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ORIGINAL ARTICLE

Insecticidal action of synthetic girsensohnine analogues and essential oils on *Rhodnius prolixus* (Hemiptera: Reduviidae)

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Introduction: The alkaloid girsensohnine has been used as a natural model in the synthesis of new alkaloid-like alpha-aminonitriles with insecticidal effect against disease vectors.

Objective: To evaluate the biocide activity of girsensohnine analogues and essential oils of *Cymbopogon flexuosus*, *Citrus sinensis* and *Eucalyptus citriodora* in stage I and stage V *Rhodnius prolixus* nymphs.

Materials and methods: We used a topical application model in tergites and sternites, as well as exposure to treated surfaces with different exploratory doses of each of the molecules and essential oils to determine the lethal doses (LD₅₀ and LD₉₅).

Results: Analogue 3 showed the highest insecticidal activity with 83.3±16.7% of mortality when applied on tergites, 38.9±4.8% on sternites and 16.7±0% on treated surfaces in stage I nymphs at 72 hours (h) and 500 mg.L⁻¹. In stage V nymphs, the compounds induced mortality only in sternites (11.1±9.6% for analogue 6 and 5.5±4.7% for analogues 3 and 7 at 72 h and 1500 mg.L⁻¹). The lethal doses for molecule 3 on tergites in stage I nymphs were LD₅₀ 225.60 mg.L⁻¹ and LD₉₅ 955.90 mg.L⁻¹. The insecticidal effect of essential oils was observed only in stage I nymphs, with 11.1±4.8% for *C. flexuosus* when applied in sternites, while using exposure to surfaces treated it was 5.6±4.8% for *C. sinensis* applied on tergites and 8.3±0% on sternites at 72 h and 1000 mg.L⁻¹.

Conclusion: Synthetic girsensohnine analogues, and *C. flexuosus* and *C. sinensis* essential oils showed insecticidal activity in *R. prolixus*. Analogue 3 showed the greatest insecticidal activity among all molecules and oils evaluated under our laboratory conditions.

Key words: Triatominae; Chagas disease; insecticides; oils, volatile.

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Acción insecticida de análogos sintéticos de girsensohnina y de aceites esenciales sobre *Rhodnius prolixus* (Hemiptera: Reduviidae)

Introducción. El alcaloide natural girsensohnina se ha usado como modelo en la síntesis de nuevos análogos de alcaloidales alfa-aminonitrílicos con efecto insecticida en vectores de enfermedades.

Objetivo. Evaluar la actividad biocida de análogos de girsensohnina y de aceites esenciales de las plantas *Cymbopogon flexuosus*, *Citrus sinensis* y *Eucalyptus citriodora* en ninfas de estadios I y V de *Rhodnius prolixus*.

Materiales y métodos. Se empleó la aplicación tópica en terguitos, esternitos y superficies tratadas con diferentes dosis exploratorias de cada una de las moléculas y aceites esenciales para determinar las dosis letales (LD₅₀ y LD₉₅).

Resultados. El análogo 3 tuvo la mayor actividad insecticida, con una mortalidad de 83,3±16,7% en los terguitos, de 38,9±4,8 % en los esternitos y de 16,7±0 % a las 72 horas en ninfas de estadio I expuestas a superficies tratadas y 500 mg.L⁻¹. En las ninfas de estadio V solo se presentó mortalidad en los esternitos (11,1±9,6 % con el análogo 6 y 5,5±4,7 % con los análogos 3 y 7 a las 72 h y 1.500 mg.L⁻¹). Las dosis letales para la molécula 3 en los terguitos de ninfas de estadio I fueron las siguientes: DL₅₀, 225,60 mg.L⁻¹ y DL₉₅, 955,90 mg.L⁻¹. En cuanto a los aceites esenciales, el efecto insecticida solo se presentó con *C. flexuosus* (11,1±4,8%) en los esternitos de ninfas de estadio I expuestas a superficies tratadas; con *C. sinensis* (5,6±4,8%) en los terguitos y en los esternitos (8,3±0%) a las 72 horas y 1.000 mg.L⁻¹.

Author's contributions:

Vladimir V. Kouznetsov designed and synthesized the girsensohnine analogues.

Juliana Cuadros-Martínez carried out laboratory procedures, performed analyses and interpreted the data.

Jonny E. Duque performed analyses and interpreted the data.

Aurora L. Carreño participated in all stages of the research.

All authors participated in the writing of the paper.

Conclusión. Los análogos sintéticos del alcaloide girsenosina y los aceites esenciales de *C. flexuosus* y *C. sinensis* exhibieron actividad insecticida en *R. prolixus*. El análogo 3 exhibió la mayor actividad insecticida de todas las moléculas evaluadas bajo las condiciones de laboratorio.

Palabras clave: Triatominae; enfermedad de Chagas; insecticidas; aceites volátiles.

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According to the World Health Organization (WHO), Chagas disease or American trypanosomiasis, caused by the parasite *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae), affects the population in 21 Latin American countries. In places where this disease is endemic, the main mode of transmission is through contact with the feces of infected blood-sucking reduviid bugs (Hemiptera: Reduviidae: Triatominae) (1-3) when the affected person rubs and spreads them to the eyes, the mouth or an open skin lesion (4). At present, there is no vaccine against the etiological agent of the disease and existing drugs are only partially effective with severe side effects (4,5).

There are approximately 151 described species of triatomines grouped into 15 genera and five tribes (6). The most important domiciled species involved in the transmission of *T. cruzi* are: *Triatoma infestans* (Klug, 1834) (in the Southern Cone countries), *Rhodnius prolixus* Stål, 1859 (in Central America and northern South America), and *Triatoma dimidiata* Latreille, 1811, Phyllosoma complex, (7,8) (throughout the southern, central and eastern areas of the Gulf of México extending to Ecuador and northern Perú).

The estimated prevalence of trypanosomiasis in Colombia ranges from 700,000 to 1,200,000 inhabitants infected and 8 million at risk of acquiring the infection (9). The main vectors adapted to human habitations are, in order of importance, *R. prolixus* in the Andes, Caribbean, Orinoco and Amazon regions; *T. dimidiata* in the Andes, Caribbean and Orinoco regions; *T. venosa* (Stål, 1872) in the Andes and Orinoco regions; *T. maculata* (Erichson, 1878) in the Andes, Caribbean and Orinoco regions (10), and *Pastronygylus geniculatus* (Latreille, 1811) in the Andes, Caribbean, Orinoco and Amazon regions (11-13). The department of Santander is one of the main endemic zones in Colombia.

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The most effective method to prevent Chagas disease in Latin America is vector control (10) through conventional use of insecticides such as organochlorines, organophosphates, carbamates and pyrethroids (2). It is urgent to find natural active ingredients or new alkaloid-like molecules to replace these insecticides (14,15), as their constant use has generated resistance mechanisms in some Triatomine species of epidemiological importance (2). Among these new molecules, synthetic analogues of the girsenosine alkaloid structure were designed and synthesized to inhibit the activity of acetylcholinesterase (AChE).

According to Carreño, *et al.* (16), these analogues, derivatives of alpha-aminonitriles, showed insecticidal activity on larvae of *Aedes aegypti*, the vector that transmits dengue disease, and the authors suggest this is a strong indication of its possible effect on other insects of medical interest, such as triatomines. Essential oils are another potential tool for insect control, due to their selectivity and minimal environmental effects and the fact that, in some cases, their action has been effective against insects that are resistant to commercial insecticides (14).

In this context, our objective was to evaluate the insecticidal activity of synthetic analogues of the girsenosine alkaloid and of essential oils from *C. flexuosus*, *C. sinensis* and *E. citriodora* against stage I and V *R. prolixus* nymphs.

Materials and methods

Biological material

We used the reference strain of *R. prolixus* CINTROP-UIS. Adults were fed every eight days with blood of hens (*Gallus gallus*). For the bioassays we used stage I nymphs of 24 to 36 h of age and 0.2-0.3 mg of weight subjected to fasting since hatching, and stage V nymphs with 20-30 mg of weight and eight days of fasting.

Synthetic analogues and essential oils

We used the girsenosine alkaloid as a natural model in the design and synthesis of 12 new synthetic analogues (figure 1). A Strecker reaction modified from commercial and inexpensive substitute benzaldehydes (3,4-dimethoxybenzaldehyde,

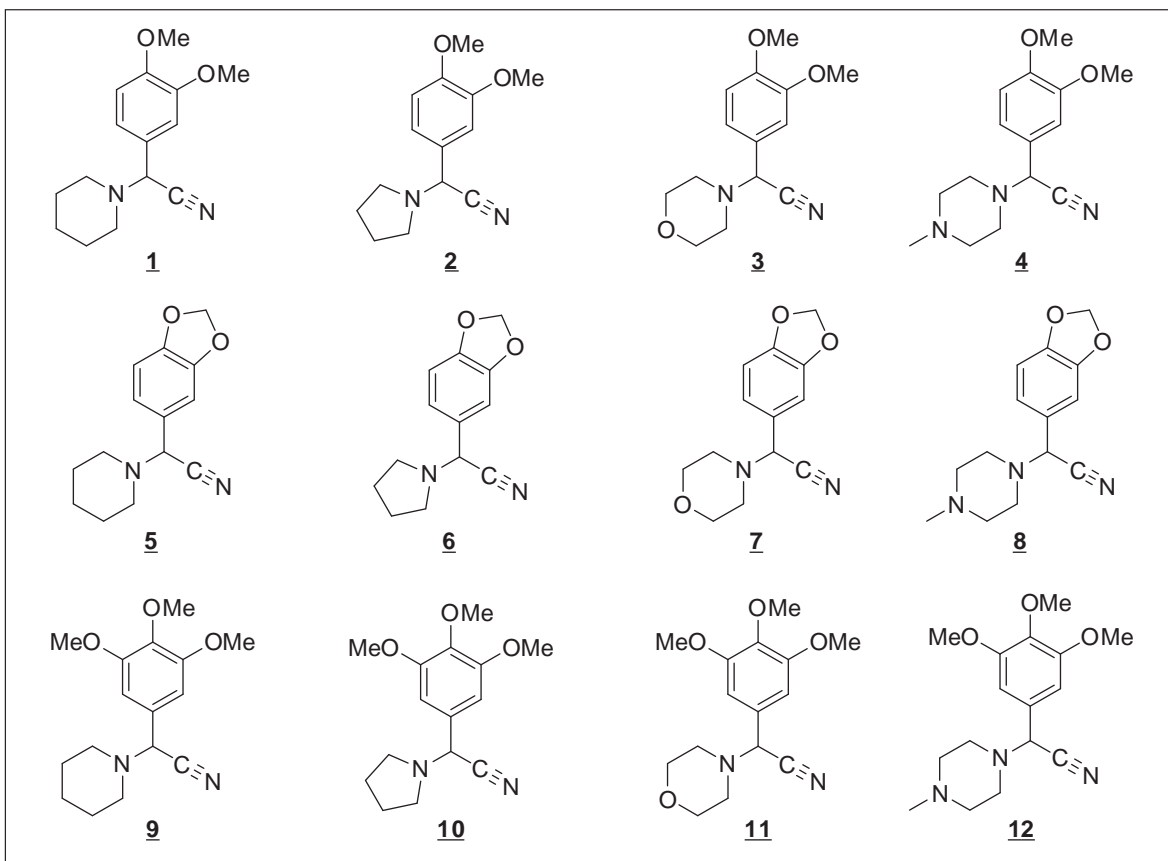


Figure 1. Synthetic alpha-aminonitriles evaluated

piperonal and 3,4,5-trimethoxybenzaldehyde), four cyclic secondary amines (piperidine, pyrrolidine, morpholine and N-methylpiperazine), and KCN and acetone cyanohydrin were employed.

In general, all molecules were prepared with moderate to excellent yields presenting well-defined melting points, and their structural elucidation was made using diverse spectroscopic techniques (FT-IR, GC-EM, ^1H NMR and ^{13}C NMR) (16). As positive controls of the analogues we used technical grade insecticides: Deltamethrin (99.5%-ChemService), pyrethroid used to control *Rhodnius prolixus* vectors in rural and peri-urban areas in Santander, and fenitrothion (98.0%-Dr. Ehrenstorfer), organophosphate used for vector control in Latin America. The essential oils of *C. flexuosus* (Poaceae) with geranial (37.5%), neral (28.2%) and geranyl acetate (10.0%) components; of *C. sinensis* (Rutaceae) with limonene (71.3%), linalol (5.4%), and beta-myrcene (5.0%), and of *E. citriodora* (Myrtaceae) with citronellal (49.3%), citronellol (13.0%), and isopulegol (12.9%) were donated by CENIVAM-UIS (17). All plants were

collected in Santander. Following the protocol by Stashenko, *et al.* (18), we extracted and analyzed essential oils using microwave assisted hydrodistillation.

Exploratory doses

The exploratory doses used to evaluate each of the molecules were the following: for stage I nymphs, 25, 125, 500 $\text{mg}\cdot\text{L}^{-1}$, and for stage V nymphs, 800, 1,000, 1,500 $\text{mg}\cdot\text{L}^{-1}$; essential oils doses for stage I nymphs were 30, 300, 1,000 $\text{mg}\cdot\text{L}^{-1}$, and for stage V nymphs, 2,000, 2,800, 3,500 $\text{mg}\cdot\text{L}^{-1}$, which were used for the negative controls with acetone as solvent in all dilutions. The doses for the positive controls were deltamethrin (0.3, 0.7 and 500 $\text{mg}\cdot\text{L}^{-1}$) and fenitrothion (0.09, 0.1 and 500 $\text{mg}\cdot\text{L}^{-1}$) for stage I nymphs, and deltamethrin (1.0, 3.0 and 800 $\text{mg}\cdot\text{L}^{-1}$) and fenitrothion (0.45, 0.5 and 800 $\text{mg}\cdot\text{L}^{-1}$) for stage V nymphs.

Topical application

We used a modified protocol (19) where nymphs were treated topically with the 12 synthetic girsogenine analogues, the three essential oils and the

two commercial insecticides diluted in acetone and applied with a Hamilton 5 and 25 μl microsyringe fitted with a repetitive discharger. We applied the treatments on tergites and sternites of each stage I and stage V nymphs by applying 0.1 and 0.5 μl of the experimental dose, respectively. We designed the experiments for the bioassay with arthropods (20). We repeated all doses four times with 12 insects per replicate, while the complete bioassay was replicated on three different days. After treatment, we placed the nymphs in 1/2 oz. plastic cups with folded paper inside and covered with perforated lids that would allow the nymphs to breathe. Mortality was recorded 2, 12, 24, 48 and 72 h following treatment.

Exposure to treated surfaces

The experiments followed the protocol (19) with modifications. We used the experimental doses in 6 cm Petri dishes provided with filter disks (Munktell) and we impregnated their surfaces evenly with a micropipette following a spiral pattern from the center toward the outside. A volume of 437 μl of the solution was used, calculated according to the protocol. After drying the impregnated surfaces, 12 nymphs were exposed per dose, i. e., 48 nymphs per bioassay, covering the Petri dishes with perforated cling wrap. Mortality was recorded 0.5, 1, 2, 12, 24, 48 and 72 h following treatment.

Lethal dose bioassays

We chose the molecule with the highest percentage of insecticidal activity from among all the molecules evaluated (synthetic analogues and essential oils) in stage I and stage V *R. prolixus* nymphs. Based on these results, we conducted bioassays with multiple asymmetrical doses whose dose-response mortality results were used to determine the LD_{50} and LD_{95} values.

Statistical analysis

We evaluated dose-response mortality data from each bioassay using the Kolmogorov-Smirnov test; the normality value indicated a non-normal distribution of the data. We analyzed the results using Kruskal-Wallis (KW) and Mann-Whitney (MW) non-parametric tests, with p values <0.05 considered as significant; we used multiple-comparisons tests when data were significantly different. For the molecule with the highest mortality rate, probit analyses were conducted to determine the statistical parameters, lethal doses (LD_{50} and LD_{95}) and their respective confidence intervals. The statistical programs used were Statistics, v11, and Probit (21,22).

Ethics statement

The study was approved by the Ethics Committee of CEINCI-UIS, minute N° 12 of May 23, 2014.

Results

The synthesized analogues and essential oils induced differentiated mortality in nymph stages at 72 h in all experiments.

Evaluation of girsensohnine analogues in stage I *R. prolixus* nymphs

We observed insecticidal activity in 11 analogues when we used the topical application on tergites, in 12 on sternums and in six on treated surfaces, with molecule 3 showing the highest mortality rate (figure 2). Among the analogues evaluated, the mortality in tergites was significantly different (Kruskal-Wallis: H (14 N=45)=36.51205 p=0.0009) (figure 2a). The topical application on sternites resulted in significantly low mortality (Kruskal-Wallis: H (14 N=45)=33.98065 p=0.0021) compared to tergites (table 1). The same effect was observed on treated surfaces (Kruskal-Wallis: H (14 N=45)=41.89138 p=0.0001) (figure 2c). As expected, positive (delta-methrin and fenitrothion) and negative controls (acetone) had 100% and 0% mortality rates, respectively (figure 2). When comparing the mortality rates in the two types of treatment, tergites and sternites, there were significant differences (Mann-Whitney, MW, $p<0.05$) in synthetic analogues 3, 5 and 12. When comparing the three types of treatment, MW $p<0.05$ differences were observed in six synthetic molecules (2, 3, 5, 6, 11 and 12), and molecule 3 showed the highest mortality rate in each of the treatments (table 1), with LD_{50} 225.60 mg.L^{-1} and LD_{95} 955.90 mg.L^{-1} in topical application on tergites at 72 h and 500 mg.L^{-1} (table 2).

Evaluation of girsensohnine analogues in stage V *R. prolixus* nymphs

Mortality in stage V nymphs at 72 h and 1,500 mg.L^{-1} was low and only evidenced in sternites with $11.1\pm 9.6\%$ for analogue 6 and $5.5\pm 4.7\%$ for analogues 3 and 7, registering significantly different action (Kruskal-Wallis: H (14 N=45)=36.64743 p=0.0008) (figure 3). There was no mortality with the topical application on tergites and surfaces treated with the 12 girsensohnine synthetic analogues.

Evaluation of essential oils in stage I and V *R. prolixus* nymphs

We observed insecticidal activity in stage I nymphs with *C. flexuosus* ($11.1\pm 4.8\%$) on sternites, and exposed to treated surfaces at 72 h and 1,000 mg.L^{-1} .

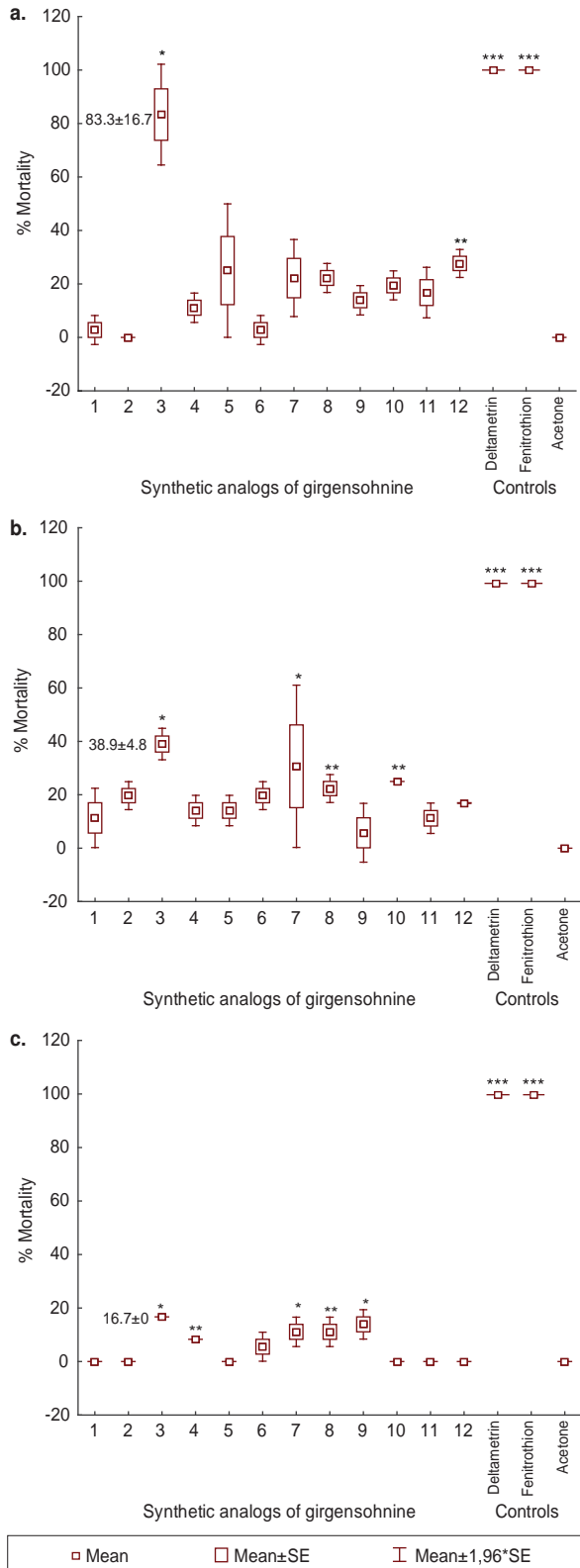


Figure 2. Insecticidal activity of each of the 12 synthetic analogues at 72 h and 500 mg.L⁻¹ in stage I *Rhodnius prolixus* nymphs. **a.** Topical application on tergites. **b.** Topical application on sternites. **c.** Exposure to treated surfaces. *: Statistically significant values

However, with *C. sinensis* at the same dose, low mortality was evidenced in tergites (5.6±4.8%) and sternites (8.3±0%). For stage V nymphs the mortality was low (8.3±0%) and it was observed only with *C. sinensis* in tergites. On the other hand, we registered no mortality with the topical application of *C. sinensis* and *E. citriodora* on sternites nor with the exposure to treated surfaces (table 1).

Discussion

Girgensohnine analogues

Synthetic analogues are hydrophobic molecules that, when applied topically, can easily cross the exoskeleton of stage I nymphs, a property that may be responsible for the insecticidal activity observed both in tergites and sternites. Hydrophobic substances penetrate the insect cuticle more easily than hydrophilic substances because they are mostly made of lipids and waxes (23-25), which are more related to the substances evaluated in our study.

However, other intrinsic and extrinsic factors (size, age, nutritional state, humidity and temperature) (26-28) can change the evaluation of the molecules. Another fact to consider is cuticle sclerotization, as nymphs of 24 to 36 hours of age have semi-sclerotized cuticle and, thus, higher susceptibility (28). As other authors have mentioned, differences in cuticle thickness (29) in stage I and V nymphs can affect the susceptibility to external stimuli; for instance, Reyes, *et al.* (30), observed that the pyrethroid insecticides (beta-cypermethrin and fenitrothion) were more effective in stage I *T. dimidiata* and *T. maculata* nymphs than in stage V ones. Cáceres, *et al.* (31), found that stage I *R. pallenscens* nymphs were sensitive to deltamethrin and lambda-cyhalothrin in different geographical zones. In another study, Germano found similar results (23) in toxicity bioassays with deltamethrin in different stages of development in *T. infestans*.

Insecticides penetrate insects until they reach their target in three ways: through the cuticle, by ingestion and by inhalation (26,32). Penetration occurs through the cuticle via articular and inter-segmental membranes, and through spiracles. We found the highest insecticidal activity for analogue 3 when it was evaluated on tergites of stage I nymphs, suggesting that penetration occurs through the thinner cuticle of this stage in dorsal, ventral and lateral positions (28,32). On stage V nymphs molecules penetration was observed only in sternites, with lower insecticide activity given cuticle characteristics in this nymphal stage.

Table 1. Mortality caused by the 12 synthetic analogues and three essential oils applied using three types of treatment against stage I *Rhodnius prolixus* nymphs at 72 h and 500 mg.L⁻¹

Molecules	Tergite mortality (% ± SD)	Sternite mortality (% ± SD)	Mann-Whitney probability test (p<0.05)	Mortality from exposure to treated surfaces (% ± SD)	Kruskal-Wallis probability test (p<0.05)
1	2.8 ± 4.8	11.1 ± 9.6	0.238594	0	0.195
2	0	19.4 ± 4.8	1.000000	0	0.0457*
3	83.3 ± 16.7	38.9 ± 4.8	0.046302*	16.7 ± 0	0.0234*
4	11.1 ± 4.8	13.9 ± 4.8	0.238594	8.3 ± 0	0.195
5	25 ± 22	13.9 ± 4.8	0.046302*	0	0.0234*
6	2.8 ± 4.8	19.4 ± 4.8	1.000000	5.6 ± 4.8	0.0498*
7	22.2 ± 12.7	30.6 ± 26.7	0.512691	11.1 ± 4.8	0.5501
8	22.2 ± 4.8	22.2 ± 4.8	0.796254	11.1 ± 4.8	0.067
9	13.9 ± 4.8	8.3 ± 8.3	0.653095	13.9 ± 4.8	0.8599
10	19.4 ± 4.8	25 ± 0	0.113847	0	0.0289*
11	16.7 ± 8.3	11.1 ± 4.8	0.345779	0	0.0446*
12	27.8 ± 4.8	16.7 ± 0	0.043115*	0	0.0226*
<i>C. flexuosus</i>	0	11.1 ± 4.8	--	11.1 ± 4.8	0.0457*
<i>C. sinensis</i>	5.6 ± 4.8	8.3 ± 0	--	0	0.0608
<i>E. citriodora</i>	0	0	--	0	--
Acetone	0	0	--	0	--

% ± SD: Mortality rate and deviation; *: Statistically significant value

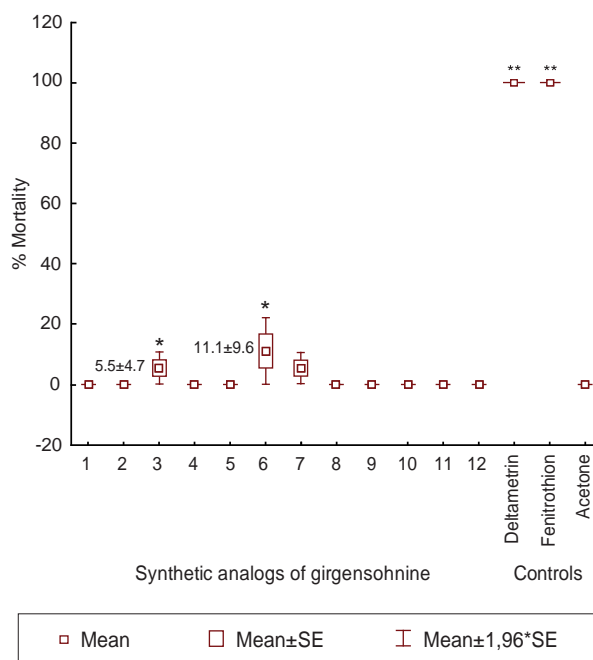
Table 2. Lethal doses LD₅₀ and LD₉₅ (mg.L⁻¹) of the molecule with the highest insecticidal activity (molecule 3) at 72 h in stage I *Rhodnius prolixus* nymphs and positive controls

Topical application on tergites	LD ₅₀	LD ₉₅	χ ²
Alpha-aminonitriles	225.60 (194.38-257.97)	955.9 (729.31-1443.64)	1.88
Deltamethrin*	0.001* (4 × 10 ⁻⁴ - 5 × 10 ⁻³)	0.01* (2 × 10 ⁻² - 4 × 10 ⁻²)	
Fenitrothion*	24* (20 - 36)	110* (100 - 260)	

Confidence intervals of 95% (in parentheses)

LD₅₀: Lethal dose causing mortality to 50% of the nymphs exposed to treatment; LD₉₅: Lethal dose causing mortality to 95% of the nymphs exposed to treatment; * χ²: Chi square; LD₅₀ and LD₉₅ of deltamethrin and fenitrothion used in the study conducted by Vivas, *et al.*

Synthetic analogues are non-volatile compounds that, when dissolved in a volatile solvent such as acetone, allow the formation of crystals, leaving residues in porous substrates such as filter paper. In some field studies (33-35), authors have explained the lack of persistence of substances on some porous surfaces by the fact that these surfaces retain particles, thus reducing their availability and insect-substance interaction. This may have occurred in the experiment with the Petri dishes in our study, as the insecticidal activity of the synthetic analogues on the exposed surfaces resulted in lower mortality rates. In this sense, Stampini, *et al.* (36), have suggested increasing the dose of the active ingredient when used on porous surfaces.

**Figure 3.** Insecticidal activity of each of the 12 synthetic analogues at 72 h and 1500 mg.L⁻¹ in stage V *Rhodnius prolixus* nymphs using topical application on sternites

*: Statistically significant values

Another factor that influenced the bioactivity of the synthetic analogues was the behavior observed in stage I nymphs: they moved toward the end of the paper, where they remained throughout the bioassay, which could have decreased their interaction with the molecules.

Although the insecticidal activity of synthetic alkaloids of girsensohnine on triatomines has not yet been reported in literature, Carreño, *et al.* (16), reported the insecticidal activity of these analogues against *Aedes aegypti*, one of dengue vectors, with analogue 1 showing the highest insecticidal activity on larvae at stages III and IV. In our study, the highest insecticidal activity and mortality rates were observed for analogue 3. The LD₅₀ and LD₉₅ of the best molecule were high in comparison with those observed by Vivas, *et al.* (37), with deltamethrin and fenitrothion in susceptible strains of *R. prolixus* from Venezuela (table 2).

Essential oils

Low insecticidal activity of essential oils on tergites and sternites was probably due to the volatility of some of their components, which, therefore, were washed away easily by acetone in its process of evaporation following topical application, thus diminishing their bioactivity. This was also the case in the assay with treated surfaces, in which the concentration of the active compounds in the Petri dish decreased.

Some authors have compared the insecticidal activity of the topical application with the exposure to surfaces impregnated with different essential oils, and they have reported low activity with the two methods. Fournet, *et al.* (38), found *Minthostachys andina* and *Hedomeam andonianum* (Lamiaceae) essential oils showed low or null insecticidal activity at 72 h in stage I and V *R. neglectus* and *T. infestans* nymphs after topical application and exposure to treated surfaces.

According to Sfara, *et al.* (14), there was low insecticidal activity in exposure to surfaces treated with five essential oils and seven monoterpenes in stage I *R. prolixus* nymphs as compared with the organophosphate insecticide used as positive control. Although the essential oils evaluated in stage I nymphs showed low insecticidal activity by contact (topical application on sternites and tergites) and exposure to surfaces (fumigant effect), when ingested mortality was much higher, as has been observed in other insects. According to Hincapié, *et al.* (39), the extracts of *Annona miricata* (Anonaceae) seeds on *Sitophilus zeamais* (Coleoptera: Curculionidae) are more effective when ingested than by contact. Ringuelet, *et al.* (40), observed that *Lippia alba* (Verbenaceae) essential oil showed greater insecticidal activity on *Tribolium castaneum* (Coleoptera: Tenebrionidae) when applied on the grain, than when using the impregnation method on filter paper.

However, some authors have reported sublethal effects of essential oils on triatomines. For instance, Lutz, *et al.* (41), found repellent activity of some monoterpenes, methyl acetate and geraniol components of essential oils in stage V *R. prolixus* nymphs. The same results were reported by Zamora, *et al.* (42), as well as an anti-feeding effect caused by the main components of *Cymbopogon winterianus* (Poaceae) essential oil on three endemic species of triatomines in Arizona.

In summary, analogues of the girsensohnine alkaloid synthesized using a modified Strecker reaction showed insecticidal activity in *R. prolixus* nymphs. The analogue that showed the greatest insecticidal activity under laboratory conditions was molecule 3, with LD₅₀ 225.60 mg.L⁻¹ and LD₉₅ 955.90 mg.L⁻¹ in stage I nymph tergites. We observed low or no insecticidal activity in stage V nymphs with the molecules evaluated. *Cymbopogon flexuosus* and *C. sinensis* essential oils insecticidal effect was observed only in stage I nymphs, but with low activity.

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Conflicts of interest

None declared.

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