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RESEARCH ARTICLE

Pharmacological evaluation of the anxiolytic-like effects of *Lippia graveolens* and bioactive compounds

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ABSTRACT

Context: *Lippia* species (Verbenaceae) are widely used in Latin America and Africa as folk medicine for their tranquilizing properties.

Objective: To evaluate the anxiolytic-like effects and safety of *Lippia graveolens* Kunth. by exploring its aqueous and organic leaf extracts and identifying the responsible chemical constituents.

Material and methods: Aqueous and organic extracts (hexane, ethyl acetate and methanol) were pharmacologically evaluated at several doses. Chemical constituents were identified using MS, NMR and GC-MS analysis. The isolated compounds (3 mg/kg, i.p.), extracts (1, 3, 10 and 30 mg/kg, i.p.), and the reference drug diazepam (0.1 mg/kg, i.p.) were assessed in CD-1 mice using experimental behavioural models: open-field, cylinder, hole-board, plus-maze and sodium pentobarbital-induced hypnosis, as well as their acute toxicity (LD_{50}).

Results: After administration of the extracts and bioactive compounds, a significant anxiolytic-like response from 1 mg/kg, i.p. was observed, resembling the effect of diazepam. Major presence of thymol (33.40%) was observed in the hexane extract; whereas for the first time in this species a p-cymene + thymol mixture (9.78%), naringenin (0.18%) and cirsimaritin (1.16%) were obtained as bioactive constituents of the ethyl acetate crude extract. Acute toxicity was calculated to be $LD_{50} = 1000$ mg/kg for the crude hexane extract, lower in comparison to the other extracts analyzed ($LD_{50} > 2000$ mg/kg).

Discussion and conclusion: Our results suggest that *L. graveolens* exerts anxiolytic-like activity involving many kinds of constituents, mainly of the terpenoid and flavonoid nature. These results reinforce the potential use of this species in the therapy of anxiety.

Introduction

People affected with anxiety disorders often face numerous barriers to take therapy, such as a lack of healthcare services or adverse reactions to anxiolytics; these negative consequences of drug use motivate further research towards the improvement in the effectiveness and tolerance of pharmacological treatment. In this regard, there is an increasing and constant interest in the medicinal plants field, emphatically in aromatic ones, due to their potential application as anxiety disorders treatment (Ballenger 1998; Gum et al. 2009).

Lippia graveolens Kunth. (Verbenaceae) is a wild and aromatic plant species distributed in Mexico and Central America (Rivera et al. 2010). In Mexico, it is widely used as a seasoning and folk remedy since ancient times by prehispanic cultures (Pascual et al. 2001). Popularly known as 'mejorana', 'orégano de monte', 'orégano' and 'Mexican oregano', it is a shrub-like plant that grows in semiarid environments (Castillo-Herrera et al. 2007). Antipyretic, analgesic, abortive, antispasmodic, anti-inflammatory, for the treatment of menstrual disorders, syphilis, gonorrhoea and diabetes are some of the medicinal activities already reported for this species (Pascual et al. 2001; Soto-Domínguez et al. 2012; Pérez 2014). Other *Lippia* species, such as *L. alba*

(Vale et al. 1999; Zétola et al. 2002), *L. javanica* (Mujovo et al. 2008) and *L. multiflora* (Abena et al. 2001; Stafford et al. 2008; Folashade & Omoregie 2012) are also known to be widely consumed as tea or essential oil in Latin America and Africa due their sedative and tranquilizing properties.

Regarding the phytochemical studies of *L. graveolens,* it has already been reported that a fractionated chloroformic leaf extract confirmed the presence of terpenes, such as thymol and carvacrol, among others; as well as flavonoids such as quercetin *O*-hexoside, scutellarein 7-*O*-hexoside, phloridzin (phloretin-2-*O*-glucoside), trihydroxy-methoxyflavone derivative and 6-*O*-methylscutellarein (Leyva-López et al. 2016). Naringenin (Domínguez et al. 1989; Martínez-Rocha et al. 2008), pinocembrim, lapachenole and ten iridoids were also isolated from the aerial parts of this species (Domínguez et al. 1989). Whereas kaempferol, isokaempferide, pilosin, cirsimaritin, naringenin, and a derivative of catechin were isolated from the stem of *L. graveolens* H.B.K. var. *berlandieri* Schauer (González-Güereca et al. 2007). Finally, the isolation of (-)(2S)-5,6,7,3',5'-pentahydroxyflavanone-7-*O*-β-D-glucopyranoside as a novel flavanone was obtained from the same variety (González-Güereca et al. 2010).

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Pharmacological studies have assessed the tranquilizing effects of the leaves of *L. alba* and *L. multiflora* (Vale et al. 1999; Abena et al. 2001; Zétola et al. 2002). In the case of *L. graveolens*, the inhabitants of many communities in Mexico have orally described that this species is prepared as an infusion or decoction by boiling approximately 3.5 g of leaves per 1 L of water for 5 min, and by drinking it during the day to get sedative and tranquilizing effects. However, scientific or ethnobotanic studies of this species supporting these specific properties still remain largely unexplored.

Despite that *Lippia* species are associated to an anxiolytic effect in folk medicine there are no supporting pharmacological studies for *L. graveolens* and its active compounds. The objective of our study is to provide scientific evidence of the anxiolytic-like effect of *L. graveolens* and to indicate the possible bioactive compounds involved.

Materials and methods

Plant material

Lippia graveolens leaves were collected in La Guía del Porvenir, Abasolo, in the state of Tamaulipas Mexico in March 2012. This species was identified by Dr José Guadalupe Martínez-Ávalos and a voucher specimen No. 1786 was deposited at the Herbarium of the Institute of Applied Ecology of the Autonomous University of Tamaulipas, Mexico.

Preparation of the extracts

The leaves (657 g) were air-dried at room temperature, pulverized in a mill and then sequentially macerated in lapses of 24 h using organic solvents (3 L) of increasing polarity (hexane, ethyl acetate and methanol). Excess of solvent was removed under reduced pressure with a rotary evaporator (R-205, Büchi Labortechnik AG, Switzerland). The yields of the organic crude extracts obtained as dry weight were 75.84 g (11.54%), 77.30 g (11.76%) and 169.12 g (25.74%), respectively. In order to elucidate its components, a portion of the hexane crude extract (10 mg) was subjected to GC-MS analysis; whereas ethyl acetate and methanol crude extracts were submitted to a fractionation. Additionally, all the crude extracts were used for pharmacological evaluation.

The aqueous extract was obtained from a decoction of the pulverized dried plant (76 g) in boiling water (500 mL) for 5 min. Afterwards, the liquid was cooled to room temperature, filtered and frozen in liquid nitrogen to be lyophilized (HETO FD3, Heto-Holten A/S, Denmark) yielding 15 g (19.73%).

Phytochemical analysis

Hexane extract

The GC-MS analysis was performed with an Agilent 7890B gas chromatograph coupled to an Agilent 5977 mass-selective detector (Agilent Technologies, Palo Alto, CA) using the following analytical conditions: an HP-5MS fused-silica capillary column (30 m × 0.25 mm i.d.) coated with a film of 5%-phenyl-95%-methyl silicone (thickness 0.25 μm); Helium gas as carrier at a flow rate of 1.4 mL/min, sample injection volume was 1.0 μL in splitless mode and the injector port temperature was 280 °C. The oven temperature program was from 30 °C (2 min) to 300 °C (3 min) at 5 °C/min. Mass spectra were recorded in SCAN mode

from 50 to 550 amu, the electron impact energy was set at 70 eV. The total run time was 59 min. The linear retention index (LRI) was calculated by injection of a mixture of *n*-alkanes, C7 to C30, under the same analytical conditions (Van Den Dool & Kratz 1963). Identification of the detected peaks were performed by careful comparison of the obtained MS spectra and LRI vs. data from computer library (NIST/EPA/NIH Mass spectral Library 2.0) and the literature (Adams 2007).

Ethyl acetate extract

The crude ethyl acetate extract was separated using a chromatographic open column packed with silica gel 60 GF$_{254}$ (Merck KGaA, Germany) in a proportion of 1:15, extract-eluent. The elution started with hexane followed by hexane-ethyl acetate mixture, ethyl acetate, ethyl acetate–methanol mixture and finally methanol. A total of 206 fractions (100 mL each) were collected and then grouped by similarity according to their profiles acquired by thin-layer chromatography (TLC). A mixture of *p*-cymene and thymol was obtained from the fractions eluted with hexane-ethyl acetate (9:1); similarly, naringenin was obtained from hexane-ethyl acetate (7:3) and cirsimaritin from hexane-ethyl acetate (6:4).

The *p*-cymene + thymol mixture was identified by ^{1}HNMR and MS EI positive, [thymol + H]$^+$ (151.1, *m/z*) and [*p*-cymene]$^+$ (134.22, *m/z*). The ^{1}H NMR spectra were recorded with an Avance DPX400 spectrometer (Bruker) at 400 MHz. Positive electrospray ionization mass spectra were obtained with a Cap LC coupled Micromass® Q-ToF Ultima ESI system (Waters Corp., Milford, MA). Combustion analysis was performed with an organic elemental analyzer (Flash 2000, Thermo Fisher Scientific Inc.).

The identified flavonoids corresponded to pure naringenin and cirsimaritin according to their ^{1}H NMR spectra in DMSO-d_6, mass spectrometry and elemental analysis (C, H, N). The cirsimaritin was recrystallized in CH$_3$OH, by slow evaporation at room temperature and analyzed by single-crystal X-ray diffraction; its crystal structure matched with that of a previous report where this flavonoid was isolated from *Salvia nubicola* Wall. ex Sweet (Lamiaceae) (Hieu-Hong et al. 2002).

Naringenin (N): white powder;^{1}H NMR (400 MHz, DMSO-d_6) δ 12.16 (s, OH, 1 H), 10.54(s broadband, OH, 1 H), 9.62 (s broadband, OH, 1 H), 7.30 (d, $J = 7.5$ Hz, 2 H), 6.77 (d, $J = 7.0$ Hz, 2 H), 5.87 (s, 2 H), 5.45 (d, $J = 10.0$ Hz, 1 H), 2.63 (d, $J = 10.0,1$ H) (a triplet signal is overlapped by water at 3.3 ppm); [13]C (100 MHz, DMSO-d_6): δ188.3, 158.8, 156.0, 155.4, 149.5, 121.6, 119.5, 106.8, 93.9, 87.5, 86.7, 71.0, 34.5. MS (FAB, *m/z*) 273 [M$^+$]. Anal. cal. for C$_{15}$H$_{12}$O$_5$ (272.25): C, 66.17; H, 4.44. Found: C, 65.98, H, 4.55, as previously reported in the literature (Kyriakou et al. 2012).

Cirsimaritin (CS): Palid yellow crystal;^{1}H NMR (400 MHz, CD$_3$OD) δ 7.84 (d, $J = 8.8$ Hz, 2 H), 6.95 (d, $J = 8.8$ Hz, 2 H), 6.61 (s, 1 H), 6.58 (s, 1 H), 4.61 (s, 3 H), 3.89 (s, 3 H); [13]C (100 MHz, CD$_3$OD): δ 184.5, 166.7, 163.0, 159.1, 154.9, 154.3, 133.1, 129.7, 123.5, 117.3, 106.0, 103.6, 95.6, 61.2, 57.3.MS (FAB, *m/z*) 315 [M$^+$]. Anal. cal. for C$_{17}$H$_{14}$O$_6$ (314.08): C, 64.97; H, 4.49. Found: C, 64.01, H, 4.62, as previously reported (Alwahsh et al. 2015).

Methanol and aqueous extracts

The methanol extract was also fractionated by an open column chromatography under the conditions mentioned for the ethyl acetate extract. A total of 275 fractions (100 mL each) showing

similar chromatographic profile were pooled in 12 fractions. Chromatographic analysis of these fractions confirmed the presence of complex mixtures of flavonoids that did not allow the separation of pure compounds; it was the same case for the aqueous extract.

Animals

Male CD-1 mice (25–30 g) were obtained from the animal housing facility of the Faculty of Medicine in the Universidad Nacional Autónoma de México, Mexico City. Groups of 10 animals were kept in acrylic boxes with *ad libitum* access to food and water in a controlled environment at 22 °C and a 12 h light/dark cycle. All experimental procedures followed the protocol approved by the local Animal Ethics Committee (S/N-902-03) in compliance with national (NOM-062-ZOO-1999) and international regulations on the care and use of laboratory animals.

Drugs

Sodium pentobarbital (PISA Agropecuaria, Mexico City) was dissolved in saline solution (s.s., 0.9% NaCl). Diazepam (Sigma-Aldrich Co., St. Louis, MO), hexane and ethyl acetate crude extracts, as well as the *p*-cymene + thymol mixture, were suspended in 0.5% Tween 80 in s.s. The methanol and aqueous extracts, as well as cirsimaritin and naringenin, were dissolved in distilled water. All the treatments were prepared the same day of the experiments and administered intraperitoneally (i.p.) in a volume of 10 mL/kg body weight. Animals from the vehicle group received only s.s. in the same volume and route of administration.

Pharmacological evaluations

Diazepam was chosen as the anxiolytic reference drug. Each group of six animals received an injection of the vehicle, and diazepam (0.1 mg/kg), or the hexane, ethyl acetate, methanol and aqueous crude extracts at doses of 1, 3, 10 and 30 mg/kg, i.p.

Open field test

Forty-five min after the treatment administration, and prior to the anti-anxiety assays, the ambulatory activity of each mouse was evaluated. In this test, a mouse was placed in the centre of an acrylic cage which floor is divided in 12 drawn squares (6 × 6 cm). The number of explorations was recorded for a 2 min lapse (Pérez-Ortega et al. 2008).

Hole-board test

This model consisted in an acrylic box which wood floor has evenly distributed holes of 3 cm diameter. Each mouse was placed in the centre of the box, when the animal dipped its head into a hole an event was registered, the exploratory activity was monitored for a 3 min lapse (Clark et al. 1997).

Exploratory rearing

In this test, each mouse was placed inside a glass cylinder (20 cm high × 13 cm diameter × 5 mm thick) for a 5 min lapse, the observer counted the number of occasions that each animal stood over its hind legs and touched the cylinder wall with both anterior legs (Hiller & Zetler 1996).

The elevated plus-maze

This is the most validated test to evaluate anxiolytic-like effects (Lister 1987). It was conducted in a wooden structure with four arms, two open (30 × 5 cm) and two enclosed (30 × 15 × 5 cm), extending from a central platform (5 × 5 cm) at 50 cm high from a base. Each mouse was situated in the central cross and then the number of entries and the lapse that the animal remained whether in the open or closed arms was registered for 5 min.

Sodium pentobarbital-induced hypnosis

To evaluate a central nervous system (CNS) depressant interaction, the reference drug and the extracts were administered with sodium pentobarbital (SP), 42 mg/kg, i.p. The latency to the onset of sedation and hypnosis and the sleeping time duration were recorded. Substances with CNS depressant activity increase the hypnosis duration produced by SP (González-Trujano et al. 1998).

Acute toxicity (LD$_{50}$)

The LD$_{50}$ of the extracts was estimated by a modification of the method described by Lorke (1983). Each mice of a group of three per extract received a dosage of 2000 mg/kg i.p. If death occurred, the dosage was lowered to 10, 100 and 1000 mg/kg. Mice were kept under observation for 14 days, recording mortality and weight lost. At the end of the study, animals were sacrificed and their tissues examined macroscopically.

Statistical analysis

Results are presented as the mean ± standard error of the mean (SEM.). Data were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's test to compare multiple experimental groups against the control group or Student's *t* test to determine if two sets of data were significantly different from each other by comparing vehicle vs. a treatment group. Analysis was done using the software SigmaStat for Windows Version 3.5 (Systat Software Inc). A value of $p < 0.05$ was considered significant.

Results

The GC-MS analysis showed 16 compounds in the hexane crude extract, the most abundant of them were thymol (33.40%), *m*-cymen-8-ol (16.37%), methyl salicylate (10.48%), carvacrol (6.75%) and linalool (5.17%) (Table 1). Four compounds were obtained from the ethyl acetate crude extract, which according to the spectral analysis were identified as *p*-cymene + thymol mixture (7.56 g, 9.78%), cirsimaritin (140 mg, 0.18%) and naringenin (900 mg, 1.16%).

Neither the extracts (1 to 30 mg/kg, i.p.) nor the isolated compounds (3 mg/kg, i.p.) modified the ambulatory activity compared to the vehicle group in the open-field test (Figure 1, panel A–D). Nevertheless, the hexane (Figure 1, panel A) and aqueous (Figure 1, panel D) extracts at 10 and 30 mg/kg showed a tendency to reduce this activity. Similar responses were observed in the number of rearings in the cylinder exploration (Figure 2, panel A–D), except in the ethyl acetate (Figure 2, panel B) and aqueous (Figure 2, panel D) extracts that showed a significant decrease at 30 mg/kg (Figure 2, panel B and D, respectively), and

as well as the *p*-cymene + thymol mixture at 3 mg/kg (Figure 2, panel A). In contrast, every extract and isolated compound at every dose tested showed a significant decrease in the head-dips frequency in the hole-board test (Figure 3, panel A–D), with a significant increase in the lapse of open arms exploration in the plus-maze test (Figure 4, Panel A–D). The effects produced by the crude extracts were similar to the ones observed in the presence of the reference drug diazepam at dosage of 0.1 mg/kg (Figures 1–4).

With regard to the hypnotic–pharmacological interaction, none of the tested extracts or isolated compounds modified the

onset to sedation and hypnosis induced by SP (Table 2). Conversely, all the organic and aqueous extracts at all doses tested, as well as the isolated compounds cirsimaritin, naringenin and the *p*-cymene + thymol mixture (3 mg/kg, i.p.), significantly prolonged the sleeping time in mice when compared to the vehicle group (Table 2). These effects resembled those produced by diazepam at a dosage of 0.1 mg/kg (Table 2).

Mice treated with hexane crude extract died 30 min after i.p. administration; the estimated LD_{50} was 1000 mg/kg. The aqueous, methanol and ethyl acetate extracts caused 33.3% mortality at dosage of 2000 mg/kg. Therefore, it was considered an LD_{50} > 2000 mg/kg. No significant weight loss was observed in mice surviving after treatment with the organic or aqueous extracts, these groups of mice did not show toxic effects like loss of locomotion or respiratory alteration. After the sacrifice of remaining animals, no morphological changes were observed during macroscopic examination of principal organs such as the liver, kidney, heart, stomach and the intestines.

Discussion

To the best of our knowledge, there are no previous reports of the hexane extracts of *Lippia* species. However, as a non-polar extract it contains similar constituents to those already reported in the essential oil of several species of this genus. As a matter of fact, there are reports of these essential oils evaluated for their anxiolytic-like effects due to the presence of these constituents. Therefore, in this study we considered importance to give these references due to the close chemical composition and the pharmacological activity observed in the hexane extract of *L. graveolens* described as following.

Constituents such as limonene, linalool, α-terpineol, (*Z*)-ocimenone and β-caryophyllene have been found in *L. javanica* (Manenzhe et al. 2004); whereas geraniol, myrcene, linalool,

**Table 1.** Constituents of *L. graveolens* leaf hexane extract analyzed by GC-MS.

LRI	Compound	Relative abundance (%)	Method of identification
1113	Linalool	5.17	MS
1166	Borneol	4.05	MS, RI
1178	**m-Cymen-8-ol**	16.37	MS
1186	p-Cymen-8-ol	1.10	MS, RI
1193	**Methyl-salicylate**	10.48	MS
1294	**Thymol**	33.40	MS, RI
1302	**Carvacrol**	6.75	MS, RI
1424	β-Caryophyllene	1.88	MS
1459	(E)-β-farnesene	1.63	MS
1492	Viridiflorene	0.59	MS
1500	α-Muurolene	0.54	MS, RI
1591	n.d.	5.37	–
1615	Tetradecanal	3.35	MS, RI
1640	t-Muurolol	1.13	MS, RI
1644	n.d.	0.68	–
1666	Bulnesol	3.39	MS, RI
1678	Methyl epi-jasmonate	0.54	MS
1767	Eicosane	2.69	MS
	Total identified	93.06	
	Total detected	99.11	

Compounds in major relative abundance are described in bold.
LRI: Linear retention index relative to $C_7 - C_{30}$ n-alkanes on a HP-5MS column; MS: identification by mass spectra; RI: retention index; n.d. = not determined.
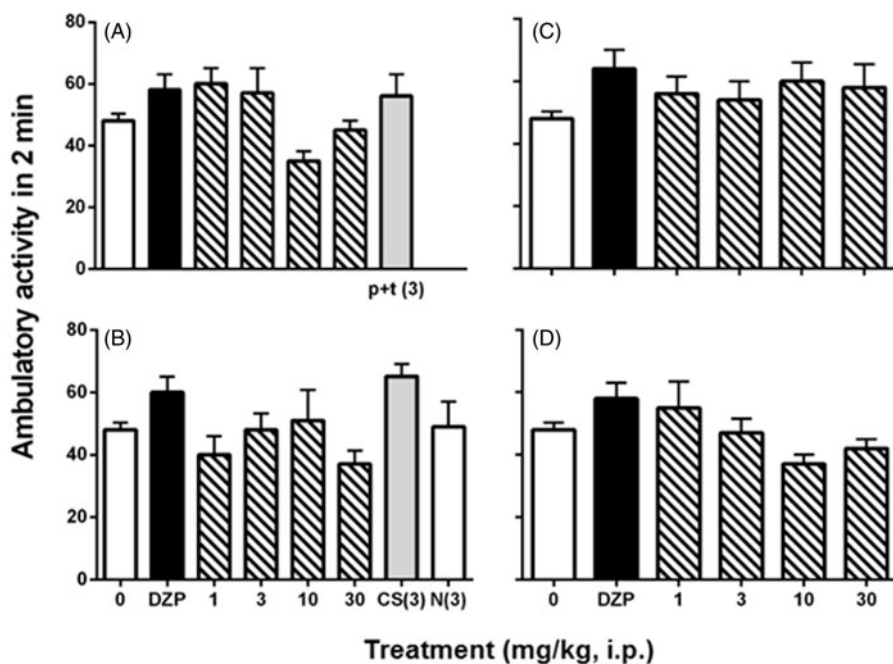
Figure 1. Pharmacological evaluation of the effects in the ambulatory activity (2 min) in mice receiving several doses (1, 3, 10 and 30 mg/kg, i.p.) of (a) hexane, (b) ethyl acetate, (c) methanol or (d) aqueous crude extracts in comparison to the control group (0), bioactive constituents such as mixture *p*-cymene + thymol (**p** + **t**, 3 mg/kg, i.p.), cirsimaritin (**CS**, 3 mg/kg, i.p.) and naringenin (**N**, 3 mg/kg,i.p.), and the reference drug diazepam (**DZP**, 0.1 mg/kg, i.p.). Bars represent the mean ± SEM of six animals.
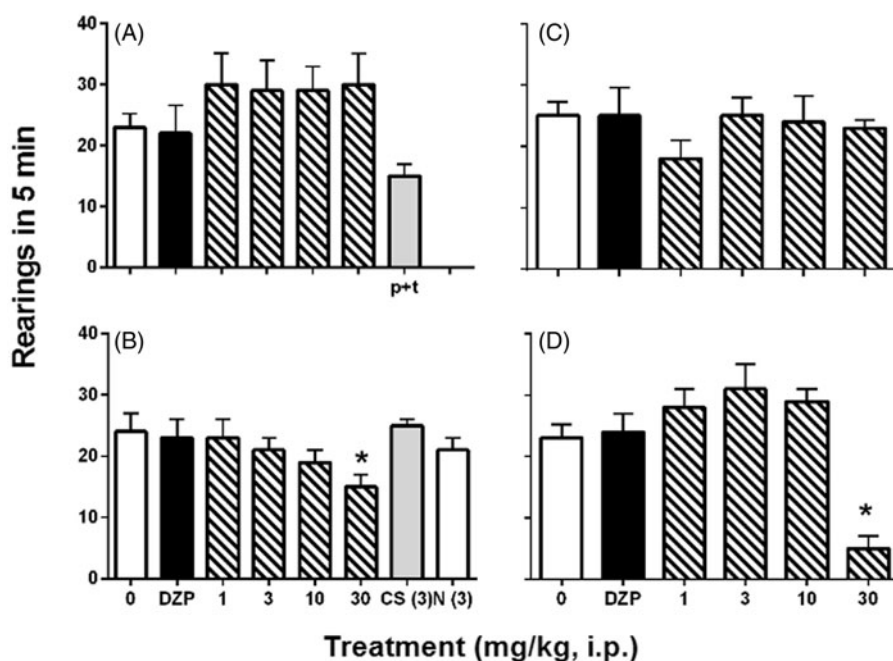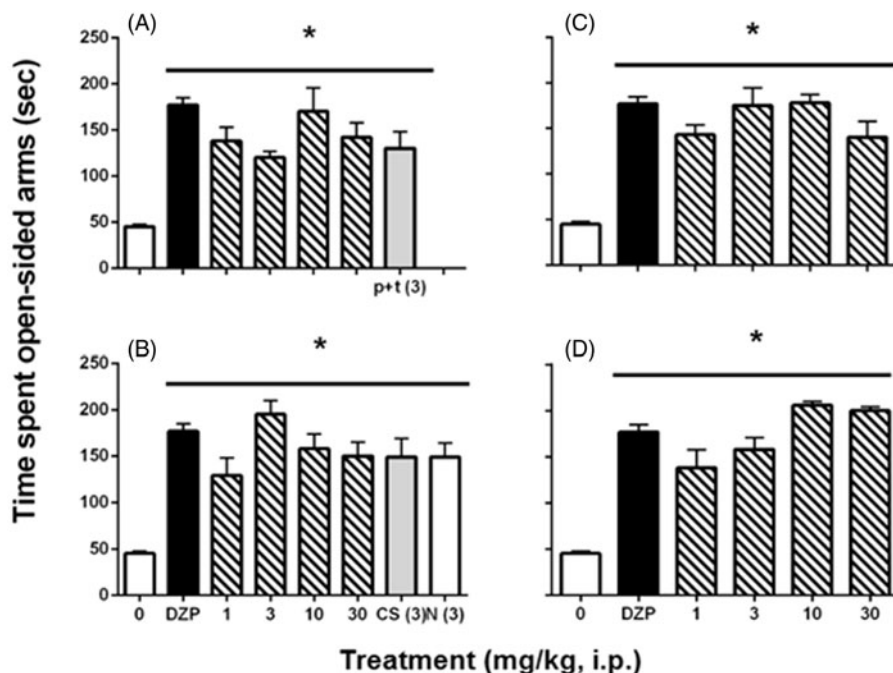
Figure 2. Pharmacological evaluation of the effects in the cylinder exploration (5 min) in mice receiving several doses (1, 3, 10, and 30 mg/kg, i.p.) of (a) hexane, (b) ethyl acetate, (c) methanol or (d) aqueous crude extracts in comparison to the control group (**0**), bioactive constituents such as mixture p-cymene + thymol (**p + t,** 3 mg/kg, i.p.), cirsimaritin (**CS**, 3 mg/kg, i.p.) and naringenin (**N**, 3 mg/kg,i.p.), and the reference drug diazepam (**DZP,** 0.1 mg/kg, i.p.). Bars represent the mean ± SEM of six animals. $p < 0.05$, *ANOVA followed by Dunnett's test. #Student's t test.
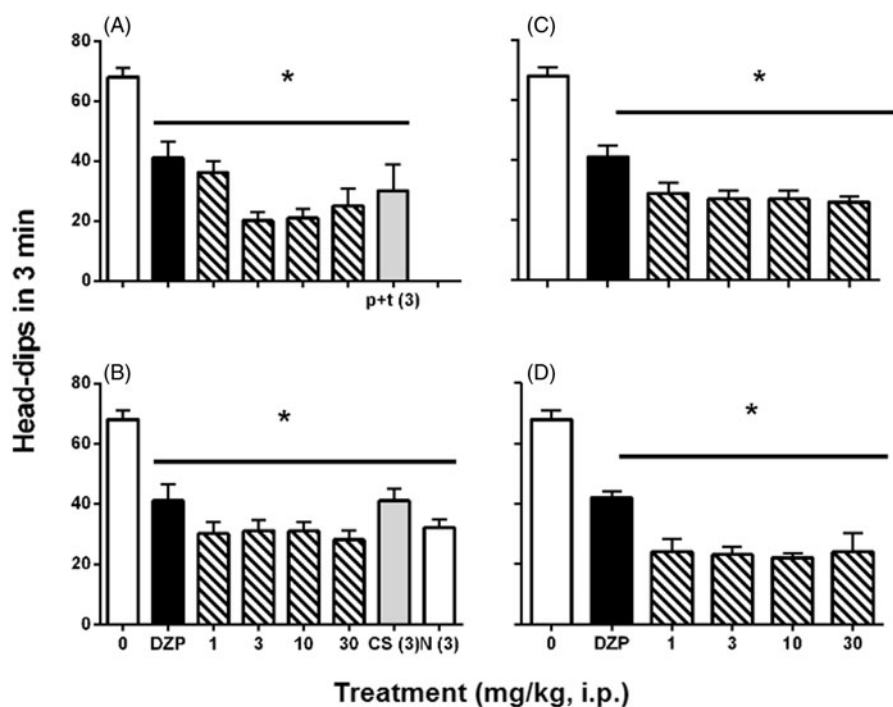
Figure 3. Anxiolytic-like effects in the hole-board exploration (3 min) in mice receiving several doses (1, 3, 10 and 30 mg/kg, i.p.) of (a) hexane, (b) ethyl acetate, (c) methanol or (d) aqueous crude extracts in comparison to the control group (**0**), bioactive constituents such as mixture p-cymene + thymol (**p + t,** 3 mg/kg, i.p.), cirsimaritin (**CS**, 3 mg/kg, i.p.) and naringenin (**N**, 3 mg/kg,i.p.), and the reference drug diazepam (**DZP,** 0.1 mg/kg, i.p.). Bars represent the mean ± SEM of six animals. *$p < 0.05$, ANOVA followed by Dunnett's test.

limonene, thymol, carvacrol, farnesol, ρ-cymene, α,β-caryophyllene, caryophyllene oxide and α-pinene have been described in the essential oil of *L. multiflora* (Folashade & Omoregie 2012). Compounds such as citral, α-myrcene, limonene, β-caryophyllene, caryophyllene oxide, carvacrol, linalool, α-, β-myrcene, *p*-cymene and thymol have been assessed as possible responsible for the anxiolytic and sedative-like effects of the essential oils of *L. alba* and *L. multiflora* (Vale et al. 1999, 2002; Abena et al. 2001; Pascual et al. 2001; Zétola et al. 2002; Hennebelle et al. 2008; Folashade & Omoregie 2012).

Figure 4. Anxiolytic-like effects in the plus-maze exploration (3 min) in mice receiving several doses (1, 3, 10 and 30 mg/kg, i.p.) of (a) hexane, (b) ethyl acetate, (c) methanol or (d) aqueous crude extracts in comparison to the control group (**0**), bioactive constituents such as mixture *p*-cymene + thymol (**p + t**, 3 mg/kg, i.p.), cirsimaritin (**CS**, 3 mg/kg, i.p.) and naringenin (**N**, 3 mg/kg,i.p.), and the reference drug diazepam (**DZP,** 0.1 mg/kg, i.p.). Bars represent the mean ± S.E.M. of six animals. $*p < 0.05$, ANOVA followed by Dunnett's test.

Table 2. Pharmacological interaction of the *L. graveolens* leaf organic and aqueous extracts and its bioactive compounds on the sodium pentobarbital-induced hypnosis in mice.

Treatment	Dose (mg/kg, i.p.)	Latency to sedation	The onset (min) Hypnosis	Duration of hypnosis (min)
Vehicle (0.9% saline solution)	–	1.23 ± 0.12	2.97 ± 0.32	11.93 ± 1.67
Diazepam	0.1	1.10 ± 0.08	2.00 ± 0.12	40.80 ± 3.27^b
Hexane	1	1.59 ± 0.13	3.61 ± 0.42	26.14 ± 5.65^a
	3	1.10 ± 0.21	3.80 ± 0.39	25.60 ± 2.33^a
	10	1.14 ± 0.21	3.78 ± 0.39	25.62 ± 2.33^a
	30	1.52 ± 0.09	4.01 ± 0.19	47.54 ± 6.66^b
p-Cymene + thymol	3	1.46 ± 0.12	4.03 ± 0.44	39.06 ± 3.83^b
Ethyl acetate	1	1.48 ± 0.14	3.11 ± 0.45	25.74 ± 2.21^b
	3	1.46 ± 0.21	4.00 ± 0.80	36.88 ± 2.57^b
	10	1.39 ± 0.18	2.84 ± 0.32	25.57 ± 4.28^b
	30	1.30 ± 0.21	2.80 ± 0.30	38.93 ± 5.71^b
Cirsimaritin	3	1.53 ± 0.10	2.95 ± 0.14	30.99 ± 1.72^b
Naringenin	3	1.10 ± 0.04	3.00 ± 0.26	27.32 ± 2.57^b
Methanol	1	1.79 ± 0.09	3.04 ± 0.20	25.03 ± 6.09^{ab}
	3	1.09 ± 0.11	2.75 ± 0.31	38.12 ± 4.86^b
	10	1.41 ± 0.23	2.61 ± 0.22	43.81 ± 1.35^b
	30	1.30 ± 0.21	2.75 ± 0.30	35.18 ± 6.22^b
Aqueous	1	1.35 ± 0.15	4.08 ± 0.60	25.23 ± 3.64^b
	3	1.49 ± 0.18	3.38 ± 0.23	27.81 ± 2.79^b
	10	1.40 ± 0.10	3.33 ± 0.30	34.48 ± 3.10^b
	30	1.52 ± 0.15	2.91 ± 0.31	40.32 ± 5.61^b

Data represents the mean ± SEM of six animals. $p < 0.05$.
[a]Student's *t* test.
[b]ANOVA followed by Dunnett's test.

Citral, α-myrcene and limonene, identified in *L. alba* as responsible for its anxiolytic and sedative-like effects of its essential oil (Vale et al., 2002), were assessed in the open-field, rotarod, plus-maze and sodium pentobarbital-hypnosis potentiation tests in mice at doses of 100 and 200 mg/kg (Vale et al. 1999,

2002). In the case of *L. graveolens,* the GC-MS profile of its essential oil has reported that carvacrol, *p*-cymene and thymol are some of its main constituents (Turgut & Silva 2005; Calvo-Irabien et al. 2009, 2014; Rivero-Cruz et al. 2011). In our investigation, we found that the *p*-cymene and thymol mixture were partially responsible for the effects of *L. graveolens* hexane extract. Moreover, thymol, *m*-cymen-8-ol, methyl salicylate, carvacrol and linalool were also identified as the main constituents of this hexane crude extract. The literature search yielded no previous records of anxiolytic effect of the *p*-cymene + thymol mixture; nevertheless, it has been reported that thymol, as individual compound, possesses this activity (Singh & Prasad 2014) and it is a possible allosteric regulator of human GABA$_A$ receptors (Priestley et al. 2003). On the basis of that, the mode of action of thymol is different to that of pentobarbital and propofol, which both are GABA receptor agonists. The synergy between the *p*-cymene + thymol mixture and pentobarbital observed in the hypnotic-response in this study might be related to this allosteric GABA$_A$-activation. Yet, it is not possible to suggest a specific union site for thymol in the GABA$_A$ receptor, because other possible mechanisms of action may be involved in this activity. In spite of the expected loss of volatile components during the drying process of herbs and spices, it has been reported that main constituents of *L. graveolens* might vary using different methods during postharvest drying. However, this variation was no significant on the qualitative composition or in the relative abundance of these main components in *L. graveolens* leaves (Calvo-Irabien et al. 2009). These results support the presence of such constituents in our studied species.

In our experimental exploration, not only the *p*-cimene + thymol mixture but also cirsimaritin and naringenin were partially responsible for the effects of *L. graveolens*. Regarding to the flavonoids such as apigenin, luteolin, naringenin, quercetin and their glycosylated derivatives have been identified in the aqueous or hydroalcoholic extract from leaves of *L. balansae,*

L. lasiocalycina, *L. lupulina*, *L. salviaefolia*, *L. sidoides*, *L. velutina*, *L. alba*, *L. multiflora* and *L. graveolens* (Abena et al. 2001; Zétola et al. 2002; Hennebelle et al. 2008; Funari et al. 2012). From the thin-layer chromatogram (data not shown), it was evident that the ethyl acetate extract shared components with the crude extracts of hexane and methanol, such as thymol and the flavonoid aglycones naringenin and cirsimaritin. Individual compounds were not isolated from the methanol and aqueous extracts given that both showed complex mixtures of flavonoids. Phytochemical analysis of a hydroalcoholic extract of *L. graveolens* has demonstrated the presence of 23 flavonoids such as galangin, apigenin, apigenin 7-*O*-glucoside, scutellarein, scutellarein 7-*O*-hexoside, luteolin, luteolin 7-*O*-glucoside, quercetin, pinocembrin, naringenin, eriodictyol, eriodictyol 7-*O*-glucoside and phloretin (Long-Ze et al. 2007).

Regarding the constituents of the ethyl acetate crude extract that showed anxiolytic-like response in this study, there are no reports for the *Lippia* species. However, it is known that flavonoids can modulate inhibitory neurotransmission; apigenin has been considered as a partial agonist that binds to the benzodiazepine site on the $GABA_A$ receptor (Viola et al. 1995; Avallone et al. 2000). This might not be the only mechanism associated to CNS inhibitory effect that is addressed to this group of compounds; for example, apigenin is also capable of inhibiting the N-methyl-D-aspartate (NMDA) receptor (Losi et al. 2004). Preliminary data have reported the anxiolytic and sedative-like effects of naringenin and its glycoside (naringin) in mice (Fernández et al. 2006, 2009; Anderson et al. 2012). Naringin (30 mg/kg, i.p.) reduced the exploratory behaviour in the hole-board test and the spontaneous locomotor activity (Fernández et al. 2006), whereas significantly increased in the lapse in the open arms test at 1 and 3 mg/kg, i.p. (Fernández et al. 2009). In the case of naringenin, it did not show effect on the spontaneous locomotor activity at a dosage of 15 mg/kg, i.p. (Fernández et al. 2006). These reports are in agreement with our study, where naringenin produced anxiolytic-like behaviour in the plus-maze and hole-board tests without altering the exploratory activity in open-field and cylinder tests. Our study showed that a similar anxiolytic-like response was obtained in the presence of cirsimaritin isolated from a crude ethyl acetate extract of *L. graveolens*; this result supports the reported effects of this compound isolated from an ethyl acetate fraction of the ethanol crude extract of *Rosmarinus officinalis* L. (Lamiaceae) (Abdelhalim et al. 2015). Moreover, the anxiolytic-like activity of cirsimaritin has been also associated to a $GABA_A$ receptor involvement in behavioural models in mice (Abdelhalim et al. 2015), and also in *in vitro* studies suggesting its effects as positive modulator of this neurotransmission (Kavvadias et al. 2003; Abdelhalim et al. 2014). The pharmacological interaction observed in the pentobarbital-induced hypnosis test corroborated the depressant activity of this species.

Finally, according to the LD_{50} calculated in this study, polar extracts of *L. graveolens* represents less risk of toxicity than non-polar extract. Our results are in part in agreement to those reporting low or not toxicity for the aqueous extract of this species using *Artemia salina*, as an *in vitro* analysis, and exploring qualitative- and quantitatively several tissues such as the liver and kidney of mice administrated with doses until 5000 mg/kg, via oral (Soto-Domínguez et al. 2012).

In conclusion, the anxiolytic-like effects of the aqueous and organic extracts obtained from *L. graveolens* leaves are confirmed in this study, its isolated constituents (*p*-cymene, thymol, cirsimaritin and naringenin), belonging to different chemical groups,

are partially responsible for the observed effects. This work gives evidence that supports the traditional use of *L. graveolens* as tranquilizer, suggesting its potential in the anxiety therapy.

Disclosure statement

The authors declare that there are no conflicts of interest.

References

Abena AA, Atipo-Ebata JK, Hondi AT, Diatewa M. 2001. Psychopharmacological properties of crude extract and essential oil of *Lippia multiflora*. Encephale. 27:360–364.

Abdelhalim A, Chebib M, Aburjai T, Johnston GAR, Hanrahan JR. 2014. GABAA receptor modulation by compounds isolated from *Salvia triloba* L. Adv Biol Chem. 4:148–159.

Abdelhalim A, Karim N, Chebib M, Aburjal T, Khan I, Johnston GAR, Hanrahan JR. 2015. Antidepressant, anxiolytic and antinociceptive activities of constituents from *Rosmarinus officinalis*. J Pharm Pharm Sci. 18:448–459.

Adams RP. 2007. Identification of essential oil components by gas chromatography and mass spectroscopy. Chicago (IL): Allured Publishing Corp.

Alwahsh MA, Khairuddean M, Keng-Chong W. 2015. Chemical constituents and antioxidant activity of *Teucrium barbeyanum* Aschers. Rec Nat Prod. 9:159–163.

Anderson W, Barrows M, Lopez F, Rogers S, Ortiz-Coffie A, Norman D, Hodges J, McDonal K, Barnes D, McCall S, et al. 2012. Investigation of the anxiolytic effects of naringenin, a component of *Mentha aquatica*, in the male Sprague-Dawley rat. Holist Nurs Pract. 26:52–57.

Avallone R, Zanoli P, Puia G, Kleinschnitz M, Schreier P, Baraldi M. 2000. Pharmacological profile of apigenin, a flavonoid isolated from *Matricaria chamomilla*. Biochem Pharmacol. 59:1387–1394.

Ballenger J. 1998. Comorbidity of panic and depression: implications for clinical management. Int Clin Psychopharmacol. 13:S13–S17.

Calvo-Irabien LM, Yam-Puc JA, Dzib G, Escalante-Erosa F, Peña-Rodríguez LM. 2009. Effect of postharvest drying on the composition of Mexican oregano (*Lippia graveolens*) essential oil. J Herbs Spices Med Plants. 15:281–287.

Calvo-Irabien LM, Parra-Tabla V, Acosta-Arriola V, Escalante-Erosa F, Díaz-Vera L, Dzib G, Peña-Rodríguez LM. 2014. Phytochemical diversity of the essential oils of Mexican oregano (*Lippia graveolens* Kunth) populations along an edapho-climatic gradient. Chem Biodivers. 11:1010–1021.

Castillo-Herrera GA, García-Fajardo JA, Estarrón-Espinosa M. 2007. Extraction method that enriches phenolic content in oregano (*Lippia graveolens* H.B.K.) essential oil. J Food Process Eng. 30:661–669.

Clark G, Koster A, Person DW. 1997. Exploratory behavior in chronic disulfoton poisoning in mice. Psychopharmacologia. 20:169–171.

Domínguez XA, Sánchez V, Suárez M, Baldas JH, González MR. 1989. Chemical constituents of *Lippia graveolens*. Planta Med. 55:208–209.

Fernández SP, Wasowski C, Loscalzo LM, Granger RE, Johnston GAR, Paladini AC, Marder M. 2006. Central nervous system depressant action of flavonoid glycosides. Eur J Pharmacol. 539:168–176.

Fernández SP, Nguyen M, Yow TT, Chu C, Johnston AR, Hanrahan JR, Chebib M. 2009. The flavonoid glycosides, myricitrin, gossypin and naringin exert anxiolytic action in mice. Neurochem Res. 34:1867–1875.

Folashade OK, Omoregie HE. 2012. Essential oil of *Lippia multiflora* Moldenke: a review. Pharm Sci. 2:15–23.

Funari CS, Eugster PJ, Martel S, Pierre-Alain C, Jean-Luc W, Dulce Helena SS. 2012. High resolution ultra high pressure liquid chromatography-time-of-flight mass spectrometry dereplication strategy of the metabolite profiling of Brazilian *Lippia* species. J Chromatogr A. 1259:167–178.

González-Güereca MC, Soto-Hernández M, Kite G, Martínez-Vázquez M. 2007. Antioxidant activity of flavonoids from the stem of the Mexican oregano (*Lippia graveolens* HBK var. *berlandieri* Schauer). Rev Fitotec Mex. 30:43–49.

González-Güereca MC, Soto-Hernández M, Martínez-Vázquez M. 2010. Isolation of (-)(2S)-5,6,7,3′,5′-pentahydroxyflavanone-7-*O*-β-D-glucpyranoside, from *Lippia graveolens* H.B.K. var. *berlandieri* Shauer, a new anti-inflammatory and cytotoxic flavanone. Nat Prod Res. 24:1528–1536.

González-Trujano ME, Navarrete A, Reyes B, Hang E. 1998. Some pharmacological effects of the ethanol extract of leaves of *Annona diversifolia* on the central nervous system in mice. Phytother Res. 12:1–3.

Gum AM, King-Kallimanis B, Kohn R. 2009. Prevalence of mood, anxiety, and substance-abuse disorders for older Americans in the National Comorbidity survey-replication. Am J Geriatr Psychiatry. 17:769–781.

Hennebelle T, Sahpaz S, Joseph H, Bailleul F. 2008. Ethnopharmacology of *Lippia alba*. J Ethnopharmacol. 116:211–222.

Hieu-Hong CN, Parvez M, Shaiq AM, Ahmed S, Ahmed W. 2002. Cirsimaritin. Acta Crystallogr. E58:o285–o287.

Hiller K, Zetler G. 1996. Neuropharmacological studies on ethanol extracts of *Valeriana officinalis*: behavioral and anticonvulsant properties. Phytother Res. 10:145–151.

Kavvadias D, Monschein V, Sand P, Riederer P, Chreier P. 2003. Constituents of Sage (*Salvia officinalis*) with *in vitro* affinity to human brain benzodiazepine receptor. Planta Med. 69:113–117.

Kyriakou E, Primikyri A, Charisiadis P, Katsoura M, Gerothanassis IP, Stamatis H, Tzakos AG. 2012. Unexpected enzyme-catalyzed regioselective acylation of flavonoid aglycones and rapid product screening. Org Biomol Chem. 10:1739–1742.

Leyva-López N, Nair V, Younga-Bang W, Cisneros-Zevallos L, Heredia JB. 2016. Protective role of terpenes and polyphenols from three species of oregano (*Lippia graveolens, Lippia palmeri* and *Hedeoma patens*) on the suppression of lipopolysaccharide-induced inflammation in RAW 264.7 macrophage cells. J Ethnopharmacol. 187:302–312.

Lister RG. 1987. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology. 92:180–185.

Long-Ze L, Mukhopadhyay S, Robbins RJ, Harnly MJ. 2007. Identification and quantification of flavonoids of Mexican oregano (*Lippia graveolens*) by LC-DAD-ESI/MS analysis. J Food Compost Anal. 20:361–367.

Lorke D. 1983. A new approach to practical acute toxicity testing. Arch Toxicol. 54:275–287.

Losi G, Puia G, Garzon G, de Vuono MC, Baraldi M. 2004. Apigenin modulates GABAergic and glutamatergic transmission in cultured cortical neurons. Eur J Pharmacol. 502:41–46.

Manenzhe JN, Potgieter N, van Ree T. 2004. Composition and antimicrobial activities of volatile components of *Lippia javanica*. Phytochemistry. 65:2333–2336.

Martínez-Rocha A, Puga R, Hernández-Sandoval L, Loarca-Piña G, Mendoza S. 2008. Antioxidant and antimutagenic activities of Mexican oregano (*Lippia graveolens* Kunth). Plant Foods Hum Nutr. 63:1–5.

Mujovo SF, Hussein AA, Meyer JJM, Fourie B, Tshilidzi M, Lall N. 2008. Bioactive compounds from *Lippia javanica* and *Hoslundia opposita*. Nat Prod Res. 22:1047–1054.

Pascual ME, Slowing K, Carretero E, Sánchez MD, Villar A. 2001. *Lippia*: traditional uses, chemistry and pharmacology: a review. J Ethnopharmacol. 76:201–214.

Pérez RM. 2014. Effect of Mexican oregano (*Lippia graveolens* Kunth) on streptozotocin induced diabetic mice and its role in regulating carbohydrate metabolism enzymes and their inhibitor effect on the formation of advances glycation end products. Annu Res Rev Biol. 4:3470–3491.

Pérez-Ortega G, Guevara-Fefer P, Chávez M, Herrera J, Martínez A, Martínez AL, González-Trujano ME. 2008. Sedative and anxiolytic efficacy of *Tilia americana* var. *mexicana* inflorescences used traditionally by communities of State of Michoacan, Mexico. J Ethnopharmacol. 116:461–468.

Priestley CM, Williamson EM, Wafford KA, Sattelle DB. 2003. Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human GABA(A) receptors and a homo-oligomeric GABA receptor from *Drosophila melanogaster*. Br J Pharmacol. 140:1363–1372.

Rivera G, Bocanegra-García V, Monge A. 2010. Traditional plants as source of functional foods: a review. J Food. 8:159–167.

Rivero-Cruz I, Duarte G, Navarrete A, Bye R, Linares E, Mata R. 2011. Chemical composition and antimicrobial and spasmolytic properties of *Poliomintha longiflora* and *Lippia graveolens* essential oils. J Food Sci. 76:309–317.

Singh BS, Prasad KM. 2014. To evaluate anti-anxiety activity of thymol. J Acute Dis. 3:136–140.

Soto-Domínguez A, García-Garza R, Ramírez-Casas Y, Morán-Martínez J, Serrano-Gallardo LB. 2012. The aqueous extract from oregano (*Lippia graveolens* HBK) from the north of Mexico has antioxidant activity without showing a toxic effect *in vitro* and *in vivo*. Int J Morphol. 30:937–944.

Stafford GI, Pedersen ME, van Staden J, Jäger AK. 2008. Review on plants with CNS-effects used in traditional South African medicine against mental diseases. J Ethnopharmacol. 119:513–537.

Turgut DN, Silva VR. 2005. Effect of water stress on plant growth and thymol and carvacrol concentrations in Mexican oregano grown under controlled conditions. J Appl Hort. 7:20–22.

Vale TG, Matos FJA, Lima TCM, Viana GSB. 1999. Behavioral effects of essential oils from *Lippia alba* (Mill) N.E. Brown chemotypes. J Ethnopharmacol. 67:127–133.

Vale TG, Furtado EC, Santos JG, Viana GSB. 2002. Central effects of citral, myrcene and limonene, constituents of essential oil chemotypes from *Lippia alba* (Mill) N.E. Brown. Phytomedicine. 9:709–714.

Van Den Dool H, Kratz PD. 1963. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J Chromatogr. 11:463–471.

Viola H, Wasowski C, Levi de Stein M, Wolfman C, Silvera R, Medina AE. 1995. Apigenin, a component of *Matricaria recutita* flowers, is a central benzodiazepine receptors ligand with anxiolytic effects. Planta Med. 61:213–216.

Zétola M, de Lima TCM, Sonaglio D, González- Ortega G, Limberger RP, Petrovick PR, Bassani VL. 2002. CNS activities of liquid and spray-dried extracts from *Lippia alba* (Verbenaceae) (Brazilian false melisse). J Ethnopharmacol. 82:207–215.