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Research article

The Botanical Monoterpenes Linalool and Eugenol Flush-Out Nymphs of *Triatoma infestans* (Hemiptera: Reduviidae)

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Abstract

Monoterpenes are the main components of essential oils. Some members of this chemical family present insecticidal activity. *Triatoma infestans* (Klug) is the main vector of Chagas disease in Argentina, Bolivia, Paraguay, and Perú. The objective of this work was to evaluate the effect of six monoterpenes (1,8-cineole, eugenol, linalool, menthol, α-terpineol, and thymol) on the locomotor and flushing out activity of *T. infestans.* A video tracking technique was used to evaluate the locomotor activity of nymphs exposed to different concentrations of these chemicals applied as films on filter paper. Papers treated with acetone alone were used as negative controls, while solutions of tetramethrin were applied as positive controls. Only linalool and menthol produced hyperactivation. Flushing out was assessed under laboratory conditions using a standardized aerosolization method. All monoterpenes were applied at 1.5 g/m^3. 1,8-Cineole, α-terpineol, and thymol flushed out 10% or less nymphs. The average flushing out produced by eugenol was 36.7%. Values of median flushing out time (FT_{50}) could only be calculated for linalool and menthol (16.67 and 42.98 min, respectively). The FT_{50} value for the positive control tetramethrin (applied at 0.006 g/m^3) was 8.29 min. Following these results, the flushing out activity of a mixture of linalool and eugenol was evaluated. The FT_{50} of this 2:1 linalool:eugenol mixture was 40.73 min. Finally, flushing out assays performed in semifield conditions showed similar results to those obtained at the laboratory.

Key words: triatomines, chagas disease, linalool, eugenol, locomotor activity

Chagas disease is the most severe parasitic disease of the American continent. It is caused by the protozoan *Trypanosoma cruzi* (Chagas) and transmitted to humans and other vertebrates by insects of the Triatominae family (Stevens et al. 2011). The triatomine *Triatoma infestans* (Klug) is the most important vector of *T. cruzi* in Argentina, Bolivia, Paraguay, and Perú (Schofield and Gorla 2010). With 1,500,000 people infected with Chagas disease in Argentina (3.6% of total population) and 2,240,000 people exposed to it in endemic areas, it is the main endemic disease of the country (World Health Organization [WHO] 2015). Although several indexes related to entomological surveillance and vector control have improved in several Argentinian provinces during the years 2009 and 2010, the levels considered acceptable have still not been reached in others (Ministerio de Salud de la Nación 2014).

Timed manual capture is the most frequently used method to establish if a rural dwelling is infested with triatomines, either using a flushing out agent or not. A flushing out agent is a chemical that makes the insects leave their refuges, exposing them to the view of the sanitary personnel in charge of the capture. Hence, optimal flushing out agents are essential for implementing strategies in both the eradication and reinfestation phases of Chagas disease control (Gürtler et al. 2001).

In Argentina, the use of a 0.2% tetramethrin spray followed by manual captures has been the standard method used by sanitary agents during decades (Schofield 1978, Gürtler et al. 1993, Rabinovich et al. 1995). A house is considered positive (infested) when the evaluator finds adults, nymphs, eggs, exuviae, or recent traces of triatomine feces. Tetramethrin is a noncyanopyrethroid, with low toxicity in *T. infestans* (Casabé et al. 1988) and a low hyperactivant effect compared to cyanopyrethroids like deltamethrin or cypermethrin (Alzogaray et al. 1997, Alzogaray and Zerba 2001a). This is considered an advantage, because it allows capturing healthy insects for further studies. However, despite its low toxicity in triatomines, aerosolized tetramethrin can sometimes cause knock-

down before flushing out because sanitary agents apply subjective quantities (Nelson 1988). In turn, the abundance of insects is underestimated and thus decreases the probability of capturing healthy individuals (U.S. Environmental Protection Agency [EPA] 2010).

In recent years, pyrethroid resistance has been reported in *T. infestans* populations from Argentina and Bolivia (Vassena et al. 2000, Picollo et al. 2005, Roca-Acevedo et al. 2013). Individuals resistant to pyrethroid-induced knock-down are also resistant to hyperactivation (Sfara et al. 2006). Moreover, the use of conventional insecticides as pyrethroids is controversial because of the associated environmental and health risks (Isman 2006). For these reasons, it is important to identify alternative flushing out agents to replace tetramethrin.

Monoterpenes are the most representative components in essential oils, constituting 90% of them and showing a great variety of structures (Bakkali et al. 2008). Some monoterpenes show a broad spectrum of insecticidal activity, and are generally less toxic to mammals and less persistent in the environment than conventional insecticides (Isman 2000). The monoterpenes eugenol and linalool have shown lethal effects against many entomological pests like stored-grain coleopterans (Rozman et al. 2007, Ogendo et al. 2008), flies (Isman 2006, Tarelli et al. 2009), German cockroaches (Alzogaray et al. 2013, Yeom et al. 2013), and lice (Toloza et al. 2006). They have also shown hyperactivant, repellent, and knock-down effects on first-instar nymphs of *T. infestans* and *Rhodnius prolixus* (Moretti et al. 2013). Despite these characteristics, monoterpenes and botanical insecticides in general, have had a poor penetration in the market. This is attributed mainly to their low residuality, relatively slow action (compared to conventional insecticides such as pyrethroids), and variable efficacy against different pests (Isman 2008).

As a first step in exploring the potential of several monoterpenes for detecting domestic infestation by triatomine bugs, the aim of this work was to assess, under both laboratory and semifield conditions, the hyperactivant and flushing out activities of these compounds and compare them to the effects produced by tetramethrin.

Materials and Methods

Biological Material

Fifth-instar nymphs of *T. infestans*, 7–15 d old and starved since last ecdysis, were used in all the experiments. They were provided by the Centro de Referencia de Vectores (Santa María de Punilla, Córdoba, Argentina) and kept in a breeding chamber in the Centre for Research in Pests and Pesticides (CIPEIN) at 26 ± 2 °C, 60–90% RH, and a photoperiod of 12:12 (L:D) h.

Chemicals

1,8-Cineole (99%), eugenol (98%), linalool (97%), menthol (99%), α-terpineol (99%), and thymol (99.5%) were bought from Sigma Aldrich (Buenos Aires, Argentina). Tetramethrin (92%) was acquired from Sumitomo Chemical Co. (Osaka, Japan). Acetone for analysis was bought from Merck (Darmstadt, Germany).

Recording Equipment

A black and white closed-circuit video camera (VC 1910, Sanyo Electrical Co., Tokyo, Japan) and an image analyzer (Videomex V, Columbus Instruments, Columbus, OH) were used to quantify locomotor activity. The video camera captures the image of the insects placed on a circular piece of treated filter paper. The image analyzer converts the analogue signal input from the video camera into digital data with a resolution of 256 × 192 pixels and an acquisition and processing speed of 30 frames per second. On the screen, the video signal colors are inverted, i.e., white objects appear black and black ones, white. Therefore, the presence of insects on the filter paper is determined by visual contrast between the subjects (white) and the paper background (black), and is scored as the number of enlightened pixels. To quantify nymph movement, Videomex-V uses Multiple Zone Motion Monitor software that compares consecutive frames captured by the camera and records the number of pixels that change from "on" to "off" or vice versa. The sum of pixels that change during the experimental time is called motion (M). The software also calculates the average number of pixels that remain "on" during the experiment. This parameter is called area (A) and represents the average area occupied by the insects on the video image. The experimental arena was illuminated with a cold light lamp (22 watts; Luxa, Shangai, China) located at the zenith. Temperature was maintained at 26 ± 2 °C. Each set of data was imported and processed on a personal computer.

Quantification of Locomotor Activity

The experimental arena was a single filter paper circle 11 cm in diameter (101 FAST, Hangzhou Xinxing Paper Industry and Co., Ltd., Fuyang, China). The circle was impregnated with 0.74 ml of a solution of monoterpene in acetone using a pipette. Papers impregnated with acetone alone were used as negative controls, while solutions of tetramethrin were applied as positive controls.

After allowing the solvent to evaporate for 10 min, the filter paper circle was placed on a horizontal surface and surrounded by a glass ring (2.5 cm high, 10 cm in diameter). A nymph was then placed in a plastic vial (5.5 cm high, 2.5 cm in diameter) which was held a few millimeters above the experimental arena and gently inclined downward so that the nymph carefully slid down into the center of the arena. All trials lasted for 10 min and the design was completely randomized.

The concentrations used in the assays were chosen after preliminary tests. A 500 mg/ml mother solution was prepared for each compound. Subsequently, a serial dilution (10x) was performed of each mother solution and the effect of the different solutions was evaluated on the locomotor activity of nymphs. Thus, the minimum concentration that increased the locomotor activity was identified (except for monoterpenes that did not modify locomotor activity). After these preliminary results, it was decided to work with the following concentrations: 0.5, 5.0, 50.0, and 500.0 mg of monoterpene/ml (corresponding to 3.9, 39, 390, and 3,900 µg of monoterpene/cm^2, respectively); and 0.05, 0.5, 5.0, 50.0, and 500.0 mg of tetramethrin/ml (corresponding to 0.039, 0.39, 3.9, 39, and 390 µg of tetramethrin/cm^2, respectively). Each experiment was independently replicated six times (replicates were performed on different days and each insect was only used once and then discarded).

Because of the changes in nymph position, the number of pixels varies during the experimental time. To standardize the data for the size of the nymphs, we calculated the quotient M/A = *Locomotor Activity* (Alzogaray et al. 1997).

Flushing Out Under Laboratory Conditions

Bioassays were performed inside a glass chamber (70 by 70 by 70 cm), illuminated by two cold light tubes of 20 watts each (Osram, Buenos Aires, Argentina) placed externally at the upper rear corner (Fig. 1). Light intensity was 680 lux. In the front panel there were two holes (5 and 15 cm in diameter) used to aerosolize the flushing out agents inside the chamber. Room temperature was kept at 26 ± 2 °C, and RH varied between 60 and 90%. A black

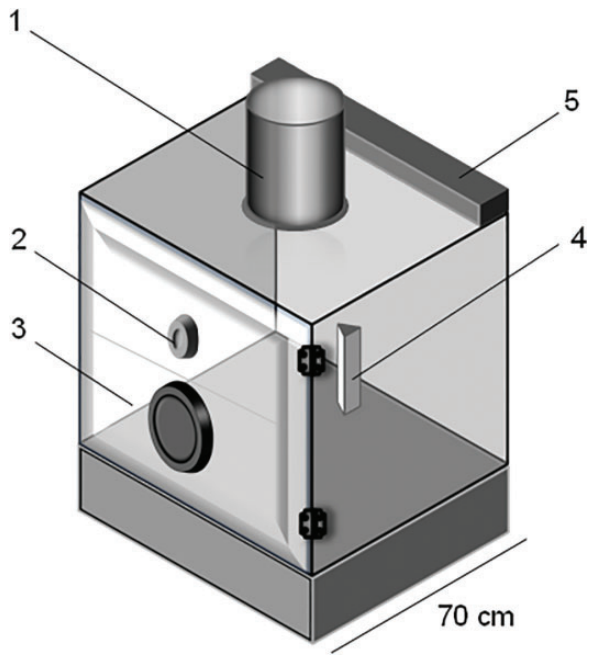

Fig. 1. Glass chamber used for flushing out bioassays. 1, Exhaust fan (it is used to exhaust the contaminated air out of the glass chamber after each assay); 2, hole sealed with a rubber stopper; 3, front panel; 4, black cardboard triangular hollow prism (insects refuge); 5, two cold light tubes.

cardboard shaped as a triangular hollow prism (3 by 15 cm high) with both ends open, was located vertically inside the glass chamber (5 cm from the back wall and equidistant from the lateral walls). Ten fifth-instar nymphs were gently released inside the black cardboard refuge and were allowed 15 min of familiarization. After that, 1 ml of monoterpene in acetone was aerosolized through the front hole of the chamber using a glass sprayer. Compressed nitrogen was used as the carrier (3.5–3.8 psi). The hole was sealed with a rubber stopper and the number of insects leaving the refuge was recorded every 1 min during half an hour.

The monoterpenes used in this assay were the same as for the locomotor activity bioassays, but only the highest concentration of each compound was tested for flushing out (500 mg/ml, equivalent to 1.47 g/m^3). One ml of acetone alone was aerosolized as a negative control, while a solution of 0.2% tetramethrin in acetone (0.006 g/m^3) was used as a positive control. Trials were always carried between 9 and 12 am, coinciding with the moment when the insects remain inside their refuges during the light phase of the photoperiod (Lazzari 1992, Lorenzo and Lazzari 1998). Three or four independent replicates were performed for each treatment. When flushing out values at the end of the assay were higher than 50%, the values of median flushing out time (FT_{50}) were calculated.

Flushing Out Under Semifield Conditions

Bioassays were carried out in a location that belongs to the experimental field of the Centro de Referencia de Vectores (Santa María de Punilla, Córdoba, Argentina). Experimental walls (0.26 m^2) were built with mud bricks (27 by 13 by 6 cm) within an area delimited by a wire fence. Each wall was considered an experimental unit. The position of the wall as well as the cardinal orientation of the treated side and the treatment used in each case were randomly assigned.

A group of nine third-instar nymphs and ten fifth-instar nymphs were released in the center of the top area of the wall. Within a few seconds the insects found refuge inside the crevices between the bricks. To prevent the insects from escaping, the wall was surrounded by a metal mesh cage with an aluminum frame (1.5 by 1.5 by 1.5 m). The top area of the cage was covered by a plastic sheet (2 by 2 m) to protect it from the rain. The assays were carried out the following day.

The experimental solutions were applied using a 200-ml plastic sprayer that ejects ~0.3 ml per puff (La Casa de los Mil Envases S.A., Ciudad Autónoma de Buenos Aires, Argentina). The sprayer was located 20 cm from the wall and one puff was administered in each vertical crevice between adjacent bricks. One side of the wall (0.26 m^2) was covered by a total of 20 puffs. The following treatments were tested: acetone alone (negative control), tetramethrin alone (negative control), linalool alone, eugenol alone, and a mixture linalool:eugenol 2:1.

The number of insects that came out completely (full body) from within the crevices was registered for half an hour following the application of the treatment. Once the assay was over, the wall was completely disassembled and each brick was carefully examined until all remaining insects were recovered. The bricks were discarded and the next test was performed on a wall made with new bricks.

Three (acetone alone and tetramethrin) or four (monoterpenes) independent replicates were carried out for each treatment. The assays were performed on December 10th, 11th, and 12th 2013. Temperature varied between 21.4 and 31.3 °C; and RH, between 41 and 65%.

The results are expressed as the average number of nymphs flushed out per wall after 30 minutes.

Statistical Analysis

The results from locomotor activity and flushing out assays were analyzed using one-way ANOVA. The flushing out percentages from the semifield experiment were arcsine square root transformed before analysis. When $P < 0.05$, Fisher's LSD test was used to detect significant differences between pairs of treatments.

Median flushing out time (FT_{50}) values and their 95% confidence limits (95% CL) were calculated using the statistical software for correlated data developed by Throne et al. (1995). Differences between values of FT_{50} were considered significant ($P < 0.05$) when their respective 95% CL did not overlap.

Results

Effect of Monoterpenes on Locomotor Activity

Figure 2 shows the locomotor activity of fifth-instar nymphs of *T. infestans* exposed to different concentrations of five monoterpenes. Within the range of concentrations tested, linalool and menthol were the only monoterpenes to significantly increase locomotor activity compared to acetone (negative control; ANOVA; linalool: $F = 10.188$, df = 4, 66, $P < 0.001$; menthol: $F = 4.125$, df= 4, 66, $P = 0.005$). The two highest concentrations of linalool applied (390 and 3,900 μg/cm^2) produced a significantly higher effect than acetone alone (negative control; Fisher's LSD test, $P < 0.001$). Menthol significantly hyperactivated the nymphs but only at a concentration of 3,900 μg/cm^2 (Fisher's LSD test, $P = 0.005$). Compared to acetone alone, the positive control tetramethrin produced a significant hyperactivation at a concentration of 3.9 μg/cm^2 (Fisher's LSD test, $P < 0.001$). Although the highest concentrations of the remaining monoterpenes produced a slight increase in locomotor activity, it was not significantly different from the positive controls (ANOVA; $P > 0.05$ in all cases).

Flushing Out Under Laboratory Conditions

Figure 3 and Table 1 show the results of the laboratory flushing out bioassays. No flushing out was observed with the negative control acetone. During the first 10 min following the application of

tetramethrin, linalool, or menthol, the nymphs left the shelter at an approximately constant rate. This effect continued during the next 10 min, although at a slower rate. Finally, between 21 and 30 min after application, the percentage of insects flushed out by these monoterpenes hardly varied.

1,8-Cineole, α-terpineol, and thymol produced very weak flushing out, most of it during the first 10 min after application and never higher than 10%. After this initial period of time, flushing out by these compounds remained mostly unchanged during the rest of the assay.

A very different profile of flushing out was observed after application of eugenol: it caused rapid initial flushing out (an average of 30.0% in <30 s), after which almost no insects left the refuge.

The FT_{50} values for linalool and menthol (1.5 g/m^3) were 16.67 and 42.98 min, respectively (Table 1). The effect of linalool was greater than that of menthol, but there was no significant difference between their values of FT_{50} (based on nonoverlapping of 95% CL; $P > 0.05$). The FT_{50} for the positive control tetramethrin (0.006 g/m^3) was 8.29 min. Compared to tetramethrin, both monoterpenes only produced a weak flushing out, and much higher concentrations were needed to obtain a similar effect to the pyrethroid.

Taking these results into account, we studied the flushing out effect of different mixtures of linalool and eugenol. Only the combination linalool:eugenol 2:1 produced >50% flushing out and allowed calculating the value of FT_{50} (40.73 min).

Based on these results, we decided to carry out assays under semifield conditions using linalool and eugenol alone as well as a mixture linalool:eugenol 2:1.

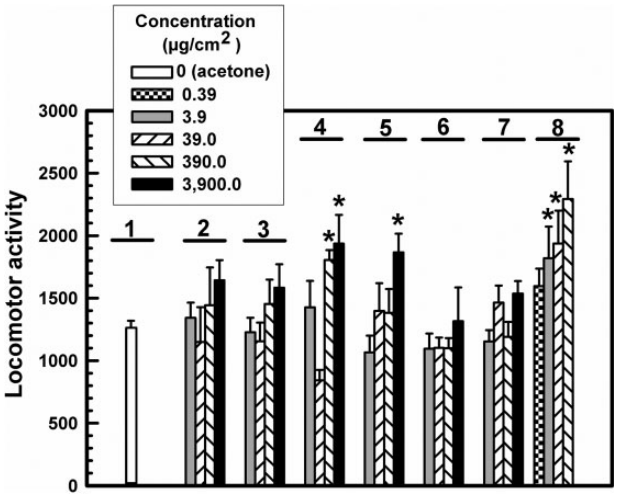

Fig. 2. Locomotor activity of fifth-instar nymphs of *T. infestans* exposed to monoterpenes. 1, Acetone alone (negative control); 2, 1,8-cineole; 3, eugenol; 4, linalool; 5, menthol; 6, α-terpineol; 7, thymol; 8, tetramethrin (positive control). Locomotor activity = M/A, where M is the nymph's movement (expressed in pixels) and A is the area occupied by the nymph (expressed in pixels). Each bar represents the mean of six independent replicates. Vertical lines are SE. Inside each group, bars marked with an asterisk are significant different from the respective control (Fisher's test, $P > 0.05$).

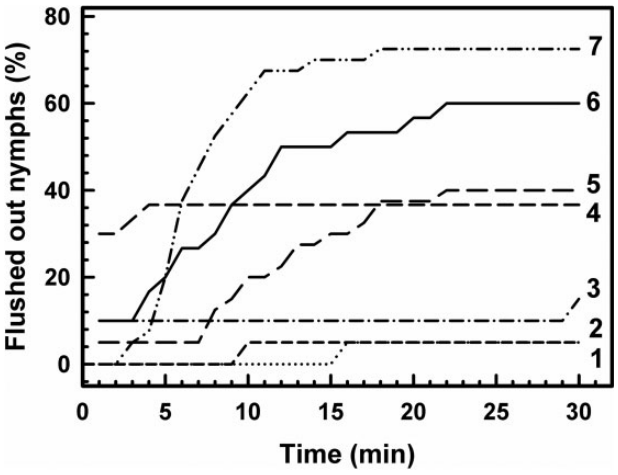

Fig. 3. Flushing out by monoterpenes on fifth-instar nymphs of *T. infestans* in laboratory conditions. 1, α-terpineol; 2, 1,8-cineole; 3, thymol; 4, eugenol; 5, menthol; 6, linalool; 7, tetramethrin (positive control). Each line represents the mean of three or four independent replicates.

Flushing Out Under Semifield Conditions

In general terms, the results of the semifield assays (Fig. 4) reproduced the flushing out pattern observed at the laboratory (Table 2). No nymphs left the refuges in treatments using only acetone, so this negative control was not included neither in Fig. 4 nor in the ANOVA. The differences between the remaining treatments were significant (ANOVA; $F = 4.488$, df = 3, 12, $P = 0.025$). Tetramethrin produced the best result (39.4% flushing out). The effects of linalool and eugenol pure or mixed were similar, showing not significant differences (Fisher's LSD test; linalool vs. eugenol: $P = 0.951$; linalool vs. mixture: $P = 0.501$; eugenol vs. mixture: $P = 0.462$). Compared to tetramethrin, they showed a significantly lower effect even though they were applied at a much higher concentration (Fisher's LSD test; linalool vs. tetramethrin: $P = 0.008$; eugenol vs. tetramethrin: $P = 0.007$; mixture vs. tetramethrin: $P = 0.019$).

Discussion

Flushing out activity on *T. infestans* and *R. prolixus* has been reported for several pyrethroids (Wood et al. 1993) and for compounds delivered by the Brindley's gland of triatomines (Minoli et al. 2013). In the present work, the flushing out effect of monoterpenes on insects was evaluated for the first time. Flushing out activity was observed when using pure linalool and eugenol or a mixture of both (2:1), although this effect was lower than that of tetramethrin.

Locomotor activity is a complex behavioral trait implicated directly or indirectly in almost all the insects' activities and regulated

Table 1. Flushing out time 50% for monoterpenes on fifth-instar nymphs of *T. infestans* in laboratory conditions

Treatment	Concentration (g/m^3)	*n*	Slope (SE)	FT_{50} (95% CL) (min)
Linalool	1.5	30	1.47 (0.38)	16.67ab (8.63–39.98)
Menthol	1.5	40	1.33 (0.24)	42.98a (23.97–87.09)
Linalool:eugenol (2:1)	1.5	30	1.02 (0.3)	40.73a (21.65–87.84)
Tetramethrin	0.006	40	3.23 (0.58)	8.29b (6.48–10.9)

n, number of nymphs assayed. FT_{50}: Time required to flushing out 50% of the nymphs. 95% CL, 95% confidence limits. In the last column, values followed by different letters are significantly different (based on nonoverlapping of their confidence limits, $P < 0.05$).

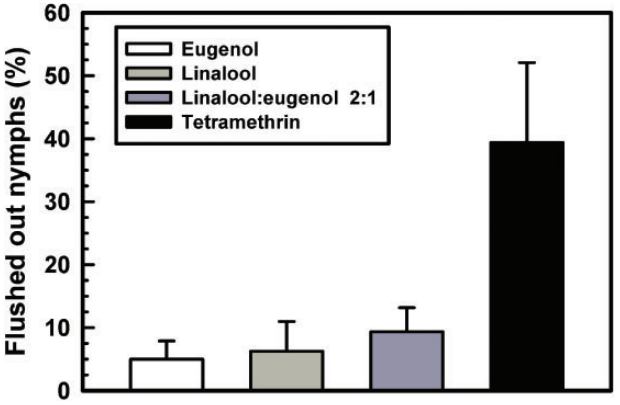

Fig. 4. Flushing out by monoterpenes on nymphs of *T. infestans* in semifield conditions. Each bar represents the mean of three or four independent replicates. Vertical lines are SE. Bars marked with different letters are significantly different (Fisher's test, $P > 0.05$).

Table 2. Summary of the flushing out effect on *T. infestans* produced of linalool and eugenol pure and mixed (2:1) in laboratory and semifield conditions

Flushing out[a] (%)		
Treatment	Laboratory conditions	Semifield conditions
Acetone (negative control)	0	0
Linalool	60.0a	6.25a
Eugenol	36.7a	5.0a
Linalool:eugenol (2:1)	44.0a	9.3a
Tetramethrin (positive control)	72.5b	39.4b

[a] Percentage (%) of insects flushed out at 30 min after the application of the compounds. In each column, values followed by different letters are significantly different (Fisher's LSD test, $P < 0.05$).

by neurophysiological mechanisms (Ridgel and Ritzmann 2005). It is not easy to define per se and is often confused with locomotion (basic neural oscillations controlling reflex behavior and walking activity; Martin 2004). Locomotor hyperactivation is recognized as the first symptom of intoxication with pyrethroids in insects (Gammon 1978, Miller and Adams 1982, Alzogaray et al. 1997). Hyperactivity was also reported in first-instar triatomines exposed to monoterpenes (Moretti et al. 2013, 2014). In contrast with attraction and repellence, hyperactivation is nondirectional. When a hyperactivant compound is applied to insects located in hiding places, they leave their refuges randomly, making it easier to estimate their presence and abundance. This phenomenon is called flushing out and is used for detecting domestic infestation by triatomine bugs in rural houses (Pinchin et al. 1980, Gualtieri et al.1985).

Of the six monoterpenes studied in the present work, linalool was the greatest hyperactivant followed by menthol. The remaining monoterpenes produced only a slight effect on locomotor activity that was not significantly different from the negative controls. Confirming that flushing out is a consequence of hyperactivation, linalool produced the highest flushing out effect, followed by menthol. On the other hand, the flushing out capacity of the other monoterpenes was very weak.

Pyrethroid insecticides alter the function of voltage-gated sodium channels (Casida and Durkin 2013). In insects, the exposure to these insecticides induces a rapid increase in locomotor activity (Gammon 1978, Alzogaray et al. 1997, Alzogaray and Zerba 2001a, 2001b). *Triatoma infestans* nymphs resistant to the knock-down effect of pyrethroids were also resistant to hyperactivation, confirming that an increase in locomotor activity is part of the characteristic sequence of toxicological symptoms of these insects instead of a behavioral response to a sensory stimulus (Sfara et al. 2006).

Some monoterpenes also produce hyperactivity in insects. Menthyl acetate increased locomotor activity in first-instar nymphs of *Blattella germanica* (Alzogaray et al. 2013), whereas carvacrol, (-)-carveol, geraniol, and other alcohol monoterpenes caused hyperactivation in first-instar nymphs of *R. prolixus* and *T. infestans* (Moretti et al. 2013). The primary site of action of most members of this chemical family is still unknown, but there are evidences that some of them are neurotoxic. It has been suggested that the octopamine receptor could be the site of action for eugenol and terpineol (Enan 2001), and the tyramine receptor for thymol (Enan 2005). The neurotoxic effect of these monoterpenes might be causing the locomotor activity response in the insects. The differences observed between the magnitude of the effects of the six monoterpenes studied here could be due to differences in the toxicokinetic and toxicodynamic processes involved in the insect's organism.

Pyrethroid-resistant triatomines are found in a large region of Argentina and Bolivia (Vassena et al. 2000, Picollo et al. 2005, Roca-Acevedo et al. 2013). It is thus necessary to identify alternative hyperactive agents to detect their presence in rural homes. These agents are useful for determining whether an insecticide treatment must be applied, for assessing the effectivity of the treatments and establishing the presence of reinfestation. In the present study the flushing out pattern of tetramethrin, linalool, eugenol, and a mixture linalool:eugenol 2:1 was similar under both laboratory and semifield conditions. The effect of the monoterpenes was lower in the semifield conditions, but this could be due to the differences between the two methods used. Although this factor was not quantified, it is evident that the devices used in each case to nebulize the solutions generated drops with different sizes. This could produce an important qualitative and quantitative difference in the way the solutions reach the insects. This variable could be improved, for example, by applying the substances with an aerosol that would reduce the size of the drop considerably, increasing the reach of the hyperactivants in refuges and the respiratory system of the insects.

Contrary to the other monoterpenes assessed in this study that had a much slower activity, eugenol flushed out an important number of nymphs in <30 s. Identifying the cause of this phenomenon could help find a better way to exploit the flushing out effect produced by this monoterpene.

Compared to tetramethrin, pure linalool and its 2:1 mixture with eugenol produced low flushing out. While interpreting these results, it is important to bear in mind that tetramethrin belongs to the pyrethroid family, which are the most effective insecticides known to this day. Until alternative insecticides as toxic as they be identified, we probably won't find flushing out agents that are as effective either.

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