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Artículo / Article

Effects of essential oils from two *Lippia* species on growth of phytopathogenic fungi

[Efecto de los aceites esenciales de dos especies de *Lippia* sobre el crecimiento de hongos fitopatógenos]

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Abstract: Chemical characterization of the essential oils of two *Lippia* species by GC-MS and NMR spectroscopy revealed that limonene (84.3%) and β-caryophyllene (6.1%) were the most abundant components in *Lippia turbinata* while (6S,7S,10S)-trans-davanone (99.1%) predominated in *Lippia integrifolia*. Antifungal activity of the essential oils was determined by headspace volatile exposure assay against the fungal phytopathogenic *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Rhizoctonia solani*. The essential oil of *L. turbinata* showed potent antifungal activity against the panel of fungi tested while that the oil of *L. integrifolia* significantly inhibited the mycelial growth of *S. rolfsii* and *R. solani*.

Keywords: *Lippia turbinata*, *Lippia integrifolia*, essential oils, chemical composition, antifungal activity

Resumen: La caracterización química de los aceites esenciales de dos especies de *Lippia* por cromatografía gaseosa-espectrometría de masas (CG-EM) y espectroscopia de RMN reveló que limoneno (84,3%) y β-cariofileno (6,1%) fueron los componentes más abundantes de *Lippia turbinata* mientras que (6S,7S,10S)-trans-davanona (99,1%) predominó en *Lippia integrifolia*. La actividad antifúngica de los aceites esenciales se determinó por el ensayo de exposición a los vapores frente a los hongos fitopatógenos *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* y *Rhizoctonia solani*. El aceite esencial de *L. turbinata* mostró una potente actividad antifúngica frente al panel de hongos ensayados, mientras que el aceite de *L. integrifolia* inhibió significativamente el crecimiento micelial de *S. rolfsii* y *R. solani*.

Palabras clave: *Lippia turbinata*, *Lippia integrifolia*, aceites esenciales, composición química, actividad antifúngica

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INTRODUCTION

Lippia is a genus which belongs to the Verbenaceae family; it is represented by more than 200 species, distributed mainly in South and Central America, and Africa (Sanders, 2001). Several species of the genus *Lippia* are used in folk medicine mostly in dermatological, gastrointestinal and respiratory affections. In addition, most of these species are used as food condiment and flavoring (Pascual *et al.*, 2001). In Argentina, the two most widely used native species are *Lippia integrifolia* (Griseb.) Hieron ("incayuyo", "té del inca" or "inca yerba"), and *Lippia turbinata* Griseb. ("Poleo","té del país" or "té criollo"). In recent years, these species have become subject of scientific interest in view of other potential uses of its essential oils, for the most part, as antimicrobial (Bluma & Etcheverry, 2008; González & Marioli, 2010; Lima *et al.*, 2011; Passone *et al.*, 2013; Pérez-Zamora *et al*., 2016), and antioxidant agents (Quiroga *et al.*, 2013; Barbieri *et al*., 2016).

To our knowledge, the biological activities of the oils of *L. turbinata* and *L. integrifolia* collected in Salta province, Argentina, have not yet been explored. Therefore, the present work was undertaken with the main objective to investigate the effects of the essential oils on the radial growth of three phytopathogenic fungi, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Rhizoctonia solani*, by headspace volatile exposure assay. Previous reports on *L. integrifolia* and *L. turbinata* EOs have indicated antifungal activity (Bluma & Etcheverry, 2008; Lima *et al.*, 2011; Passone *et al.*, 2013), but they were performed on different fungal species from those assayed herein.

MATERIAL AND METHODS

Plant Material

Aerial parts of *L. integrifolia* and *L. turbinata* were collected at the flowering stage in Salta, Argentina. Voucher specimens were deposited at the Museo de la Facultad de Ciencias Naturales, Universidad Nacional de Salta, Salta, Argentina. Plant identification was made according to voucher specimen numbers MCNS 11880 for *L. integrifolia* and MCNS 11879 for *L. turbinata*.

Isolation of the essential oils

Dried aerial parts were subjected to hydrodistillation for 4 h in a Clevenger type apparatus. The essential oils obtained were stored at 4º C until further analysis.

GC-FID and GC/MS analysis

GC/MS analyses of the EOs were performed with a Perkin-Elmer Series Clarus 600 gas chromatograph equipped with a flame ionization detector (FID) and Clarus 600 T mass spectrometer detector, and a DB-5 MS fused-silica cap. column (60 m, 0.25 mm, 0.25 µm). The oven temperature was programmed isothermal at 60º C for 5 min, then rising from 60 a 240º C at 4º C/min, and isothermal at 240º C for 10 min; injector and detector temp.250º C; transferline temp., 200º C; carrier gas, He (49.6 psi); column head pressure, 15 psi. The split-injection mode was selected, and the ionization was carried out in the mass spectrometer under vacuum by electron impact (ionization energy, 70 eV). The chromatograms were acquired (scan mode, m/z 25-350) with a scan time of 0.2 s and an inter-scan time of 0.1 s. Diluted samples (1/100, v/v, in EtOH) of 1.0 µL were injected in the split mode (split ratio 50/1).

The constituents were identified by comparing their retention indices and mass spectra with those found on the NIST 02 library and in the literature (Adams, 2001). Retention indices were determined in relation to a homologous series of n-alkanes ($C_7 - C_{16}$ on DB-5) under the same operating conditions. The relative amounts of individual components were calculated on the GC-peak area (FID response) without using correction factors.

NMR Spectroscopy

NMR spectra were recorded on a Bruker Avance 400 (^{1}H at 400 MHz and ^{13}C at 100 MHz) spectrometer with TMS as internal reference. The sample was dissolved in $CDCl_3$. ^{1}H NMR and ^{13}C NMR analyses were performed on the whole sample, without any previous fractionation. The identification was based on comparison of the signals in the oil spectrum with reference spectra compiled in the laboratory spectral library and the literature (Kubeczka & Formacek, 2002; Skakovskii *et al*., 2010; Marcial *et al*., 2016).

Limonene: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.40 (m, H-6), 4.70 (br s, H-10), 1.97 (m, H-2), 1.81-1.76 (m, H-3), 2.10 (m, H-4), 2.05-1.93 (m, H-5), 1.65 (s, H-7), 1.73 (br s, H-9).^{13}C NMR (100 MHz, $CDCl_3$): δ 133.7 (s, C-1), 30.6 (t, C-2), 27.9 (t, C-3), 41.1 (d, C-4), 30.8 (t, C-5), 120.6 (d, C-6), 23.2 (q, C-7), 150.3 (s, C-8), 20.9 (q, C-9),108.3 (t, C-10).

(6*S*,7*S*,10*S*)-trans-davanone: ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.75 (d, *J*= 1.1, H-1), 5.33 (m, H-3), 3.21

(dd, *J*= 6.9, 18.1, H-4a), 3.28 (dd, *J*= 6.9, 18.1, H-4b), 2.70 (dq, *J*=6.9, 8.5, H-6), 4.05 (ddd, *J*= 6.9, 8.4, H-7), 1.66 (m, H-8a), 1.70 (m, H-8b), 1.71 (m, H-9a), 1.86 (m, H-9b), 5.80 (dd, *J*= 10.6,17.2, H-11), 4.95 (dd, *J*=9.1, 1.6, H-12a), 5.13 (dd, *J*= 17.3, 1.6, H-12b), 1.62 (br s, H-13), 1.01 (d, *J*= 6.9, H-14), 1.29 (s, H-15). ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.7 (q, C-1), 135.0 (s, C-2), 116.1 (d, C-3), 42.7 (t, C-4), 211.9 (s, C-5), 51.2 (d, C-6), 80.5 (d, C-7), 29.3 (t, C-8), 36.8 (t, C-9), 83.0 (s, C-10), 143.6 (d, C-11), 111.3(t, C-12), 18.1 (q, C-13), 12.8 (q, C-14), 27.2 (q, C-15).

Antifungal Activity Assay

The fungi tests were field isolates maintained on potato dextrose agar (PDA) plates (*Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Rhizoctonia solani*). Inoculated plates were sealed with parafilm and incubated at 20º C until confluent growth across the agar surface occurred. Plates were then stored at 4º C. Antifungal activity was determined by headspace volatile exposure assay.

To agar plates containing 5 mL PDA in glass Petri dishes (Ø= 50 mm) placed with the lid upside down was added essential oil in a small jar plugged with cotton having no direct contact with the PDA agar plates (Vilela *et al.*, 2009). Oil doses were 0 (control), 10 and 20 µL/Petri dish. At the center of each dish a 5 mm diameter disk of fungal species cultivated for not less than 7 days was placed. The plates were sealed with polyethylene film and incubated in the dark at 22 ± 2º C. Five replicates were prepared for each treatment. The oil samples and controls were randomly arranged. The colony diameter was measured after 3 days for *S. sclerotiorum* and *S. rolfsii*, and after 7 days for *R. solani*. The percentage inhibition of growth was calculated for each colony according to the following formula: % Inhibition= [(*C*-*T*)/*C*] x 100, where *C* is the average diameter of the colonies of control, in mm, and *T* is the average diameter of the colonies developed in each treatment. The fungicidal or fungistatic activity of the vapor of *L. turbinata* originating from different application concentrations was confirmed by transferring half of the mycelial plug onto fresh PDA agar after 3 days for *S. sclerotiorum* and *S. rolfsii*, and after 7 days for *R. solani*. The activity of EO was regarded as fungicidal if 100% growth inhibition after initial exposure was maintained following transfer of the inoculated plug to fresh agar.

Statistical analysis

The statistical significance of assay results was determined by variance analysis with the Infostat Programme. Differences between means were tested by Kruskal-Wallis test; differences between experiment and control were significant with a value of $p \leq 0.05$.

RESULTS AND DISCUSSION

Hydrodistillation of the dried aerial parts of *L. integrifolia* and *L. turbinata* gave yellow essential oils in yields of 0.76% and 0.32% (w/w) respectively. Table 1 shows the components of EOs. The major component of *L. integrifolia* was davanone (99.1%), while the essential oil of *L. turbinata* was characterized by a high percentage of monoterpenes, limonene (84.3%) being the main component.

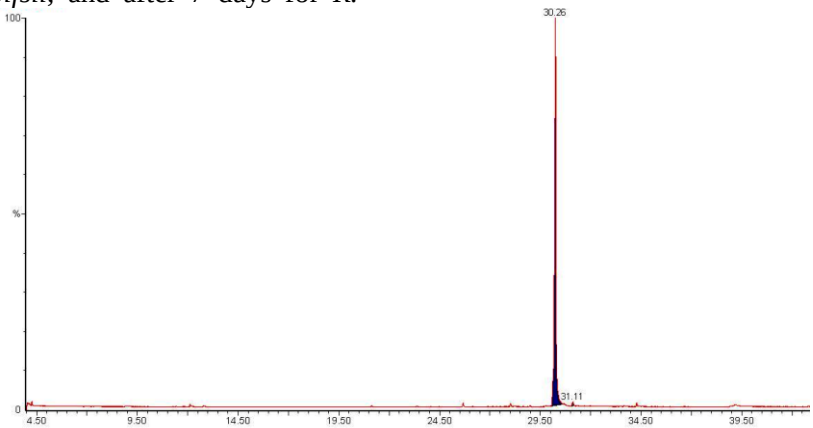

Figure 1
Chromatogram of *Lippia integrifolia* essential oil

A recent study reported that essential oil composition of *L. integrifolia* collected from different locations in Argentina had different chemical constituents. According to this report, five chemotypes were observed: *trans*-davanone chemotype, lippifolienone chemotype, β-davanone-2-ol chemotype, spathulenol/bicyclogermacrene chemotype, and *trans*-nerolidol chemotype (Marcial *et al.*, 2016). In this study, the high percentage of davanone (99.1%) clearly indicates that the plant belongs to *trans*-davanone chemotype. In addition, the specific optical rotation recorded for essential oil of *L. integrifolia* { $[\alpha]_D$ (20º C) + 65.9 (c 1.0 in $CHCl_3$) } was in agreement with those reported for (6*S*,7*S*,10*S*)-trans-davanone (Marcial *et al.*, 2016).

In this work, at the doses tested (Table 2), the essential oil of *L. integrifolia* showed significant antifungal activities (inhibition % > 60%) against *R. solani* and *S. rolfsii*, while not affecting the growth of *S. sclerotiorum*.

In a previous study, essential oil of *L. integrifolia* with borneol, terpinen-4-ol and lippifoli-1(6)-en-5-one as major constituents has shown, *in vitro*, an interesting spectrum of antifungal properties (Lima *et al.*, 2011). However we did not find in the literature any report of antifungal properties of essential oils of *trans*-davanone chemotype.

Table 1
Composition of the essential oils extracted from aerial parts of *Lippia turbinata* and *Lippia integrifolia*.

Components	RI[a]	Peak area (%		Identification
		L. integrifolia	*L. turbinata*	
Davanone	1588	99.1	-	MS, RI, ^{1}H and ^{13}C NMR
Camphene	954	-	Tr	MS, RI
Limonene	1034	-	84.3	MS, RI, ^{1}H and ^{13}C NMR
Camphor	1146	-	1.5	MS, RI
Carvone	1245	-	1.1	MS, RI
Piperitenone oxide	1369	-	1.3	MS, RI
β – caryophyllene	1419	-	6.1	MS, RI
Total		99.1	94.3	

[a] Retention indices on a DB-5 column; tr = trace amount (< 0.05%)

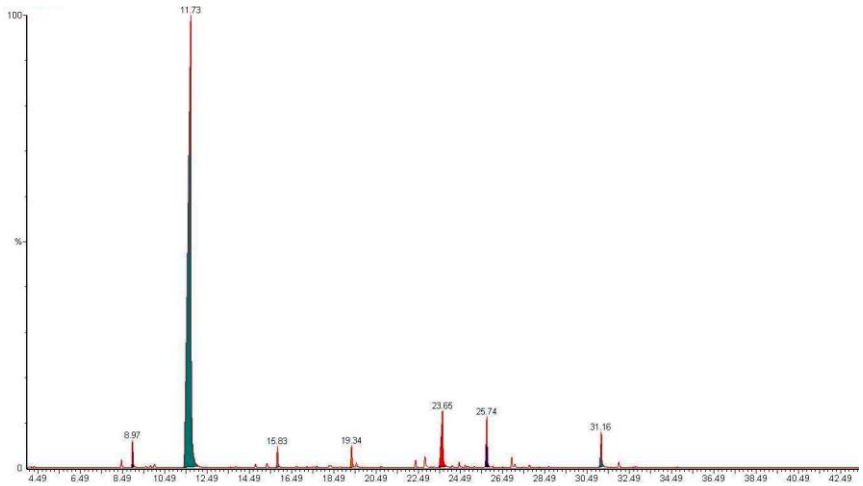

Figure 2
Chromatogram of *Lippia turbinata* essential oil

Table 2
Growth inhibition of fungal species by essential oils of *Lippia turbinata* and *Lippia integrifolia.*

Essential Oils	Dose (µL of oil/petri dish)	*Sclerotium rolfsii*	*Sclerotinia sclerotiorum*	*Rhizoctonia solani*
		Growth mm	Growth mm	Growth mm
L. turbinata	0	45.0 ± 0.0	45.0 ± 0.0	45.0 ± 0.0
	10	0.0 ± 0.0* (100)	0.0 ± 0.0 a* (100)	0.0 ± 0.0 (100)
	20	0.0 ± 0.0* (100)	0.0 ± 0.0* (100)	0.0 ± 0.0 (100)
L. integrifolia	0	45.0 ± 0.0	45.0 ± 0.0	44.8 ± 0.4
	10	15.6 ± 8.7* (65.3)	38.4 ± 7.2 (14.7)	10.4 ± 1.8* (76.9)
	20	12.7 ± 7.2* (71.8)	33.1 ± 17.8 (26.4)	9.6 ± 3.5* (78.7)

The growth of fungal species is given as mean ± SD of five replicates. *Significant differences, compared to the control for $p \leq 0.05$, according to Kruskal-Wallis's test. Figures in parenthesis indicate percent inhibition

Moreover, the essential oil of *L. turbinata* caused complete growth inhibition of all the pathogens tested via a fungicidal mode. It is highly probable that the antifungal activity of *L. turbinata* essential oil is attributed to its high limonene content (84.3%). This compound evidences fungicidal activity against *Trichophyton rubrum*, inducing changes in cell membrane integrity and metabolic activity (Chee *et al.*, 2009). Recently, it has been reported that limonene may show antifungal activity by inhibition of pectin methyl esterase (PME) and cellulase enzymes (Marei *et al.*, 2012). In previous studies, other authors (Bluma & Etcheverry, 2008; Passone *et al*., 2012; Passone *et al.*, 2013) have reported antifungal properties for this oil, finding piperitenone oxide (48.6%) and limonene (24.5%) as major components, while in our study, piperitenone oxide only represented 1.6% of the oil. They evaluated the antifungal activity against *Aspergillus flavus* and *Aspergillus parasiticus*. In general, EO composition showed significant chemodiversity according to extraction method and geographical origin (Bakkali *et al*., 2008), showing the importance of analyzing plants from different locations.

CONCLUSIONS

The composition of our *L. turbinata* sample agrees with that in previous reports of plants collected from Cordoba province, Argentina (Quiroga *et al.*, 2013), regarding the presence of limonene as a major constituent (% > 75). On the other hand, our findings are in agreement with those of the previous chemical composition analysis of plants of *L. integrifolia* from the same province (Marcial *et al.*, 2016). It should be noted that in our oil, content of (6*S*,7*S*,10*S*)-trans-davanone (99.1%) is the highest reported so far for this ketone in any other essential oil.

In our study, *L. turbinata* essential oil was highly effective in vapour phase against *S. sclerotiorum*, *S. rolfsii* and *R. solani*, and its activity was higher than *L. integrifolia* EO. In addition, this is the first report on the biological activities of *trans*-davanone chemotype from *L. integrifolia*.

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