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Phythochemical screening and antimicrobial activity of essential oil from *Lippia gracillis*

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Abstract: The chemical composition of the essential oil obtained from the fresh and dried leaves of *Lippia gracillis* Schauer, Verbenaceae, was analyzed by gas chromatography and gas chromatography/mass spectrometry (GC/MS). The yield of essential oil extracted from the dried leaves was significantly higher ($p<0.05$) when compared to the fresh leaves. Seventeen components were identified. The monoterpenes and sesquiterpene hydrocarbons with 96.26% (w/w) of the total oil obtained of fresh leaves and 86.99% (w/w) of the total oil obtained of dried leaves were the principal compound groups. Thymol was observed dominant (44.42%; 21.3%), followed by carvacrol (22.21%; 21.30%), *p*-cymene (6.23%; 8.58%), α-pinene (5.65%; 19.42%), β-caryophyllene (5.61%; 3.57%) and other minor constitutes, respectively. Microbiological results obtained by agar diffusion method, micro dilution method and minimum inhibitory concentration (MIC) showed that the essential oil has a relevant antimicrobial activity against *E. coli* (ATCC 10536), *E. coli* (Ec 27), *Pseudomonas aeruginosa* (ATCC 15442), *S. aureus* (ATCC 12692) and *S. aureus* (Sa 358), with their inhibition zones ranging from 9 to 13 mm and the MIC ranging from 64 to 512 μg/mL.

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Introduction

The increasing resistance developments with available antimicrobials have drawn the attention of the scientific community to search for new and effective drugs from natural origin (Botelho et al., 2007a). Essential oils exert many properties such as antinociceptive (Franco et al., 2011), anticonvulsant (Oliveira, 2009), cardiovascular effects (Menezes et al., 2010), antimicrobial activity against cariogenic bacteria (Botelho et al., 2009a) and fungal filaments as well (Botelho et al., 2007b). Some studies point out that those plant-derived essential oils may be an effective alternative to overcome microbial resistance (Botelho et al., 2008).

These components are very versatile and they were mainly used for flavouring, fruit beverages, confectioneries, soft drinks, perfume, soaps, cosmetics, household products and pharmaceutical industry (Botelho et al., 2007b). They were also used in medical treatments and were known for exhibiting antimicrobial properties such as antifungal, antibacterial, antiviral and antiparasite (Fuselli et al., 2008; Botelho et al., 2007c; Botelho et al.,2009a).

Lippia gracillis Schauer, Verbenaceae, popularly known as "alecrim-de-tabuleiro", is a typical shrub commonly grown in the Northeast of Brazil. This species produces an essential oil (EO) rich in thymol and carvacrol, which has a potent antimicrobial activity against fungi and bacteria. It is one of the widely used substances in Brazilian traditional medicine, in the Northeast of Brazil for skin cuts, insect bites and sore throat (Botelho et al., 2008).

Previous studies have described the larvicidal property of *Lippia* essential oil (Carvalho et al., 2003).

Recently, quinones from *Lippia sidoides* EO have been described to possess cytotoxic activity (Costa et al., 2001). Scientific evidence also suggests that the essential oil may be useful for oral hygiene and in the prevention of dental disorders such as caries and gingivitis (Botelho et al., 2007b; Botelho et al., 2009a). However, the *in vitro* activity against pathogens related to other diseases has not been so far reported. Thus, any treatment that would eliminate or substantially reduce colonization by these types of bacteria would likely have a strong impact and it would be beneficial for human health.

The aim of this study was to evaluate the chemical composition and the antibacterial activity of *Lippia gracillis* essential oil (LGEO) extracted from fresh and dried leaves against pathogens related to many human infections such as food spoilage, food safety and persistent hospital infection, since these organisms have now gained more importance due to increased concerns about safety in food and better quality of life. In addition, we studied the phytochemical composition of the essential oils by GC-MS analysis.

Material and Methods

Plant material and extraction

Leaves of *Lippia gracillis* Schauer, Verbenaceae, were collected in the month of January/2009 in the Medicinal Garden of the Regional University Cariri in Crato (CE). A representative sample was classified and deposited in the Herbarium Prisco Bezerra of the Universidade Federal do Ceará, under number 44456.

The leaf essential oil was extracted using a modified Clevenger apparatus (Botelho et al., 2007c) by the hydro-distillation technique. The essential oils extracted from fresh leaves (FL LGEO, 77 g) and dried leaves (DL LGEO, 35.6 g) were obtained by hydrodistillation using Clevenger type apparatus for a period of 2 h, resulting in a yielding of 0.56 and 2.64% respectively. Then the oil were collected, dried with anhydrous sodium sulfate (Na_2SO_4), filtered and stored under refrigeration until analysis. After extraction, the volume of *L. gracillis* essential oil (LGEO) obtained in both extractions was measured and the essential oil conditioned in hermetically sealed glass containers with rubber lids, covered with aluminum foil to protect the contents from light and kept under refrigeration at 8 °C until used.

GC-MS analysis

The composition of the essential oil was investigated by GC and GC/MS. The analytical GC was carried out using a spectrometer Shimadzu GC-17A/IN QP5050A system (GC/MS): DB-5HT capillary column (30 m x 0.251 mm), carrier gas: helium 1.7 mL/min; column pressure 107.8 kPa, linear velocity, 47.3 cm/sec, total flow 24 mL/min carrier flow of 24 mL/min, injector temperature 270 °C, detector temperature 290 °C, column temperature 60 (2 min) -180 °C (1 min) at 4 °C/min, then 180 to 260 °C to 10 °C/min (10 min). Operating under an ionization energy of 70 eV (electron impact ionization). The identification of components was based on spectral fragmentation, using standard computer library Wiley 229, plus two other arguments: the retention indices and comparison with literature data (Botelho et al., 2007c).

GC-MS analysis was carried out on the same chromatograph equipped with a Hewlett-Packard MS computerized system (Palo Alto, California, USA), Model 5971A, ionization voltage 70 eV, electron multiplier 1300 V, ion source temperature 280 °C, mass range *m/z* 35-450, scanning interval 0.5 s, scanning speed 1000 amu/s. GC conditions were the same as above. Identification of components was based on computer matching with NIST107 and NIST21 libraries and comparisons of the Kovats index with those reported in literatures.

Antimicrobial assay

Strains

The essential oil was tested towards eight micro-organisms *S. flexineri* (ATCC 12022), K. pneumoniae (ATCC 10031), *B. cereus* (ATCC 33018), *E.coli* (ATCC 10536), *S.aureus* (ATCC 12692) and *P. aeruginosa* (ATCC 15442) and two clinical isolates, *S. aureus* (Sa 358) and *E. coli* (Ec 27). These micro-organisms were provided by the Fiocruz and the Federal University of Paraíba.

Antimicrobial screening by the disk diffusion method

The antimicrobial activity of LGEO against pathogens was determined by standard Disk Susceptibility Tests (Botelho et al., 2007c) tested against *Bacillus cereus* (ATCC 33018), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 15442), *Shigella flexineri* (ATCC 12022), *Klebsiella pneumoniae* (ATCC 10031) and *Sthaphylococcus aureus* (ATCC 12692) by using the agar diffusion method (Romeiro, 2001). The clinically isolated bacterial and inoculum of all strain was obtained from fresh colonies grown on Müeller Hinton agar plates. Each strain was inoculated into 5 mL of Müeller Hinton broth in order to obtain a concentration of 1.5 x 108 CFU/mL (0.5 MacFarland) and incubated at 37 °C for 24 h. After this period, they were replicated on Petri dish containing Müeller Hinton agar (MH). The plates containing the microorganisms were then perforated and the cavities were filled with 25 µL of the oil solutions at

10, 5, 2.5, 1.25, 0.6 and 0.3% concentrations. Trials were performed in triplicate and commercial antibiotic disks of chloramphenicol (30 µg/disk) and tetracycline (30 µg/disk) were employed as positive controls, while DMSO was served as negative control. Halos of inhibition were measured 24 h after initial exposure. The results were expressed in milimilters: <9 mm, inactive; 9-12 mm, partially active; 13-18 mm, active; >18 mm, very active (Rios et al., 1998).

Antimicrobial testing (quantitative antimicrobial evaluation)

The antibacterial activities of essential oils of *L. gracillis* were evaluated by broth microdilution, based on document M7-A6 (NCCLS, 2003) to determine the minimum inhibitory concentration (MIC). Gram-negative and Gram-positive bacteria were tested concomitantly with five others bacterial strains, three strains default, *E. coli* (ATCC 10536), *S. aureus* (ATCC 12692) and *P. aeruginosa* (ATCC 15442) and two clinical isolates, *S. aureus* (Sa 358) and *E. coli* (Ec 27). Before the tests, the bacterial strains were inoculated in Brain Heart Infusion (BHI) 3.8% during 24 h at 35±2 °C. After this period, the microorganisms were diluted at 1:10 in BHI broth to obtain 10% final concentration of 104 cells/mL (Botelho et al., 2007c). The samples to be tested were prepared in advance to obtain an initial concentration of 100 mg/mL, and observed the following ratio: 100 mg of sample solubilized in 1 mL of dimethyl sulfoxide (Merck, Darmstadt, Germany). From this merger, was made a 1:10 dilution in sterile distilled water (10 mg/mL), and then it was diluted in the same way to 1024 µg/mL. After wards, serial dilutions were 1:1 in distilled water, resulting in concentrations from 512 to 4 µg/mL.

Samples of essential oils obtained were prepared from fresh and dried leaves and used small volumes distributed in sterile microplate wells. The samples were prepared in double concentration (1024 µg/mL) compared with the initial concentration and defined volumes of 100 mL and then were serially diluted 1:2 in 10% Brain Heart Infusion broth. Each well with 100 µL of culture medium was placed a sample of bacterial suspension diluted at 1:10. Resazurin (0.01%) was used as revealing reagent in the volume of 25 µL/well. The negative control was performed with BHI, while the positive control was broth plus inoculum. The minimum inhibitory concentration (MIC) was defined as the lowest concentration able to completely inhibit microbial growth in microdilution wells detected at the naked eye. The reading of the results for MIC determination was considered positive for the wells that kept the blue color and those who had negative staining red.

Statistical analysis

Each parameter was tested in at least triplicate. Conventional statistical methods were used to calculate means and standard deviations. Statistical significance was determined by analysis of variance and subsequent Dunnett's t-test ($p<0.05$). The analysis was performed using SPSS statistical software.

Results and Discussion

Analysis by GC/MS of essential oil of fresh leaves (FL LGEO) allowed the identification of a total of seventeen different compounds, as revealed by Table 1, the principal components of the FL LGEO were thymol was observed dominant (44.42%), followed by carvacrol (22.21%), *p*-cymene (6.23%), α-pinene (5.65%), β-caryophyllene (5.61%) and other minor constitutes, which represented 96.26% of the total essential oil, distributed as thirteen monoterpenes and four sesquiterpenes.

Table 1. Chemical composition of essential oil of *Lippia gracillis* essential oil from fresh and dried leaves.

Components	[b]KI_{Exp}	FL LGEO		DL LGEO	
		[a]RT	(%)	[a]RT	(%)
α-thujene	927	7.66	0.47	-	-
α-pinene	931	10.96	5.65	10.64	19.42
β-pinene	965	12.43	0.33	12.42	0.83
myrcene	992	12.69	1.63	12.68	2.00
α-terpinene	1012	13.78	0.61	13.78	0.79
p-cymene	1028	13.88	6.23	13.87	8.58
limonene	1033	14.25	0.39	14.24	0.83
γ-terpinene	1288	16.45	2.83	15.25	3.51
4–terpineol	1181	19.48	3.27	19.48	0.40
thymol-methyl ether	1230	21.29	0.63	21.27	3.46
thymol-methyl ether	1230	21.29	0.63	21.27	3.46
carvacrol-methyl ether	1248	21.67	0.74	21.66	0.64
thymol	1288	23.14	44.42	23.01	21.30
carvacrol	1296	23.45	22.21	23.36	20.85
β-caryophylene	1414	28.43	5.61	28.40	3.57
α-humulene	1434	29.44	0.45	29.44	0.27
δ-cadinene	1525	31.31	0.26	31.31	0.54
caryophillene oxide	1577	33.37	0.51	-	-
Total		96.26		86.99	

[a]RT: retention time; [b]relative retention indices experimental: *n*-alkanes were used as reference points in the calculation of relative retention indices. FL LGEO: Fresh leaves of *Lippia gracillis* essential oil. DL LGEO: Dried leaves of *Lippia gracillis* essential oil.

The yield of *L. gracillis* oil extracted from the dried leaves was considerably higher ($p<0.05$) than that obtained from fresh leaves, ranging in average of 2.64 and 0.56% respectively. The volatile constituents often

represent a mixture of monoterpene and sesquiterpene hydrocarbons and their oxygenated derivatives including: aldehydes, ketones, acids, alcohols and esters (Mondello et al., 2005). Generally, volatile essential oils containing raging from 85-99% and 1-15% non-volatile components (Njoroge et al., 2005). Regarding the chemical composition of DL LGEO, using the same analytical process, we could identify 86.99% of its chemical constitution, corresponding to fifteen compounds (Table 1). Of this total are reported: non-oxygenated monoterpenes, oxygenated monoterpenes and non-oxygenated sesquiterpenes. In this volatile oil were found in greater quantity thymol (21.30%), carvacrol (20.85%), α-pinene (19.42%) and *p*-cymene (8.58%).

In the preliminary test of susceptibility for evaluation of antibacterial activity, essential oils extracted from fresh and dried leaves presented activity against *S. aureus, B. cereus, S. flexineri, P. aeruginosa* and it proved ineffective against *E. coli* and *K. pneumoniae*. These results agree with those in the literature that report a minor susceptibility of Gram-negative compared to the plant extracts (Burt, 2004). The largest diameters of inhibition zones were observed against *S. aureus,* inhibited at concentrations of 10 and 5% with inhibition halos of 12 and 9 mm respectively (Table 2).

A recent study showed the promising anti-staphylococcal property of *L. sidoides* essential oil, thus it is reasonable to speculate the possibility of the rational use of this substance as an alternative antibacterial agent (Oliveira et al., 2006), in this study the findings does not present statistically significance differences between the activity of the oils tested against *S. aureus* e *P. aeruginosa* at 10% concentration. However, when compared to standard antibiotics, the diameters of inhibition zone presented a considerably higher activity of both oils against *P. aeruginosa*, when compared to chloramphenicol.

The data indicated that the two types of essential oil exhibited varying levels of antimicrobial activity against the investigated microorganisms Table 3. Through antimicrobial screening, the essential oil from dried leaves (DL LGEO) was more effective against *S. flexineri* and *B. cereus* when compared to the fresh leaves essential oil (FL LGEO). This activity probably is related to large amounts of sesquiterpenoids oxygen present in the dried leaves essential oil (Botelho et al., 2007c).

The *in vitro* antimicrobial activity of LGEO against the microorganisms and its potentials activity were qualitatively and quantitatively assessed by microdilution methodology and MIC values. The data obtained from this method indicated that the LGEO displayed a variable degree of antimicrobial activity on different tested strains (Table 4). The highest activity of this essential oil was observed against *E. coli* (ATCC 10536), with a MIC of 64 µg/mL for the FL LGEO and 128 µg/mL for the DL LGEO.

Through the statistical analysis it was shown that the values of the antimicrobial activity of the essential oil extracted from the fresh leaves presented a statistical significant difference when compared to the dried leaves activity. The essential oil also exhibited moderate activity against *E. coli*, evidenced by the MIC of 128 and 256 µg/mL, respectively.

Results of the MIC are presented in Table 4. The data indicated that the two types of essential oil exhibited varying levels of antimicrobial activity against the investigated microorganisms. The inhibitory properties of the essential oil from fresh leaves were observed within a range of MIC concentrations ranging from 64 to 512 µg/mL. *P. aeruginosa* (ATCC 15442) showed similar susceptibility to the investigated oils, with MIC of 512 µL/mL.

The chemical compositions of essential oils in the present study, was consistent with other reports (Packer & Luz, 2007; Costa et al., 2008; Botelho et al., 2009b). In fact, essential oils contents and compositions could differ greatly even in the same genus, as well as in different ripening stage and different organs (Tirado et al.,1995; Stashenko et al., 1996). In *Lippia sidoides* essential oil from Fortaleza, Ceará, Brazil (Botelho et al., 2007c), monoterpene hydrocarbons constituted 97.5% in the essential oils, with carvacrol (11.3%),

Table 2. Antimicrobial activity in millimeters of *Lippia gracillis* essential oil from fresh leaves (FL LGEO), determined by direct contact method.

Microorganism	Inhibition zones (mm ± SD)				
	FL LGEO 5%	FL LGEO 5%	TE (30µg)	CHL (30µg)	DMSO (5%)
E. coli 25922	-	-	-	7+0.47	-
S. aureus 12692	9+0.96	12.33+0.47^{c}	20+0.96	18+0.47	-
P. aeruginosa 15442	-	11.33+0.96^{b}	13+0.96	6+0.96	-
S. flexineri 12022	-	11+0.47^{a}	21+0.47	14+0.96	-
K. pneumoniae 10031	-	-	-	9+0.96	-
B. cereus 33018	-	11++0.96d	22+0.47	21+0.47	-

-: no inhibition zone was observed; $^{a\text{-}d}$Stand for the significance of difference ($p<0.05$) based on Tukey's test; CHL: chloramphenicol and TE: tetracycline; FL LGEO: Fresh leaves of *Lippia gracillis* essential oil; DL LGEO: Dried leaves of *Lippia gracillis* essential oil.

Table 3. Antimicrobial activity in millimeters of *Lippia gracillis* essential oil from dried leaves (DL LGEO), determined by direct contact method.

Microorganism	Inhibition zones (mm ± SD)				
	DL LGEO 5%	DL LGEO 5%	TE (30µg)	CHL (30µg)	DMSO (5%)
E. coli 25922	_	_	_	6+0.96	_
S. aureus 12692	_	12.33+0.47	19+0.47	18+0.96	_
P. aeruginosa 15442	_	11.33+0.47	_	6+0.96	_
S. flexineri 12022	_	12+0.96	14	12+0.47	_
K. pneumoniae 10031	_	_	9+0.96	15+0.47	_
B. cereus 33018	_	13+0.96	7+0.96	23+0.96	_

-: no inhibition zone was observed; a-dStand for the significance of difference (p<0.05) based on Tukey's test; CHL: chloramphenicol and TE: tetracycline; FL LGEO: Fresh leaves of *Lippia gracillis* essential oil; DL LGEO: Dried leaves of *Lippia gracillis* essential oil.

Table 4. Antimicrobial activity expressed as minimum inhibitory concentration (µg/mL) of LGEO against tested bacteria.

Microorganism	MIC (µg/mL)	
	FL LGEO*	DL LGEO#
E. coli (ATCC 10536)	64	128
E. coli (Ec 27)	128	256
P. aeruginosa (ATCC 15442)	512	512
S. aureus (ATCC 12692)	128	256
S. aureus (Sa 358)	512	512

*FL LGEO: Fresh leaves of *Lippia gracillis* essential oil; #DL LGEO: Dried leaves of *Lippia gracillis* essential oil.

α-terpinene (1.8%), and α-pinene (0.5%) as the main compounds, while contents of esters (0.4%), alcohols (0.3%) and ketones (0.1%) with a minor concentration when compared to LGEO, represented 1.06%, 0.74%, and 0.59%, respectively.

Other interesting topic to notice is when carvacrol and thymol contents where compared, the data reach a statistical significant differences when compared fresh with dried leaves essential oils.

The application of the essential oils in fine perfumery is very interesting, therefore, the LGEO constituents might be valuable for the flavouring of foods, chewing gums, sweets, teas and energy drinks. In cosmetics, the investigated agents may contribute with characteristic that might be used in shampoos, soaps, shower gels, body lotions and tooth pastes (Ngassoum et al., 2004; Fisher & Phillips, 2008).

The LGEO presented significant activity against *S. aureus* and *E. coli.* These activities might be produced by a single major compound or by the synergistic or antagonistic effect of various minor compounds present in the tested oils.

Thymol, which was found to be in appreciable amounts in the LGEO, it has been reported to have a wide range of antibacterial and antifungal activity (Pattnaik et al.,1997; Botelho et al. 2007c; Fuselli et al., 2008). Botelho et al. (2007c) have shown that *C. albicans,* was susceptible to *Lippia sidoides* essential oil and pure thymol and carvacrol. They also tested the antimicrobial activity of *Lippia sidoides* leaf oil (56.7%) thymol and (16.7%) carvacrol as the major components against cariogenic bacteria and fungi, found either similar activity. Other than thymol and carvacrol, minor constituents such as α-pinene and *p*-cimene present in the essential oil of *Lippia gracillis* might also contributed to the antimicrobial activity, such as α-terpineol that has been reported to inhibit the growth of quite a number of bacteria and fungi that include *E. coli, S. aureus, Bacillus* spp. and *C. albicans* (Magwa et al., 2006).

The antimicrobial activity of oil from *S. officinalis* and *S. triloba* was suggested to be the presence of α-pinene, β-pinene, α-terpineol, terpinen-4-ol, β-caryophyllene, α-phellandrene and *ρ*-cymene (Dorman & Deans, 2000). α-pinene and β-pinene were active against the fungi *Verticillium fungicola* and *Trichoderma harzianum* and the bacterium *Pseudomonas tolaasii* (Sokovic & Van Griensven, 2006).

The antifungal effect of *M. glyptostroboides* essential oil against tested fungi could be attributed to presence of α-pinene, α-thujene and caryophyllene oxide (Bajpai et al., 2007). The antifungal activity of α-pinene was reported when *Pistacia lentiscus* essential oil was evaluated (Matasyoh et al., 2007).

It is well established that microbes can negatively affect the host health status. *E. coli* and *S. aureus* are the most common bacteria that infect humans through generations. During the microbial invasion the released metabolism byproducts can facilitate invasion

and can cause significant tissue damage followed by other vascular phenomena mediated by inflammatory compounds (Botelho et al., 2007a).

Within the limitation of this study, the findings suggest that the essential oils tested presented a relevant antimicrobial activity against *E. coli, P. aeruginosa* and *S. aureus*. Among the microorganisms related to gastrointestinal infection, *E. coli* presented the highest sensitivity to the tested compounds.

Our present results demonstrated that essential oil extracted from LGEO was rich in thymol, carvacrol, myrcene, α-pinene and other components, which plays positive roles in inflammatory diseases and also has a financial interesting for the cosmetic industry for different applications in perfumes, creams and soaps. The essential oil also showed a wide spectrum of antimicrobial activity against the tested microorganisms suggesting that this essential oil would be a natural flavor additive substituting chemicals in food preservation.

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