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Insecticidal activity of three essential oils against two new important soybean pests: *Sternechus pinguis* (Fabricius) and *Rhyssomatus subtilis* Fiedler (Coleoptera: Curculionidae)

[Actividad insecticida de tres aceites esenciales contra dos nuevas plagas importantes de la soja: *Sternechus pinguis* (Fabricius) y *Rhyssomatus subtilis* Fiedler (Coleoptera: Curculionidae)]

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Abstract

In the present study, the essential oils from *Eucalyptus globulus* Labill., *Citrus sinensis* L. Osbeck and *Citrus limon* L. were analysed by GC-MS (gas chromatography–mass spectrometry) and evaluated for their toxic effects on *Sternechus subsignatus* and *Rhyssomatus subtilis*, two important pest of soybean in South America. Contact toxicity assayed by impregnation on filter paper discs showed that these oils caused significant mortality of the test insects (100% of mortality at 5 $\mu\text{L}/\text{cm}^2$). Eucalyptus oil (98.9 % of 1,8-cineole) had the greatest contact toxicity ($\text{LD}_{50} = 0.40$ and $0.84 \mu\text{L}/\text{cm}^2$ for *S. pinguis* and *R. subtilis*, respectively), whereas orange (87.6 % of limonene) and lemon oils (62.5 and 12.6 % of limonene and β pinene, respectively) were less toxic ($\text{LD}_{50} > 1 \mu\text{L}/\text{cm}^2$ for both insects). All the responses were found dose-dependent. *Rhyssomatus subtilis* was more resistant than *Sternechus pinguis* to the toxicity of the essential oils studied.

Keywords: *Sternechus subsignatus*; *Rhyssomatus subtilis*; essential oils; contact toxicity; soya pest.

Resumen

En el presente estudio se analizaron por CG-EM (cromatografía de gases-espectrometría de masas) los aceites esenciales de eucalipto (*Eucalyptus globulus* Labill.), naranja (*Citrus sinensis* L. Osbeck) y limón (*Citrus limon* L.) y se evaluaron sus efectos tóxicos contra dos especies de plagas importantes para la soja en Sud América: *Sternechus subsignatus* y *Rhyssomatus subtilis*. La toxicidad por contacto, ensayada por discos de papel de filtro impregnados, mostró que estos aceites causaron una mortalidad significativa de los insectos testeados (100% de mortalidad a 5 $\mu\text{L}/\text{cm}^2$). El aceite de eucalipto (98.9 % de 1,8-cineol) presentó la mayor toxicidad por contacto ($\text{LD}_{50} = 0.40$ y $0.84 \mu\text{L}/\text{cm}^2$ para *S. pinguis* y *R. subtilis*, respectivamente), mientras que el aceite de naranja (87.6 % de limoneno) y el de limón (62.5 y 12.6 % de limoneno y β pineno, respectivamente) fueron menos tóxicos ($\text{LD}_{50} > 1 \mu\text{L}/\text{cm}^2$ para ambos insectos). Todas las respuestas fueron dosis dependiente. *Rhyssomatus subtilis* fue más resistente que *Sternechus pinguis* a la toxicidad de los aceites esenciales estudiados.

Palabras Clave: *Sternechus subsignatus*; *Rhyssomatus subtilis*; aceites esenciales; toxicidad por contacto; plaga de la soja.

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Lista de abreviaciones:

EO - essential oil; LD₅₀ - lethal dose 50.

INTRODUCTION

Soybean today is one of the most important and extensively grown crops in the world. It accounts for 29.7% of the world's processed vegetable oil and is a rich source of dietary protein both for the human diet and for the chicken and pork industries (Graham and Vance, 2003). Although soybean production continues to grow worldwide, a major part of this production comes from only four countries: USA, Brazil, Argentina, and China (Hungria *et al.*, 2005).

Argentina exports more than 95% of its soybeans in the form of unprocessed grains, meals for animal food, edible oils and biofuel. Argentina is the world's largest exporter of soybean oil (30% of the world exports) and the second largest exporter of soybean flour (27% of the world exports). These figures allude to the rising importance of soybeans to Argentina (Penna and Lema, 2003).

The link between biodiversity reduction caused by the monoculture expansion and increased insect pest outbreaks and disease epidemics is well established. In poor and genetically homogenous landscapes insects and pathogens find ideal conditions to thrive. This leads to the increased use of pesticides, which after a while are no longer effective due to the development of pest-resistance or ecological upsets typical of the pesticide treadmill. Besides, human activity is rapidly altering the chemical composition of the air in ways that may profoundly affect the interactions between insects and plants (Hamilton *et al.*, 2005).

Yet another variable factor is the clearance that increases the number of pest insects. Crop pests are showing increased geographical range, increased numbers of generations, and higher densities (Parmesan, 2007).

The monocultures and the genetic uniformity bring as consequence the invasion of herbivorous insects that are considered to be plagues (Bertonatti and Corcuera, 2000).

Sternechus sp. and *Rhyssomatus subtilis* are pests of economic importance in many regions of Brazil and North of Argentina. These insects affect soybean crop. From the 1980s, the Brazilian soybean stalk weevil, *Sternechus subsignatus* became a plague of economic importance in areas of tillage located in region of traditional cultivation of soybean in Brazil

(Hoffmann-Campo *et al.*, 1990; Da Silva, 1996). In Argentina *Sternechus pinguis* and *Rhyssomatus subtilis* were described for the first time by Costilla and Venditti (1989) and Socías *et al.* (2009), respectively, as pests that affect soybean crop in North Argentina. These authors report the great potential damage of the insects, caused by the feeding of both adult and larvae. It expects that they expand its geographical distribution.

Some papers refer to geographical distribution, biology (Costilla and Venditti, 1989; Sosa, 2002), infestation level (Hoffmann-Campo *et al.*, 1990), damage, cultural control (crop rotation or trap crop), biological aspects (Da Silva, 1999; Sosa, 2002) or aggregation pheromone (Ambrogi and Zarbin, 2008) of *S. subsignatus* and to a lesser extent of *S. pinguis*. Moreover, there is little information on *R. subtilis* (Socías *et al.*, 2009).

Concerns over health and environmental problems associated with synthetic insecticides currently in use in agriculture have led to an intensification of efforts to find safe alternatives. Plants may provide potential alternatives to currently used insect-control agents because they constitute a rich source of bioactive chemicals. Natural pesticides based on plant-essential oils may represent alternative crop protectants. Various essential oils are documented to exhibit acute toxic effects against insects (Kim *et al.*, 2003; García *et al.*, 2007; Rajendran and Sriranjini, 2008; López and Pascual Villalobos, 2010). Eucalyptus and Citrus oils, such as lemon and orange, are widely utilized in various industries and are of low cost in our country.

At present the soybean weevils *S. pinguis* and *R. subtilis* are controlled by the following insecticides: thiamethoxan, carbosulfan, fipronil, metamidofos and chlorpyrifos (Lesche Tonet and Salvadori, 2002) and now they show resistance to them. This paper describes the chromatographic examination of three common essential oils (eucalyptus, lemon and orange), and for the first time, a bioassay for evaluating their contact toxicity against two soybean weevils with economic importance.

MATERIALS AND METHODS**Chemicals**

Technical grade chlorpyrifos was a gift from agronomist Manuel Esteve. Analytical grade n-hexane was used as the carrier. Essential oils were dissolved

in n-hexane (for application to filter paper discs) as required.

Essential oils

Lemon oil (*Citrus limon* L.) was purchased from Montreal (local market) (Droguerías Córdoba, Argentina) and eucalyptus (*Eucalyptus globulus* Labill.) and orange (*Citrus sinensis* L. Osbeck) oils were a gift from INTA Paraná, province of Entre Rios, Argentina. Eucalyptus oil was steam distilled while orange oil was cold-pressed.

Essential oils GC and GC-MS analyses. GC-FID

For the quantification of individual components, the essential oil (EO) was analyzed using a Perkin-Elmer Clarus 500 gas chromatograph equipped with a flame ionization detector (GC-FID). A capillary column DB-5 (30 m x 0.25 mm i.d. and 0.25 μ m coating thickness) was used for the separation of individual components of the EO. Helium was employed as the carrier gas with a flow rate of 1 mL/min. The temperature program was 60° C for 4 min, from 60 to 240 at 5° C/min, with a final hold time of 10 min. The injector and detector were maintained at 260 and 280° C, respectively. The sample was diluted 1:100 in n-hexane, 0.2 μ L was injected with a 1:100 split ratio (Zunino *et al.*, 2000).

GC/MS

For the determination of the composition, EO samples were diluted with hexane. The injection volume was 1 μ L. The identification of the components of the EO was realized by GC-MS. A Perkin-Elmer Q 700 GC-MS coupled with an ion trap mass detector was employed for the identification. A capillary column DB-5 (60 m x 0.25 mm i.d. and 0.25 μ m coating thickness) was used for the separation of the components. Helium was used as carrier gas with a flow rate of 0.9 mL/min. The temperature program for the oven and injector was the same as that for the GC-FID. Ionization was realized by electron impact at 70 eV. Mass spectral data were acquired in the scan mode in the m/z range 35-450. Retention indices (RI) of the sample components were determined on the basis of homologous n-alkane hydrocarbons under the same conditions. The compounds were identified by comparing their retention indices and mass spectra with published data (Adams, 2007) and libraries NIST and Adams. The main components were further identified by coinjection of authentic standards (SIGMA, USA). The quantitative composition was

obtained by peak area normalization, and the response factor for each component was considered to equal 1.

Insects

Sternechus pinguis (Fabricius) and *Rhyssomatus subtilis* Fiedler adults of unknown age and mating status were collected from soybean crops located in Metán and Rosario de La Frontera, Salta province (Argentina), held in plastic boxes (20 x 20 x 20 cm), fed with freshly cut soybean stem, and maintained at 25 \pm 2° C, 60 \pm 5% relative humidity, and a photoperiod of 12:12 light: dark cycle.

Because of the limited information on its biological and nutritional factors cycles required is that these insects have not yet been reared in laboratories. For this reason the insects used for bioassay were taken directly from the field with the limitations that it entails.

Contact Toxicity Assay

The insecticidal activity of three essential oils against adults of *Sternechus pinguis* and *Rhyssomatus subtilis* was evaluated by direct contact application assay (Kim *et al.*, 2003). An aliquot of 17.7, 35.5, 70.9, 141.4 and 283.6 μ L of each essential oil in 1 ml of n-hexane was applied to the filter paper discs (Whatman N° 1) of a glass Petri dish (8.5 cm diameter) to give a range of concentrations of 0.31-5 μ L/cm². Solvent was allowed to evaporate for 2 min prior to introduction of insects. Six adults of each insect were separately placed into Petri dishes. Control dishes were treated with n-hexane alone (Taponjdjou *et al.*, 2005). All treatments were replicated four times. Chlorpyrifos was used as a reference insecticide (Lesche Tonet and Salvadori, 2002). Mortality percentages were recorded after 24 h of treatment.

Statistical analysis

The percentage mortality of each insect was determined and transformed to arcsine square-root values for analysis of variance (ANOVA). Treatment means were compared and separated by Duncan's test at $p = 0.05$ (InfoStat software, 2002). Means (\pm SE) of untransformed data are reported.

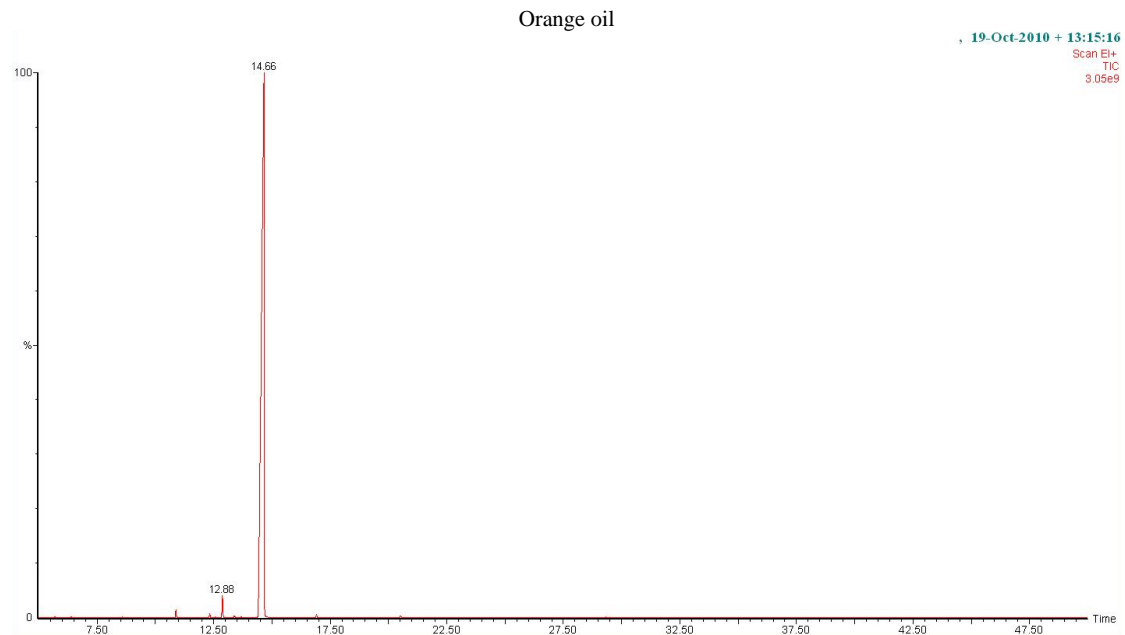
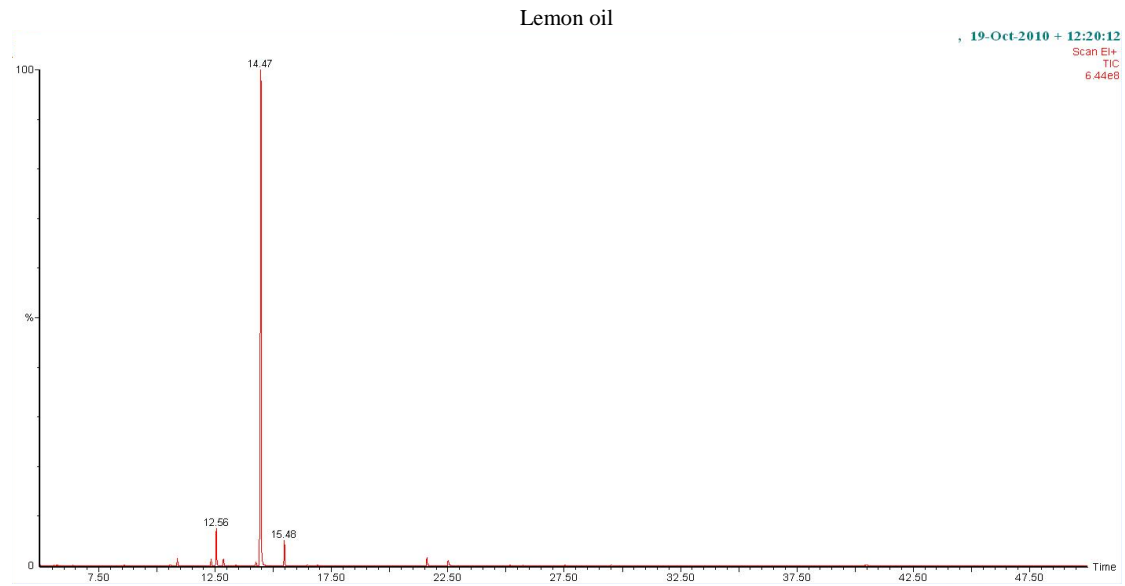
LD₅₀ values were calculated according to Finney (1971).

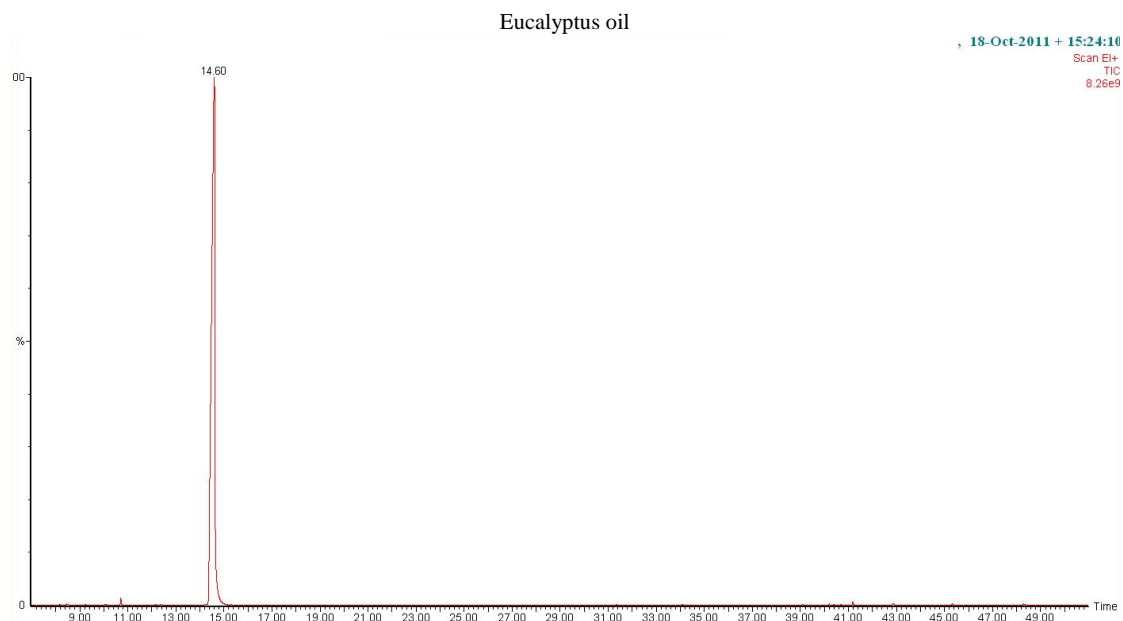
RESULTS AND DISCUSSION

Chemical constituents of the essential oils

Figure 1 shows the gas chromatograms of the three essential oils studied.

Figure 1
GC-MS chromatographic profiles of the essential oils studied. Column DB-5 (60 m x 0.25 mm i.d. and 0.25 µm coating thickness).





The composition of the eucalyptus, lemon and orange oils is described in Table 1. Eucalyptus oil presented 1,8-cineole as practically the only component (98.9%). This is higher than that reported for the same species from other regions of Argentina (Bandoni et al., 1993; Viturro et al., 2003; Gende et al., 2010). Citrus oils showed limonene as the main terpene (87.6% and

62.5% for orange and lemon oils, respectively). Besides, lemon oils showed β pinene as the second principal component (12.6%) (Table 1). These two monoterpenes also were reported as the main compounds of citrus oils (Ahmad et al., 2006; Yoon et al., 2009; Hosni et al., 2010).

Table 1

Relative percentage concentrations of the terpenoid constituents of the three essential oils studied, according to their elution order in the GC analysis.

<i>Retention index</i>	<i>Compounds</i>	<i>Lemon oil</i>	<i>Orange oil</i>	<i>Eucalyptus oil</i>
936	α -Pinene	2.5	2.1	0.4
977	Sabinene	2.2	1.2	--
983	β -Pinene	12.6	--	--
991	Myrcene	2.6	6.5	--
1006	Octanal	--	1.1	--
1010	3-Carene	--	0.9	--
1030	<i>p</i> -Cymene	1.3	--	--
1036	Limonene	62.5	87.6	--
1039	1,8-Cineole	--	--	98.9
1040	β -Phellandrene	2.1	--	--
1064	γ -Terpinene	8.5	--	0.6
1247	Neral	3.3	--	--
1277	Geranial	2.4	--	--
Total		100	99.4	99.9

Filter paper contact toxicity

The % mortalities of each insect species after 24 hours exposure to increasing dosages of volatile oils on filter paper discs are shown in Tables 2 and 3. Mortality of *R. subtilis* did not differentiate with the control at low concentrations of the essential oils, especially with *Citrus* oils. The same occurred with *S. pinguis* mortality, but only with lemon oil. Mortality increased

rapidly since dose of 0.63 $\mu\text{L}/\text{cm}^2$. The highest dose (5 $\mu\text{L}/\text{cm}^2$) of each oil was able to induce 100% mortality of both insects within 24 h of exposure (Tables 2 and 3). Moreover, eucalyptus oil induced significant mortality of both insects and approximately 100% mortality was achieved with the doses of 0.63 and 1.25 $\mu\text{L}/\text{cm}^2$ for *S. pinguis* and *R. subtilis*, respectively.

Table 2
Percentage mortality (\pm standard error) of *Sternechus pinguis* exposed to essential oils impregnated on filter paper discs at 24h of treatment.

Concentration ($\mu\text{L}/\text{cm}^2$)	Eucalyptus	Lemon	Orange	Chlorpyrifos
0.0	0 (0) a	0 (0) a	0.0 (0) a	
0.31	62.5 (17.2) b	5.6 (5.6) a	25.0 (8.3) b	
0.63	94.4 (5.6) c	25.0 (10.8) b	22.2 (5.5) b	
1.25	100.0 (0) c	33.3 (9.6) b	38.9 (14.7) b	
2.49	100.0 (0) c	87.5 (4.2) c	100.0 (0) c	
5,00	100.0 (0) c	100.0 (0) c	100.0 (0) c	
LD ₅₀ (95% CL)	0.40 (0.06-1.12)	2.05 (1.00-2.61)	1.13 (0.72-1.82)	< 0.003

Values (means \pm SE) with different letters in the same column are significantly different from each other according to Duncan's multiple range test at $P \leq 0.05$. Each datum represents the mean of four replicates, each set up with 6 adults ($n = 24$). Lethal dose (LD₅₀) and 95% confidence limits (Lower, Upper).

Comparison of LD₅₀ values for the three oils against both insect species showed that eucalyptus oil was relatively more toxic (LD₅₀=0.40 and 0.84 $\mu\text{L}/\text{cm}^2$ for *S. pinguis* and *R. subtilis*, respectively) than *Citrus* oils (LD₅₀ > 1 $\mu\text{L}/\text{cm}^2$). Besides, orange oil tended to be more toxic than lemon oil (Tables 2 and 3). The results of probit analysis showed that *S. pinguis* was comparatively more susceptible (LD₅₀ of *S.p* < LD₅₀ of *R.s*) to the toxic effect of essential oils than *R. subtilis* (Tables 2 and 3). This is in accordance with field experience, where *R. subtilis* is more resistant to commercial insecticides than *S. pinguis* (data not shown).

Eucalyptus and citrus fruit essential oils already have been studied as insecticides in several insects (Mareggiani et al., 2008; Sfara et al., 2009; Yoon et al., 2009; Pinto Junior et al., 2010). However, essential oil activity on these two insects has not been investigated.

Mechanisms of the insecticidal activity of terpenes would be given by inhibiting

acetylcholinesterase activity (Picollo et al., 2008; Abdelgaleil et al., 2009; López and Pascual-Villalobos, 2010) or octopamine receptors (Enan, 2001). The 1,8-cineol, the main component of eucalyptus, was described as the most potent inhibitor of the activity acetylcholinesterase among monoterpenoids studied (Picollo et al., 2008; Abdelgaleil et al., 2009; López and Pascual-Villalobos, 2010). This one might be one of the reasons for what eucalyptus oil was more toxic than that of the citrus fruits. However, limonene is generally known to be toxic to insects (Abdelgaleil et al., 2009; Papachristos et al., 2009 and references therein). In this study, orange oil showed higher percentage of limonene than lemon oil.

Rhyssomatus subtilis was more resistant than *Sternechus pinguis* to the toxicity of the essential oils studied.

From the results obtained in this study once again the essential oils showed good insecticidal activity. Based on these findings we are working in the

search of other essential oils with better LD₅₀. At this time our group is making the transfers of these results (in vitro experiments) to the field.

Table 3
Percentage mortality (\pm standard error) of *Rhyssomatus subtilis* exposed to essential oils impregnated on filter paper discs at 24h of treatment.

Concentration ($\mu\text{L}/\text{cm}^2$)	Eucalyptus	Lemon	Orange	Chlorpyrifos
0.0	8.3 (4.8) a	8.3 (4.8) ab	8.3 (4.8) a	
0.31	13.3 (8.2) a	0 (0) a	37.2 (14.8) ab	
0.63	46.7 (3.3) b	29.2 (13.2) bc	42.2 (13.1) ab	
1.25	94.4 (5.6) c	41.7 (9.3) c	61.1 (12.5) bc	
2.49	100.0 (0) c	86.7 (8.2) d	81.1 (1.1) c	
5.00	100.0 (0) c	100.0 (0) d	100 (0) d	
LD ₅₀ (95% CL)	0.84 (0.35-1.60)	1.74 (0.68-4.85)	1.35 (0.51-3.07)	< 0.004

Values (means \pm SE) with different letters in the same column are significantly different from each other according to Duncan's multiple range test at $P \leq 0.05$. Each datum represents the mean of four replicates, each set up with 6 adults ($n = 24$). Lethal dose (LD₅₀) and 95% confidence limits (Lower, Upper).

The advantages of using essential oils as pest management are that they can be easily extracted by steam distillation, they are safe to the user and the environment and they are volatile and can be potentially used as fumigants (Urzua *et al.*, 2011). Thus, they could be used to control a new pest that affects much of the economy of South America.

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