia annua* (51.54 μg mg^{-1} of insect) and control (acetone).

Action rate of selected essential oil on *D. hyalinata*

The analysis of *D. hyalinata* survival subjected to the control (acetone) and essential oil of *A. annua* indicated a difference between the treatments (log-rank test: $\chi^2 = 174.082$, df = 1, $p < 0.001$). *D. hyalinata* survival after treatment with the control was 100 % after 36 min; conversely, the mortality was 100 % for the same period after applying essential oil. The survival time (LT_{50}) of *D. hyalinata* was only 1 min 30 s after essential oil application (Figure 2).

Determining compounds responsible for the insecticidal activity of selected essential oil against *D. hyalinata*

Isolated and mixed application of *A. annua* components, camphor and 1,8-cineole, caused higher mortalities of *D. hyalinata* larvae than the control (acetone) did. The mixture of camphor and 1,8-cineole caused higher mortality of *D. hyalinata* than the isolated application of these two compounds. In addition, camphor and 1,8-cineole mixture caused mortality of *D. hyalinata* similar to the *A. annua* essential oil (Figure 3). Therefore, toxicity of *A. annua* essential oil to *D. hyalinata* was due to mainly camphor and 1,8-cineole. This toxicity was augmented by the synergistic action of these two compounds since they presented maximum activity applied in combination.

Selectivity to the non-target organisms

In this bioassay, mortality caused by *A. annua* essential oil to insects of *D. hyalinata* (96 %) was significantly higher ($p < 0.05$) than that caused to predator *S. saevissima* (42 %) and bee *T. Angustula* (74 %). Although mortality caused by this oil to the pest was higher than that caused to the non-target species, it caused significant mortality to predator and bee (Figure 4).

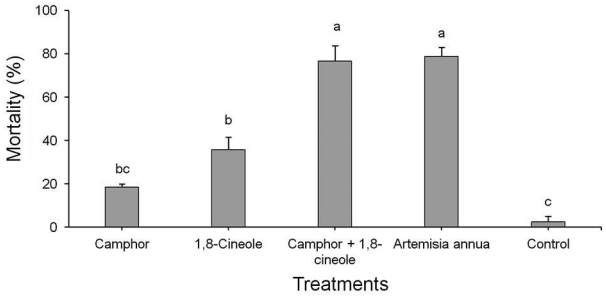

Figure 3 – Mortality (mean ± standard error) of the *Diaphania hyalinata* larvae 48 h after application of the control (acetone), essential oils *Artemisia annua* (20 μg mg^{-1} insect) and the compounds camphor (7.56 μg mg^{-1} insect) and 1,8-cineole (0.57 μg mg^{-1} insect) in the corresponded dose that they occur in the oil. Histograms followed by the same letter have averages that do not differ by the Tukey's test at $p < 0.05$.

Discussion

Variations in the chemical composition of essential oils from the *Asteraceae* plants are attributed to differences in chemotypes. In this study, the geographic location and environmental conditions were not taken into account, as the plants were grown in a greenhouse. *A.*

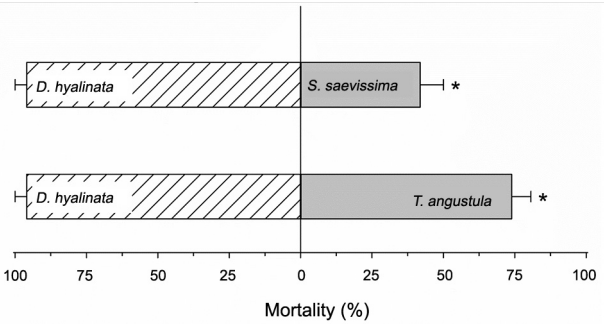

Figure 4 – Mortality (mean + standard error) caused by LD95 (51.54 μg mg^{-1} of insect) of *Artemisia annua* essential oil to the pest *Diaphania hyalinata* and to the non-target organisms *Solenopsis saevissima* and *Tetragonisca angustula*. Histogram followed by * denotes significant difference between species by the Student's t-test ($p < 0.05$).

annua essential oil caused high mortality (86 %) to *D. hyalinata* larvae, indicating its potential to control caterpillars in agricultural crops.

The chemical composition of essential oils from the *Artemisia* genus has been extensively studied in several species worldwide. Many studies have shown that the *Artemisia* species display significant intraspecific variations in terpene constituents of essential oils. In some cases, variations in volatile components of these plants may occur during plant ontogeny or growth at different altitudes. Quality and yield of essential oils from the *Artemisia* species are influenced by the harvesting season, fertilizers, and soil pH, as well as the choice and stage of drying conditions, geographic location, chemotype or subspecies, choice of plant part or genotype or extraction method (Abad et al., 2012; Rocha et al., 2014; Tariq et al., 2014; Yadav et al., 2014).

The yield of 1 % of extraction of *A. annua* essential oil agrees with reports in the literature (Radulović et al., 2013). The chemical constituents viz., 1,8-cineole, camphor, terpinen-4-ol, α-terpineol, borneol, and ethyl bornyl are described in the literature as capable of performing antibacterial, antifungal and antiviral biological activities (Abad et al., 2012; Lopes-Lutz et al., 2008; Pavela, 2015). Camphor and germacrene D are the predominant compounds recognized as a Vietnamese chemotype (Bilia et al., 2014; Brown, 2010).

A. camphorata showed an essential oil content of 2 %. The major component in Tunisia was davanone (20 %) and in our work, ascaridole (10 %) was the major component. These variations in concentration are due to differences in climate between Brazil and Tunisia (Mohsen and Ali, 2009).

A. dracunculus essential oil was rich in α-pinene (26 %), providing a yield of 1 % (Chauhan et al., 2010). Based on its chemical constitution, it is believed to belong to the Russian chemotype 2. Similar results were obtained for essential oil of *A. vulgaris*, which provided an extremely low yield, less than 1 % (Zhigzhitzhapova et al., 2016).

Essential oil activity has been extensively studied and several hundred components have been identified so far (Brown, 2010). Camphor, germacrene D, and 1,8-cineole were the major components normally found there (Ahmad Malik et al., 2009). The variability of essential oil chemical composition of *A. annua* depends on the geographical origin and plant developmental stage (Brown, 2010; Tian et al., 2015). There was a synergistic effect between camphor and 1,8-cineole, indicating that these two compounds together promote high mortality of larvae of *D. hyalinata*.

The mechanism of the physiological selectivity may be related to lower penetration rates of the insecticide through the cuticle, higher degradation rates of the insecticide and target site, as well as the relative insensitivity on natural enemies compared to *D. hyalinata* (Silva et al., 2016). Considering that lipophilicity is inversely proportional to solubility of essential oil in water, lipophilic compounds usually penetrate into the insect body at higher rates, given the similarity in their cuticles (Kumar et al., 2011; López and Pascual-Villalobos, 2010).

Lipophilic constituents of essential oils were suggested to successfully inhibit microbial growth by reacting with lipid constituents of cell membranes, making mitochondria and cell membranes more permeable, resulting in death of bacterial cells (Kumar et al., 2011). Essential oils can also inhibit the synthesis of DNA, RNA, proteins, and polysaccharides in bacterial cells (Abad et al., 2012; Demuner et al., 2011).

In addition to the efficiency and action rate of *A. annua* essential oil against *D. hyalinata* insect pests, its impact on non-target organisms is also important. In this context, mortality that this oil caused to *D. hyalinata* was greater than that caused to predator and bee. This is important because the vast majority of insecticides do not present this selectivity. However, mortality caused by *A. annua* essential oil to predator *S. saevissima* (42 %) and bee *T. angustula* (74 %) was significant. According to the classification scale of pesticide toxicity to non-target organisms of "International Organization for Biological and Integrated Control of Noxious Animals and Plants/ West Paleartic Regional Section" (IOBC/WPRS IOBC/ WPRS) (Hassan et al., 1994), mortality observed for these non-target organisms is slightly harmful (30-79 %) to them. Thus, the use of *A. annua* essential oil should follow the principles of ecological selectivity in which a product with toxicity to a non-target organism should be used in order to minimize its contact with this organism (Aguiar et al., 2016; Moreno et al., 2017; Silva et al., 2016). Therefore, it is necessary to avoid the use of *A. annua* essential oil when the plants are in the flowering stage, as it is when bees visit the plants (Silva et al., 2016). Moreover, the application of this oil should be carried out at lower air temperatures when visitation of bees to the plants is low (Silva et al., 2016).

In order to minimize the impact of *A. annua* essential oil applications on predator of *S. saevissima*, applications in the soil should be avoided, as it is the nesting

site for these natural enemies. In addition, nocturnal applications should be avoided, as it is the time of greater activity of this predator (Moreno et al., 2017; Silva et al., 2016).

Conclusion

Camphor and germacrene D accounted for more than 50 % of essential oil constituents of *Artemisia* species. This oil showed high mortality (> 80 %) to insect pest *D. hyalinata* and low mortality rates to beneficial insects. Essential oil of *A. annua* (LD_{50} = 2.27 µg mg^{-1}) is almost four times more toxic than the commercial product (Azamax) (LD_{50} = 8.35 µg mg^{-1}). Therefore, *A. annua* essential oil is clearly a potential effective insecticide for the control of melonworm insect, evidenced by the mortality of 77 % of the insects due to the synergism between camphor and 1,8-cineole. *A. annua* oil presented potential to control melonworm moth infestations, with fast action, efficiency and selectivity to beneficial insects, especially the pollination agent (bee).

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