

Antibacterial activity of essential oils of *Aloysia polystachya* and *Lippia turbinata* (Verbenaceae)

[Actividad antibacteriana de los aceites esenciales de *Aloysia polystachya* y *Lippia turbinata* (Verbenaceae)]

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Abstract: *Aloysia polystachya* and *Lippia turbinata* are medicinal and aromatic plants. Nevertheless, there are few reports in literature concerning the biological properties of species that grow in northeastern Argentina. The antibacterial activity and the chemical composition of both essential oils were evaluated in this work. The extraction was performed by steam distillation and their volatile compounds were determined by gas chromatography/mass spectrometry. The antibacterial activity was evaluated by disc diffusion and broth microdilution assay. The main compounds were carvone (78.9%) and limonene (14.2%) in *A. polystachya* and carvone (80.77%), limonene (8.73%), β -caryophyllene (2.13%) and 1,8-cineole (1.70%) in *L. turbinata*. Both essential oils were bactericide against *Escherichia coli* ATCC 35218 and clinical isolates of *Enterobacter cloacae* and *Klebsiella pneumoniae*. Essential oil of *A. polystachya* was also bactericide against *Staphylococcus aureus* ATCC 29212, *S. aureus* ATCC 25923 and clinical strain of *S. aureus* methicillin susceptible.

Keywords: aromatic plants; terpene; carvone; limonene; beta-caryophyllene; 1,8-cineole

Resumen: *Aloysia polystachya* y *Lippia turbinata* son plantas medicinales y aromáticas. Hay pocos informes en la literatura sobre las propiedades biológicas de especies que crecen en el nordeste de Argentina. La actividad antibacteriana y la composición química de ambas especies se evaluaron en este trabajo. La extracción se realizó por destilación con vapor y sus compuestos se determinaron por cromatografía gaseosa/espectrometría de masa. La actividad antibacteriana fue evaluada por difusión en discos y microdilución en caldo. Los principales compuestos fueron carvona (78.9%) y limoneno (14.2%) en *A. polystachya* y carvona (80.77%), limoneno (8.73%), β -cariofileno (2.13%) y 1,8-cineol (1.70%) en *L. turbinata*. Ambos aceites esenciales fueron activos contra *Escherichia coli* ATCC 35218 y aislamientos clínicos de *Enterobacter cloacae* y *Klebsiella pneumoniae*. El aceite esencial de *A. polystachya* fue bactericida contra *Staphylococcus aureus* ATCC 29212, *S. aureus* ATCC 25923 y aislamientos clínicos de *S. aureus* sensible a metilicina.

Palabras clave: plantas aromáticas, terpenos, carvona, limoneno, beta-cariofileno, 1,8-cineol.

Recibido | Received: July 25, 2015

Aceptado | Accepted: September 2, 2015

Aceptado en versión corregida | Accepted in revised form: February 17, 2016

Publicado en línea | Published online: July 30, 2016

Declaración de intereses | Declaration of interests: To Secretariat of Research, Science and Technology of Universidad Nacional de Chaco Austral for financial support.

Este artículo puede ser citado como / This article must be cited as: CM Pérez-Zamora, CA Torres, MI Aguado, AJ Bela, MB Nuñez, C Bregni. 2016. Antibacterial activity of essential oils of *Aloysia polystachya* and *Lippia turbinata* (Verbenaceae). **Bol Latinoam Caribe Plant Med Aromat** 15 (4): 199 – 205.

INTRODUCTION

Verbenaceae family includes some 30 genera growing in tropical and subtropical regions worldwide. *Aloysia* is an amphotropical genus of 30 species native to arid, temperate and subtropical regions of North and South America. *Aloysia polystachya* (Griseb.) Moldenke is being popularly used as sedative, eupeptic and carminative agent by Argentinians and Brazilians (Souza-Pina et al., 2012). This species has few reports on its antimicrobial activity but there information of other species of the *Aloysia* genus (Souza-Pina et al., 2012). Some examples of species *Aloysia* with antimicrobial activity are *A. triphylla* (Parodi et al., 2013; Oliva et al., 2015), *A. grattissima* (Santos et al., 2013), *A. virgata* (Montanari et al., 2011). *A. polystachya* has showed absence of genotoxicity in previous reports (Ricco et al., 2010). Several authors have demonstrated repellent, insecticide and ovicidal activities and contact toxicity (Benzi et al., 2009; Werdin-González et al., 2010; Benzi et al., 2014).

The *Lippia* genus also belongs to the Verbenaceae family and contains more than 200 species distributed throughout Africa and South America. In Argentina, *L. turbinata* (Griseb.) is widely used in folk medicine as digestive, diuretic, emenagogue, tonic, sedative and in the treatment of breathing illnesses (Coll-Aráoz & Ponessa, 2007). Diuretic, emmenagogue, anti-inflammatory, hypotensive dose-dependent and antimicrobial properties were reported for this species (Alonso & Desmarchelier, 2006). Hernández et al. (2000) proved the antimicrobial activity of methanolic extract of *L. turbinata* against *Staphylococcus aureus*, *Streptococcus sp.*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Shigella sp.* The extract fractions of *L. turbinata* obtained with MeOH-CH₂Cl₂ showed growth inhibition of *Mycobacterium tuberculosis* (Wachter et al., 2001).

In spite of being a very popular plants used in folk medicine, there are few reports about the antimicrobial properties of their essential oils. Therefore, in this study, we determined the antibacterial activity and the major chemical compounds of the essential oils (EOs) of *A. polystachya* and *L. turbinata* growing in the province of Chaco -northeast of Argentina- in order to expand their medicinal application.

MATERIALS AND METHODS

Plant material

The material was collected in the summer from the Campus of the Universidad Nacional del Chaco Austral (UNCAUS), Route 95, 10 km south of Presidencia Roque Sáenz Peña, coordinates: S 26° 51' 47.9" and W 60° 26' 18.6". The specimens were deposited and conserved in the Instituto de Botánica del Nordeste (IBONE-CONICET) in Corrientes, Argentina with voucher numbers I-1 (*L. turbinata*) and I-6 (*A. polystachya*).

Essential oils extraction

Both EOs were obtained by means of steam distillation (hydrodistillation) using a Clevenger-type apparatus with a Dean Stark trap.

Bacterial strains

The bacteria used were *Staphylococcus aureus* ATCC 25923 and ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853. Clinical isolates were provided by the Hospital "4 de Junio" (Sáenz Peña city, province of Chaco). The isolates used were: *Staphylococcus aureus* (methicillin susceptible and methicillin resistant), *Klebsiella pneumoniae* and *Enterobacter cloacae*.

Antimicrobial test

The antibacterial activity of the EOs was assayed using first the agar disc diffusion method and then the broth microdilution method according to CLSI (2006a, 2006b). Sterile filter discs with 6 mm in diameter (OXOID, Great Britain) were impregnated with 15 µL of EO and serial dilutions in dimethyl sulfoxide (DMSO), ranging from 1.82 to 29.13 µL/mL of the EOs, were prepared in a 96 well microtitre plate, including a growth control, a solvent control (Mueller Hinton Broth + Tween 80 + DMSO) and sterility control (Mueller Hinton Broth + Tween 80 + tested oil). To determine MBC, 10 µL of each culture medium was taken from each well and inoculated in Mueller Hinton Agar for 16-20 hours at 37° C. The MBC was defined as the lowest concentration of the essential oil at which 99.99% or more of the initial inoculum was killed. The number of surviving organisms was determined by viable count.

Essential oils analysis

The main components of the essential oils were separated and identified by gas chromatography and mass spectrometry (GC/MS) in a Clarus 600 equipment, Perkin Elmer (Instituto Multidisciplinario de Biología Vegetal (IMBIV) Universidad Nacional de Córdoba), under the following conditions: DB5 column (60 m x 0.25 mm, ID x 0.25 µm), carrier: helium (49.6 psi), injector: 250° C. Program data: 60° C initial temperature (5 min), ramp: 5° C/min, final temperature 240° C (10 minutes). The samples were injected diluting 1/100 µL of ethanol, injection volume 1 µL in injection mode split (50/1). Chromatogram was obtained in mode "scan", from 50 to 350 m/z (scan time: 0.2 s, inter-scan time: 0.1

s). Major components of the essential oils were obtained using TurboMass 5.4.2 program. The retention index was calculated using a homologous series of n-alkanes C7-C17. Compounds were identified by comparison of their retention indices, mass spectral libraries (NIST MS Swarch 2.0) and with the data of literature (Sawamura, 2010; El-Sayed, 2014). The analysis was performed relative percent of components as mean values (n = 3) calculated from the peak area.

RESULTS**Antimicrobial activity**

The antibacterial activity against pathogenic bacteria is presented in Table 1.

Table 1
Antibacterial activity of essential oils of *Lippia turbinata* and *Aloysia polystachya* against pathogenic bacteria

Microorganisms	EOs						Antibiotic	
	DD (mm)		MIC(µL/mL)		MBC(µL/mL)		DD (mm)	MIC (µg/mL)
Reference strains	LT	AP	LT	AP	LT	AP	AMP	AMP
<i>Gram positive</i>								
<i>Enterococcus faecalis</i> ATCC 29212	9	9	7.28	7.28	R	R	24-25	0.8
<i>Staphylococcus aureus</i> ATCC 25923	11	9.5	3.64	3.64	R	7.28	18-20	1.6
<i>S. aureus</i> ATCC 29213	10	8.5	7.28	7.28	R	29.13	23-21	0.8
<i>S. epidermidis</i> ATCC 12228	21	19	7.28	7.28	R	R	25-26	0.8
<i>Gram negative</i>								
<i>Escherichia coli</i> ATCC 35218	16	10	3.64	3.64	7.28	29.13	ND	202.8
<i>Pseudomonas aeruginosa</i> ATCC 27853	ND	ND	R	R	R	R	ND	ND/R
Clinical isolated strains								
<i>Gram positive</i>								
<i>S. aureus</i> (methicillin susceptible)	19	10	7.28	14.56	R	29.13	15-16	51.2
<i>S. aureus</i> (methicillin resistant)	6	10	29.13	29.13	R	R	9-9	101.4
<i>Gram negative</i>								
<i>Klebsiella pneumoniae</i>	10	10	7.28	14.56	29.13	29.13	ND	25.2
<i>Enterobacter cloacae</i>	12	12	7.28	14.56	29.13	29.13	ND	204.8

References: DD: diameter of inhibition (mm) including disc diameter of 6 mm. R: resistant, ND: not detected. AP: *Aloysia polystachya*, LT: *Lippia turbinata*, AMP: ampicillin.

Table 2
Major constituents of *A. polystachya* and *L. turbinata* essential oils

<i>A. polystachya</i> constituents	RI _c (DB5)	RI _d (DB5)	Relative area (% ± SD)
<i>Limonene</i>	1035	1036	14.2±0.11
<i>α-terpineol</i>	1200	1207	0.26±0.03
<i>Verbenone</i>	1234	1218	1.58±0.01
<i>Carvone</i>	1251	1271	84.4±0.63
<i>L. turbinata</i> constituents	RI _c (DB5)	RI _d (DB5)	Relative area (% ± SD)
<i>Limonene</i>	1036	1036	8.73±0.78
<i>1,8-cineole</i>	1039	1038	1.70±0.02
<i>α-terpineol</i>	1201	1207	0.84±0.18
<i>trans-carveol</i>	1229	1229	0.85±0.19
<i>Carvone</i>	1259	1271	80.7±4.13
<i>cis-carvyl acetate</i>	1333	1362	0.93±0.03
<i>Methyl-eugenol</i>	1398	1401	0.63±0.03
<i>β-caryophyllene</i>	1447	1467	2.13±0.18

References: RI_c: calculated retention index, RI_d: data base retention index.

Chemical analysis of the essential oils

The extraction yield of both EOs ranged between 0.7 and 1.2% v/w aird plant. The major components of EOs of *A. polystachya* and *L. turbinata* are shown in the Table 2.

DISCUSSION

Antimicrobial activity

In the agar disk diffusion test, both EOs presented antimicrobial activity against the most of the microorganisms tested. Only *P. aeruginosa* ATCC 27853 was not affected by the EOs. Ferreira-Sarrazin et al. (2012) have shown that this bacterium is the least sensitive of the Gram negative bacteria to the action of EOs.

The results mentioned above are partially agreed with Demo et al. (2005), who evaluated the antibacterial activity of EOs of these species growing in Cordoba province (Argentina). They found inhibition halos of both EOs for *S. aureus* ATCC 25212, *Bacillus cereus* and *Proteus mirabilis*, but not

for *S. epidermidis*, *E. coli* and *Klebsiella sp.* These divergences in the activities reported could be explained by the differences in the bacterial strains, and the source of plants (influence of habitat and climatic conditions, different harvest times), the naturally varying of EOs even in the same species due to the presence of chemotypes, as well as the mode of extraction of the EOs used.

Both EOs showed antimicrobial activity with MIC values between 3.64 and 7.28 µL/mL towards reference strains, except for *P. aeruginosa*. MIC values of EO of *L. turbinata* were lower than those from EO of *A. polystachya* for clinical isolates except against *S. aureus* methicillin resistant.

MBC values were observed between 7.28 and 29.13 µL/mL when EO of *A. polystachya* was tested. In this case, *Staphylococcus aureus* strains and *E. coli*, clinical isolates of *E. cloacae* and *K. pneumoniae* were affected. Furthermore, when EO of

L. turbinata was used, MBC values between 7.28 and 29.13 $\mu\text{L}/\text{mL}$ were detected but only against *E. coli*, *E. cloacae* and *K. pneumoniae*. The solvent used as the control in this test did not indicate any activity; whereas the MIC values for ampicillin reflected the resistance of *S. aureus* (methicillin susceptible and methicillin resistant), *E. coli* ATCC 35218, and *E. cloacae* to this antibiotic (CLSI, 2010). The EO of *A. polystachya* showed MBC values against six of the bacteria tested whereas the EO of *L. turbinata* showed MBC values only against three of them.

The EO of *A. polystachya* from Ribeirão Preto showed MIC values of 28.2 $\mu\text{L}/\text{mL}$ in fresh leaves and dried leaves against *E. coli*, 14.1 $\mu\text{L}/\text{mL}$ for *S. aureus* ATCC 6538 and both values were smaller than that of Gentamicin (Souza-Pina *et al.*, 2012).

However, the MIC values obtained in our assay are better and all clinical isolates were inhibited at concentrations of 7.28 to 29.13 $\mu\text{L}/\text{mL}$, including *S. aureus* methicillin resistant.

The antimicrobial activity would be related to the presence of terpenes compounds like carvone, limonene, caryophyllene and 1,8-cineole, main constituents of the EOs investigated.

The oxygenated monoterpenes (carvone, dihydrocarvone) and limonene are potential inhibitors of bacteria (Gallucci *et al.*, 2010; Porto *et al.*, 2010). Also, caryophyllene and 1,8-cineole present antimicrobial activity (de Souza *et al.*, 2005). An increase in activity dependent upon the type of alkyl substituent incorporated into a nonphenolic ring structure appeared to occur in that case. In accordance with Dorman and Deans (2000), limonene has an alkenyl substituent (1-methylethenyl), which results in increased antibacterial activity; the inclusion of a double bond increased its activity.

Chemical analysis of the essential oils

The GC/MS analysis of EO of *A. polystachya* showed five compounds (93.1%) and the major components were carvone (78.86%), and limonene (14.23%). These results are in agreement with those reported by Benzi *et al.* (2014), they identified carvone and limonene as the main components in essential oil of *A. polystachya* from Rio Negro province (Argentina). Souza-Pina *et al.* (2012) evaluated the essential oil of *A. polystachya* grown in Ribeirão Preto (Brasil) and

they observed a difference in oil composition between fresh and dried leaves; but again the main components identified were carvone (80.71 to 77.95%) and limonene (20.22 to 14.65%). Werding-González *et al.* (2010) reported carvone (83.5%) in EO of *A. polystachya* from Bahía Blanca city, Argentina. In San Luis, cis-thujone and carvone (73.4%) were detected; whereas in Salta, carvone (61.0-74.3%) was the main compound and in Córdoba, limonene (41.3%), carvone (16.5%) and mircenil acetate (9.6%) were found (Juárez *et al.*, 2011).

Cabanillas *et al.* (2003), studying *A. polystachya* native from Argentina reported that the species has two chemotypes, one with predominance of carvone and the second with α -thujone. The EO obtained in this work belongs to the first of them.

The GC/MS analysis of EO of *Lippia turbinata* showed nine compounds (93.3%) and the major components were carvone (80.77%), limonene (8.73%), β -caryophyllene (2.13%) and 1,8-cineole (1.70%). Duschatzky *et al.* (1998) identified limonene (43.3%), piperitenone oxide (24.8%), 1,8-cineole (14.7%) in EO of *L. turbinata* from San Luis. Nevertheless, Moriconi *et al.* (2009) observed in their work the major components were cis- α -bisabolene, limonene, β -caryophyllene, germacrene-D.

CONCLUSION

The EO of *L. turbinata* and *A. polystachya* presented antimicrobial activity with MIC values between 3.64 and 7.28 $\mu\text{L}/\text{mL}$ for reference strains, except for *P. aeruginosa*.

Both essential oils showed MBC values against *Escherichia coli* ATCC 35218, clinical isolates of *Enterobacter cloacae* and *Klebsiella pneumoniae*. Essential oil of *A. polystachya* showed also MBC values against *Staphylococcus aureus* ATCC 29212, *S. aureus* ATCC 25923 and *S. aureus* methicillin susceptible.

The main compounds were carvone (78.9%) and limonene (14.2%) in *A. polystachya* and carvone (80.77%), limonene (8.73%), β -caryophyllene (2.13%) and 1,8-cineole (1.70%) in *L. turbinata*. The antimicrobial activity would be related to the presence of these compounds.

In summary, our results indicate that these EOs have good antibacterial activity. They were able to inhibit the growth of several ampicillin resistant

bacteria. These results support the medicinal uses of these plants and they can make an important contribution to medicine and the primary health care.

Acknowledgments

We are grateful to Secretariat of Research, Science and Technology of Universidad Nacional de Chaco Austral for financial support.

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