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Biocidal effect of a hexane-soluble extract of *Lippia graveolens* Kunth (Verbenaceae)

[Efecto biocida del extracto hexánico de *Lippia graveolens* Kunth (Verbenaceae)]

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Abstract: *Lippia graveolens* Kunth (Verbenaceae) is an economically important shrub known in Mexico as Oregano. In this work, the biocidal effect of the hexane extract of *L. graveolens* leaves was evaluated on two crop pests. Thus, larvae of *Spodoptera frugiperda* were fed with mixtures of extract and artificial diet. The nematicidal activity was evaluated on juveniles of *Meloydogine javanica*. Regarding *S. frugiperda*, quantitative differences between treatments and control were observed in dead pupae, surviving adults, and deformed adults ($P < 0.05$). All the surviving adults from the extract treatments were deformed. Nematicidal effect was registered, the LC_{50} and LC_{90} were 0.672 (0.654-0.690) and 0.965 (0.937-0.998) mg/mL respectively. The extract was characterized by NMR and GC-MS, being thymol the most abundant component (70.6%) in addition to carvacrol (22.8%). The results suggest the consideration of the hexane extract of *L. graveolens* leaves within the alternatives for the biological control of pests.

Keywords: *Lippia graveolens*; *Spodoptera frugiperda*; thymol; carvacrol

Resumen: *Lippia graveolens* Kunth (Verbenaceae) es un arbusto con importancia económica conocido en México como Orégano. En éste trabajo se evaluó el efecto biocida del extracto hexánico de hojas *L. graveolens* sobre dos plagas agrícolas. Así, larvas de *S. frugiperda* fueron alimentadas con mezclas de dieta artificial y extracto. La actividad nematicida fue evaluada en juveniles de *Meloydogine javanica*, Respecto a *S. frugiperda*, se observaron diferencias cuantitativas entre tratamiento y control en cuanto a pupas muertas, adultos sobrevivientes y adultos deformes ($P < 0.05$). Todos los adultos provenientes de tratamientos con extracto estuvieron malformados. Hubo efecto nematicida, calculándose CL_{50} y CL_{90} de 0.672 (0.654-0.690) y 0.965 (0.937-0.998) mg/mL respectivamente. El extracto se caracterizó por RMN y CG-EM. Los compuestos más abundantes fueron timol (70.6%), ademas del carvacrol (22.8%). Los resultados sugieren considerar al extracto hexánico de hojas de *L. graveolens* dentro de las alternativas para el control biológico de plagas.

Palabras clave: *Lippia graveolens*; *Spodoptera frugiperda*; timol; carvacrol.

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INTRODUCTION

Plant extracts have been considered an alternative to pest control because of their effectiveness and versatility, their chemical diversity makes them interesting fumigants, insecticides, and repellents (Isman, 2000).

Lippia graveolens Kunth (Verbenaceae) is an aromatic shrub that grows in arid, semi-arid and sub-humid regions of México where is known as "Oregano" and marketed as a valuable food seasoning, (Calvo-Irabién *et al*., 2014). Its essential oil contains mainly thymol, carvacrol, p-cymene, linalool and β-caryophylene (Calvo-Irabién *et al*., 2014). These compounds have been associated to the wide range of biological activities reported for essential oils, such as antimicrobial and insecticidal (Gleiser & Zygadlo, 2007; Machado *et al*., 2010; Bueno-Durán *et al*., 2014). Regarding the latter, essential oils from *Lippia* sp. are lethal to *Aedes aegypti*, *Rhipicephalus (Boophilus) microplus*, *Culex quinquefasciatus,* and *Tenebrio molitor* among others (Carvalho *et al*., 2003; Gleiser & Zygadlo, 2007; Cruz *et al*., 2013; Martinez-Velazquez *et al*., 2011). However, these studies have focused in essential oils therefore the information about the hexane-soluble extract is unavailable. Aimed towards a more rational utilization of the resource, it becomes necessary to asses if the hexane extract reflects the behavior of the essential oil. The present work aimed to evaluate the effect of a hexane-soluble extract of leaves of *L. graveolens* on two important crop pests, *Spodoptera frugiperda* and *Meloydogine javanica*.

MATERIALS AND METHODS

Plant material

Leaves of *L graveolens* were collected on Casas, Tamaulipas, Mexico in May 2013. A specimen is deposited in the Herbarium of Instituto de Ecología Aplicada, Universidad Autónoma de Tamaulipas.

Obtention and characterization of extract

Dried leaves (100 g) were macerated in hexane for seven days at room temperature. The solvent was removed with a rotavapor under reduced pressure, the resulting extract was kept in the dark. A sample (30 mg) of the extract was dissolved in $CDCl_3$ (0.5 mL) to obtain its ^1H and ^{13}C NMR spectra using a 500 MHz spectrometer (Bruker). The GC–MS analysis was performed using a Hewlett Packard 6890A gas chromatograph, equipped with a HP-5 MS fused silica capillary column (30 m length; 0.25 mm i.d.; film thickness 0.33 µm), coupled to a Hewlett Packard Mass Selective Detector MSD 5970; ionization voltage 70, electron multiplier energy 2000 V. Chromatographic conditions were: initial temperature 50º C for 35 min, with a rate of 2º C/min, until a final temperature of 285º C. The transfer line was kept at 280° C. Individual components were identified by comparison of their GC retention index and mass spectra with those reported in the NIST library.

Insecticidal activity

The effect of the extract on *S. frugiperda* was evaluated using a first instar larva placed in a pot containing 10 g of artificial diet treated with 10 or 100 ppm of extract or solvent (hexane) in the case of the control; each treatment had 30 replicates. Insect rearing and experimental conditions were as previously reported (Cardenas *et al*., 2012). The experiment was daily monitored until control group reached adulthood; deformed adults, dead pupae and deformed pupae were counted. Data were compared using a chi squared test for goodness of fit, when significant differences were found, the residuals were analyzed to determine which categories were different. A confidence level of 95% was used.

Nematicidal activity

A colony of *M. javanica* was mantained on *Lycopersicum esculentum* (var. Marmande) planted in pots, 25 ± 1º C, 70% RH. Egg masses handpicked from roots of infected plants were incubated in water (25º C) for 24 h to obtain second-stage juveniles (J2). The assay was done on a 96-well plate (BD Falcon, San Jose, CA, USA) with four replicates. The extract was dissolved in distilled water with 5% DMSO-Tween (0.5% Tween 20 in DMSO). Each well contained 5 µL of the test solution, 95 µL of water and 90-150 nematodes. Controls consisted of water/DMSO-Tween. Five concentrations were tested: 1, 0.75, 0.50, 0.25 mg mL-1. The plates were covered and kept in darkness at 25º C. The dead organisms were counted after 72 h using a microscope (20x). The nematicidal activity was corrected according to Schneider-Orelli formula and expressed as lethal concentrations (LC_{50} and LC_{90}) calculated by Probit analysis.

RESULTS AND DISCUSSION

Extract analysis. The analysis by GC–MS allowed the identification of thirty-seven components in the *L. graveolens* hexane extract (Table 1); the major compounds were thymol (70.8%), carvacrol (22.7%) and p-cymene (5.8%).

Table No. 1
Chemical composition (GC-MS) of the hexane-soluble extract of *Lippia graveolens*.

RI[a]	ID	Abundance (%)
979	Cineole	0.6
986	Myrcene	1.1
1000	3-Carene	1.2
1013	p-Cymene	5.8
1018	1,8-Cineole	1.1
1021	Limonene	0.8
1077	Terpinolene	1.2
1158	4-Terpineol	1.7
1172	p-Cymen-8-ol	0.7
1190	α-Terpineol	0.7
1214	Methyl thymol	1.0
1284	Thymol	70.6
1295	Carvacrol	22.8
1330	Eugenol	0.7
1367	α-Copaene	0.8
1407	β-Caryophyllene	0.9
1414	$C_9H_{10}O_3$	1.4
1440	β-Humulene	3.0
1443	trans-¸-Bergamotene	0.9
1461	$C_{15}H_{24}$	0.8
1466	β-Selinene	1.2
1479	α-Selinene	1.1
1484	α-Muurolene	0.5
1494	Γ-Cadinene	1.3
1498	1,S-cis-Calamenene	0.8
1505	υ-Cadinene	1.1
1548	(E)-Nerolidol	0.5
1557	Caryophyllene oxide	0.8
1559	Globulol	0.5
1568	Viridiflorol	0.6
1576	Guaiol	1.2
1588	Epoxyhumulene II	2.0
1605	β-Eudesmol	3.1
1615	T-Cadinol	1.1
1620	α-Eudesmol	2.1
1622	α-Cadinol	1.2
1629	$C_{15}H_{26}O$	2.0

[a] Retention index, compounds listed in order of elution on a HP-5 MS capillary column

In the [1]H NMR thymol can be easily detected too, since spectrum signals for methyl group in addition to a methine of an isopropyl moiety groups were detected at 1.25 ppm and 3.2 ppm respectively. Besides, the sharp doublet signal at 7.1 (ppm J=8 Hz) and the broad doublet at 6.7 ppm represents aromatic hydrogen atom, among others. The [13]C NMR spectrum analysis was also concordant with the structure of thymol.

Insecticidal activity

The pupal mortality was different among treatments ($x^2_{0.05,2}$ = 6.9: P < 0.05), control group had the lowest count of dead pupae and 100 ppm the highest, extract treatments were similar (Figure No. 1).

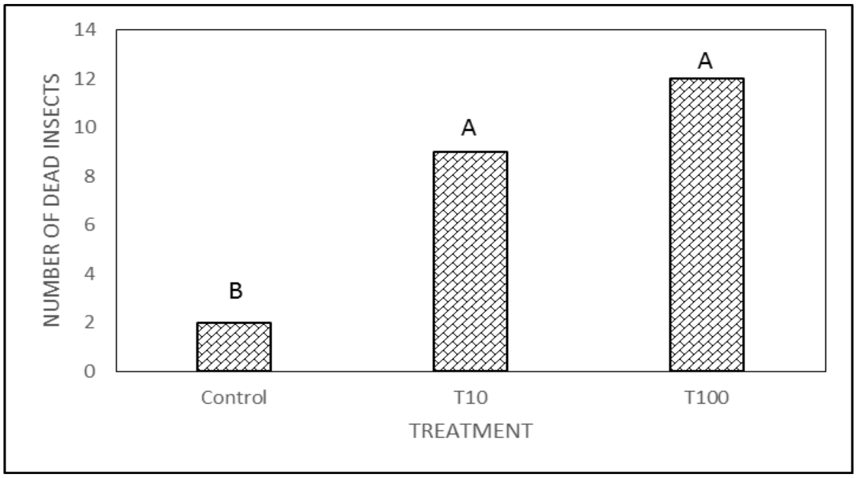

Figure No. 1
Effect of *L. graveolens* on *S. frugiperda* after feeding with mixtures containing hexane extract of leaves. Mortality of pupae

The surviving adult population was statistically different between control and treatments ($x^2_{0.05,2}$ = 6.7: P < 0.05) and extract treatments were similar although the survivor count was lower in the 10 ppm group (Figure No. 2).

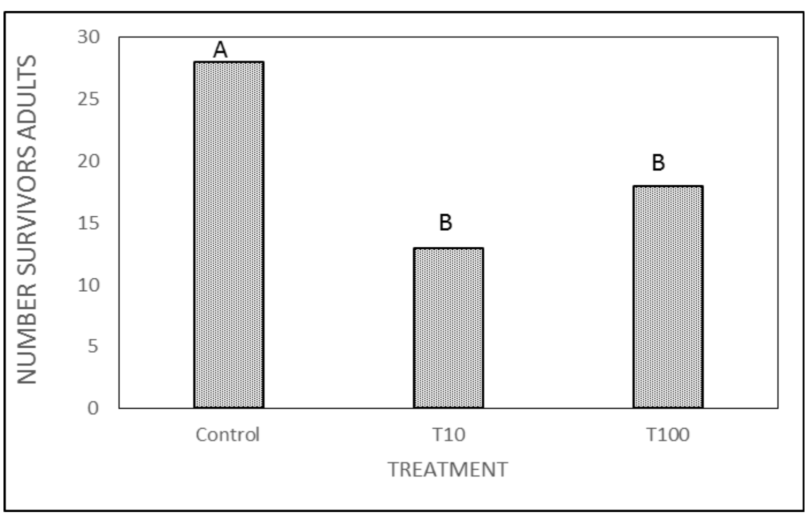

Figure No. 2
Effect of *L. graveolens* on *S. frugiperda* after feeding with mixtures containing hexane extract of leaves. Mortality of adults

Normal and deformed adults were observed (Figure No. 3). The adult count significantly differed between treatments ($x^2_{0.005,2}$ = 6.7; P < 0.05), the 100 ppm treatment had more deformed individuals whereas the control had none.

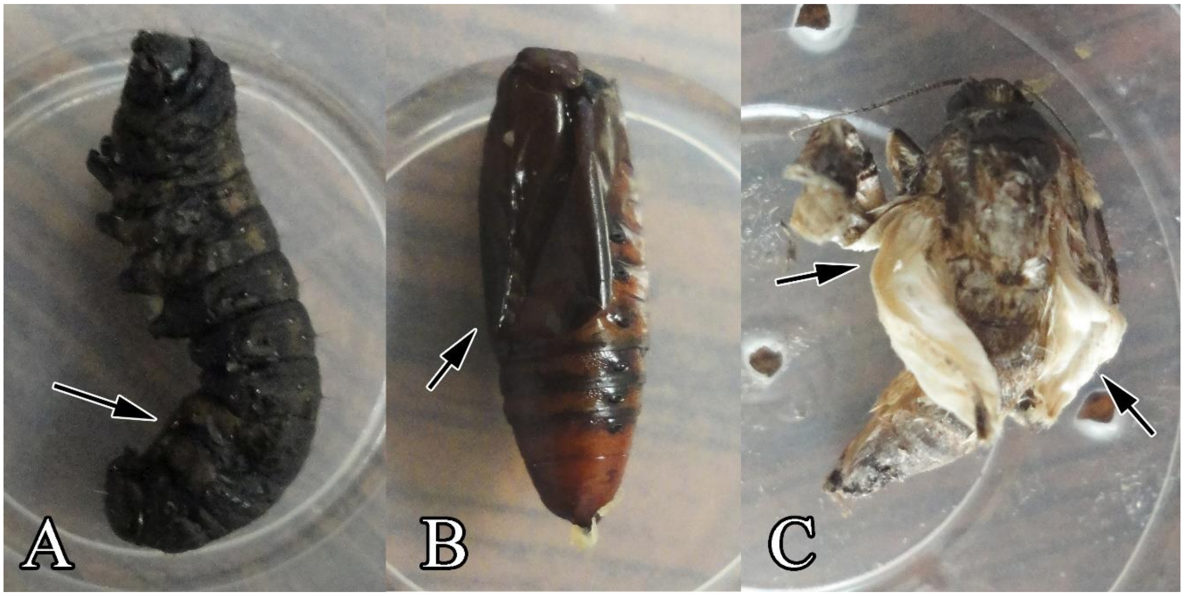

Figure No. 3
Examples of deformed (A) larvae, (B) pupa and (C) adult of the treatment group.

However, all the surviving adults were deformed in the extract treatments (Figure No. 4).

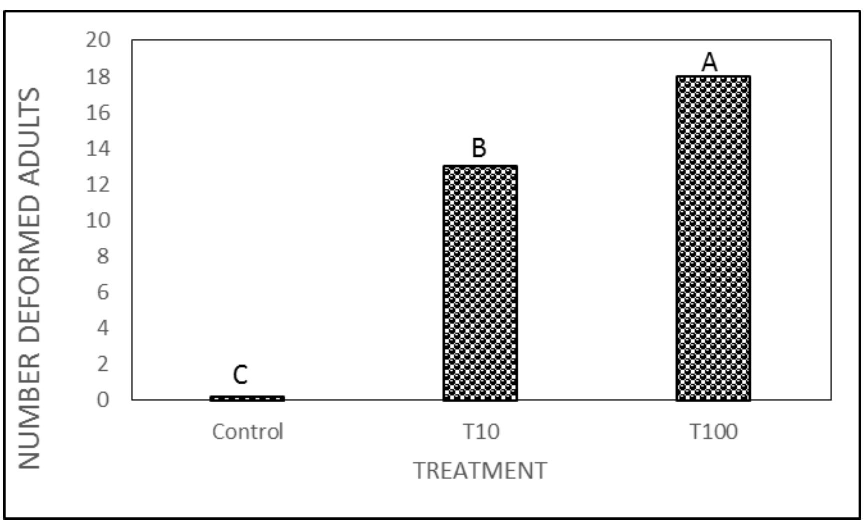

Figure No. 4
Effect of *L. graveolens* on *S. frugiperda* after feeding with mixtures containing hexane extract of leaves. Deformed adults

Nematicidal activity

The calculated LC_{50} and LC_{90} were 0.672 (0.654-0.690) mg/mL and 0.965 (0.937-0.998) mg/mL, values represent mean (confidence interval).

Thymol was the most abundant compound in the hexane-soluble extract of *L. graveolens* leaves, this agrees with the literature records, although our percentage, 70.6, doubles the mean values 33.40 and 32.59 reported by Hernández *et al*. (2009) and González-Trujano *et al*. (2017), respectively. Less compounds were detected in the same works, 12 and 16 respectively, instead of 37 in the present; moreover, from the top five abundant compounds, only carvacrol and thymol are common between profiles. Hernández *et al*. (2009) reported that hexane extracts contained a high amount of oxygenated (thymol methyl ether, carvacrol, thymol) and alkylated monoterpenes (α-thujene, α-pinene, p-cymene, 3-carene) as well as some sesquiterpenes (isocaryophyllene, α-humulene, guaia-1(5), 7(11)-diene, the authors describe qualitative and quantitative variation within and between natural populations.

Quantitative and qualitative variations of the chemical composition of *L. graveolens* are documented. Catalogued according to its most abundant compound, the existence of three chemotypes in *Lippia graveolens* has been proposed, these are "thymol" (Uribe-Hernández *et al*., 1992; Cazares *et al*., 2010; Machado *et al*., 2010), "carvacrol" (Martínez-Natarén *et al*., 2012), and "sesquiterpenes" (Martínez-Natarén *et al*., 2012), which is abundant in humid regions while the others dominate dry climates The content variation goes from 0.43% to 45.9% for thymol and 0% to 52.3% for carvacrol (Bueno-Durán *et al*., 2014). Such variability must be considered for the exploitation of the biological activity of extracts. Furthermore, the extraction with non-polar solvents allows the obtention of long chain alkanes, waxes, steroidal terpenes and fatty acids (Harborne, 1998), which could affect not only solubility but also the biological activity.

To the best of our knowledge, the effect of a hexane-soluble extract of *L. graveolens* on *S. frugiperda* has not been reported before. Mortality was higher in the extract treated groups than in the control. Extracts of the genus *Lippia* on *Spodoptera* species have been related to lethality and developmental and reproductive alterations. (Isman *et al*., 2001; Pavela, 2011; Pavela, 2014). Evidence points to terpenes such as thymol, carvacrol, carvone, citral (geranial + neral) and pulegone-1,2-epoxide as responsible of those effects (Grundy & Still, 1985; Pavela, 2014; Peixoto *et al*., 2015). The abundance of thymol in the extract and the literature evidence supports its responsibility for the observed results, nevertheless synergic or antagonistic interactions are likely occurring, the magnitude of the effect of thymol changes when is mixed with other monoterpenes, the results are related to the proportions and compounds present (Pavela, 2005).

After the exposure to monoterpenes, the relatively rapid toxicity on the insect nervous system is expressed behaviorally as fast uncoordinated movements followed by paralysis. (Isman, 2000; Enan, 2001; Waliwitiya *et al*., 2012; Peixoto *et al*., 2015). Acute lethality data of essential oils or isolated terpenes is usually obtained from topical application and fumigant assays (Hummelbrunner & Isman, 2001; Pavela, 2014). Long-term detrimental effects, on the other hand, are associated to ingestion as was the case in our experiment (Gunderson *et al*., 1985). In our assay, the insect population gradually decreased weeks after the initial exposure, the reported disruptions of reproductive and other systems may be leading to the observed results.

The characteristics of our insect assay makes the ingestion of the extract compounds more probable, although a combination with absorption via topical or aerial routes is also plausible. Furthermore, monoterpenes are volatile, therefore can reach the insect respiratory system acting as repellents, leading to starvation (Lee *et al*., 2003; Peixoto *et al*., 2015). Other types of assays are needed to discern whether the extract acted as antifeedant, antinutritional or toxic.

This is the first report of the lethal effect of *L graveolens* against *M javanica*. The lethal effect on *M. javanica* observed in the extract treated group is possibly related to the thymol and carvacrol content, as is suggested in previous findings of the nematicide action of extracts with high thymol content and thymol/carvacrol mixtures (Ntalli & Caboni, 2012; Santana *et al*., 2014). Based on the results of previous studies with *M. incognita* some authors propose that the oxygenated compounds are generally more active than hydrocarbons and that this action decreases in the order of L-carvone, pulegone, trans-anethole, geraniol, eugenol, carvacrol, thymol, terpinen-4-ol, estragole, and γeudesmol (Ntalli & Caboni, 2012).

CONCLUSION

The hexane-soluble extract of *L. graveolens* leaves is lethal to *M. javanica* and detrimental to the development of *S. frugiperda*. The extract treated insects, if able to reach adulthood, showed malformations. The effects may be attributed to the high content of thymol (70.8%) although carvacrol may be also participating. Although fast action and high mortality rate are expected traits of an insecticide, detrimental effects on the life cycle as showed here are also useful in the strategies for pest management. The hexanic extraction of *L. graveolens* is an alternative for the obtention of thymol and carvacrol with yields higher than essential oil. These data would be helpful towards a rational management of the species.

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