academicJournals

Vol. 13(30), pp. 3107-3113, 23 July, 2014
DOI: 10.5897/AJB2012.2941
Article Number: 089108B46228
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Composition and antioxidant and antifungal activities of the essential oil from *Lippia gracilis* Schauer

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Received 30 November, 2012; Accepted 3 July, 2014

In this study, the oil constituents of *Lippia gracilis* were identified by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The antioxidant and antifungal activities were also evaluated. The leaf oil showed a yield of 3.7% and its main constituents were thymol (70.3%), *p*-cymene (9.2%), thymol methyl ether (5.4%) and *p*-methoxythymol (2.7%). The thin stem oil showed a yield of 0.4% and its major components were thymol (70.1%), thymol methyl ether (4.4%), *p*-methoxythymol (4.0%), *p*-cymene (3.8%), α-humulene (2.4%) and (*E*)-caryophyllene (2.1%). The aromatic monoterpenes found in the oils showed an average of 88%. The scavenging activity of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) for the leaf oil, expressed as half maximal effective concentration (EC_{50}), was 35.7±3.3 µg/ml, indicating high antioxidant activity. The evaluation of fungicide activity for the leaf oil, using direct bioautography, showed also a significant value for lethal concentration (LC_{50} 5.0 µg/ml) against *Cladosporium sphaerospermum* and *C. cladosporioides* fungi.

Key words: Essential oil composition, thymol and carvacrol, DPPH radical scavenging and bioautography

INTRODUCTION

Lippia (Verbenaceae) comprises nearly 200 species of herbs, shrubs and small trees spread wide in South and Central America and Tropical Africa. *Lippia gracilis* Schauer [syn. *Acantholippia trifida* (Gay) Moldenke] is an aromatic shrub up to 1.5 m in height, known popularly as "vereda" or "alecrim-de-tabuleiro", growing wild in areas of savannas of North and Northeast Brazil. Its aerial parts are used to treat gastrointestinal, respiratory and cutaneous infections (Albuquerque et al., 2006).

L. gracilis occurring in Northeast Brazil have shown

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variation in the composition of its volatile constituents. The oil produced in Ceará state showed thymol (30.6%), carvacrol (11.8%) and *p*-cymene (10.7%) as main compounds (Lemos et al., 1992). In the oil obtained at Piauí state, the major components were carvacrol (47.7%), *p*-cymene (19.2%), methylthymol (6.2%) and thymol (4.8%) (Matos et al., 1999). The oil analyzed in Sergipe state was dominated by thymol (24.0%), *p*-cymene (15.9%), methylthymol (11.7%) and γ-terpinene (10.9%) (Teles et al., 2010). Previously, the oil of *L. gracilis* showed antibacterial (Mota et al., 2009) activities against *Staphylococcus aureus* and *Biomphalaria glabrata*, respectively, molluscicidal activities (Teles et al., 2010), and its methanolic extract showed antinociceptive effect in mice (Guimarães et al., 2012).

The genus *Lippia* is well-known for its aromatic properties, and more than 50 of its essential oils have been reported (Terblanché and Kornelius, 1996; Pascual et al., 2001). The main volatile constituents frequently found in the oils of *Lippia* species are thymol, carvacrol, *p*-cymene methylthymol, methylcarvacrol, γ-terpinene, 1,8-cineole and (*E*)-caryophyllene. Many *Lippia* species has shown variation in their oil composition, producing various chemical types as occur in *Lippia alba* (Matos et al., 1996; Zoghbi et al., 1998; Atti-Serafini et al., 2002), *Lippia lupulina* (Zoghbi et al., 2001), *Lippia glandulosa* (Maia et al., 2005), *Lippia origanoides* (Morais et al., 1972; Oliveira et al., 2007; Stashenko et al., 2008; Silva et al., 2009) and *Lippia grandis* (Silva et al., 1973; Maia et al., 2003; Damasceno et al., 2011).

Lately, there has been a growing interest in the search for spices, aromatic and medicinal plants as sources of natural antioxidants. The antioxidant capacity of these plants is associated with the activity of the free radical scavenging enzymes and the contents of antioxidant substances, usually phenol compounds. The use of essential oils as functional ingredients in foods, drinks, toiletries and cosmetics has become increasingly valuable also because of concern about potentially harmful synthetic additives. The oils and extracts, being biologically active natural compounds, have been proposed for the control of certain diseases and the prevention of lipid peroxidative damage implicated in various pathological disorders, such as atherosclerosis, Alzheimer`s disease, carcinogenesis and aging processes (Ruberto and Baratta, 2000; Mimica-Durik et al., 2004).

The aim of this study was to analyze the oil composition of leaves and thin stems of *L. gracilis* that occur in the eastern Brazilian Amazon, as well as to evaluate their antioxidant and antifungal and activities.

MATERIALS AND METHODS

Plant material

The specimen *L. gracilis* Schauer was collected in the locality of São Félix de Balsas, Maranhão state, Brazil, February 2011. The plant was identified and deposited (MG 200187) in the Herbarium of Museu Paraense Emílio Goeldi, Belém city, Pará state, Brazil.

Plant processing

The leaves and thin stems were air-dried separately, ground and subjected to hydrodistillation (100 g, 3 h), using a Clevenger-type apparatus. The oils were dried over anhydrous sodium sulfate, and their percentage contents were calculated on basis of the plant dry weight. The moisture content of the samples were calculated after the phase separation in a Dean-Stark trap (5 g, 30 min), using toluene.

Oil-composition analysis

The analysis of the oils were carry out on a THERMO DSQ II GC-MS instrument, under the following conditions: fused-silica capillary column DB-5ms (30 m x 0.25 mm, 0.25 μm film thickness); programmed temperature, 60-240°C (3°C/min); injector temperature, 250°C; carrier gas was helium, adjusted to a linear velocity of 32 cm/s (measured at 100°C); injection type, splitless (2 μL of a 1:1000 hexane solution); split flow was adjusted to yield a 20:1 ratio; septum sweep was a constant 10 ml/min; EIMS electron energy, 70 eV; temperature of ion source and connection parts, 200°C. The quantitative data regarding the volatile constituents were obtained by peak-area normalization using a FOCUS GC/FID operated under conditions similar to those in GC-MS, except for the carrier gas, which was nitrogen. The retention index was calculated for all the volatiles constituents using an *n*-alkane homologous series.

DPPH radical scavenging assay

A stock solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (0.5 mM) in methanol (MeOH), was prepared. The solution was diluted in MeOH (60 μM approx.) measuring an initial absorbance of 0.62±0.02 in 517 nm at room temperature. The reaction mixture was composed by 1950 μL of DPPH solution and 50 μL of the samples diluted in different methanol portions. For each sample, a methanol blank was also measured. The absorbance was measured in the reaction starting (time zero), each 5 min during the first 20 min and then at constant intervals of 10 min up to constant absorbance value. The concentration of antioxidant required for 50% scavenging of DPPH radicals (EC_{50}) was determined by linear regression using Windows/Excel. All experiments were in triplicate. Butylated hydroxyanisole (BHA) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were used as standard antioxidants. The radical scavenging activity of each sample was calculated by the DPPH inhibition percentage according to the equation IP_{DPPH} =100 (A - B) / A (where A and B are the blank and sample absorbance values in the end reaction). The radical scavenging activity, expressed as milligrams of Trolox equivalent per gram of each sample, was also calculated by means of the equation TE = (A – B)/(A – C) x 25/1000 x 250.29/1000 x 1000/10 x D (where A, B and C are the blank, sample and Trolox absorbance values in the end reaction, and D is the dilution factor) (Silva et al., 2007; Silva et al., 2011).

Antifungal bioassay

About 10 μL of the oil solutions (corresponding to 100, 50, 25, 10, 5, 1, 0.5 and 0.1 μg) were applied to pre-coated thin layer chromatographic (TLC) plates, which were developed with *n*- hexane/ethyl

acetate (8:2) and dried for complete removal of solvents. The chromatograms were sprayed with a spore suspension of the fungi *Cladosporium sphaerospermum* and *C. cladosporioides*, in glucose and salt solution and incubated for 48 h in darkness in a moistened chamber at 22°C. Clear inhibition zones appeared against a dark background indicating the minimum amount of the essential oils required. Miconazole was used as the positive control. *C. sphaerospermum* (Penzig) SPC 491 and *C. cladosporioides* (Fresen) de Vries SPC 140 have been maintained at the Laboratory of Engineering of Natural Products, Federal University of Pará, Belém city, Pará State, Brazil (Silva et al., 2011).

Statistical analysis

Samples were assayed in triplicate, and the results are shown as means ± standard deviation. Analysis of variance was conducted, and the differences between variables were tested for significance by one-way ANOVA with Tukey's post test using Minitab, version 14. Differences at $p < 0.05$ were considered statistically significant. The relationship between variables was determined by simple regression analysis.

RESULTS AND DISCUSSION

Oil-composition

The leaves and thin stems of *L. gracilis* provided oil yields of 3.7 and 0.4%, respectively, and their volatile constituents were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Individual components were identified by comparison of both mass spectra and GC-retention data with authentic compounds, which were previously analyzed and stored in the data system, or existing in commercial libraries and cited in the literature (Adams, 2007; NIST, 2005).

In total, 49 components were identified in the oils from leaves and thin stems of *L. gracilis*, comprising 99.5% of the total composition, which is listed in Table 1. Aromatic monoterpenes were the most representative class in the oils, ranging from 84.4 to 91.0%. Aliphatic monoterpenes and sesquiterpenes (hydrocarbons and oxygenated) are represented secondarily in the oils, the first varying from 4.6 to 5.2%, and the last from 3.6 to 9.4%. With a percentage above 2%, the main compounds found in the leaf oil of *L. gracilis* were thymol (73.5%), *p*-cymene (9.2%), thymol methyl ether (5.4%) and *p*-methoxythymol (2.7%) while in the thin stem oil were thymol (70.1%), thymol methyl ether (4.4%), *p*-methoxythymol (4.0%), *p*-cymene (3.8%), α-humulene (2.4%) and (*E*)-caryophyllene (2.1%).

In preliminary analysis, the oil of *L. gracilis* showed the chemical types thymol plus *p*-cymene and carvacrol plus *p*-cymene (Lemos et al., 1992; Matos et al., 1999; Teles et al., 2010). Based on the analysis of this new specimen of *L. gracilis*, we can assume that it is the chemical type thymol plus p-cymene, but with an occurrence in North Brazil. In previous works was observed that *Lippia* oils from the Brazilian Amazon showed significant amounts of thymol, carvacrol, *p*-cymene, 1,8-cineole, γ-terpinene, (*E*)-caryophyllene, citral, carvone and terpinen-4-ol (Zoghbi et al., 1998; Zoghbi et al., 2001; Maia et al., 2005; Morais et al., 1972; Silva et al., 2009; Damasceno et al., 2011). This way, one must consider that these chemical types of *L. gracilis* may result from the polymorphism of the plant, taking into account, mainly, the season time and site collection.

Thymol, carvacrol and *p*-cymene co-occur also as chief constituents in some traditional oils, such as *Monarda punctata* L., *Satureja hortensis* L. and *Thymus vulgaris* L. (Guenther, 1952; Scora, 1967). Also, it is no coincidence that co-occurs in the oil of L. gracilis the same aromatic monoterpenes, thymol, carvacrol and p-cymene. All these compounds are derived from the same biosynthetic plant process, where γ-terpinene, the cyclohexadiene constituent that occur also in the oil, is considered the initiator (Poulose and Croteau, 1978a,b). Figure 1 shows the predicted biosynthetic pathway of these aromatic monoterpenes, which on average comprises for approximately 88% of the oil composition.

Antioxidant activity

Antioxidants interact with the DPPH through the transfer of electrons or donation of hydrogen neutralizing its character of free radical (Silva et al., 2007). The leaves oil of *L. gracilis* was able to scavenging the DPPH radical, displaying a high dose-response (r^2=0.85). The half maximal effective concentration (EC_{50}) was 35.7±3.3 µg/ml, calculated by linear regression, ($p<0.05$), a significant value compared to Trolox (4.5±0.1 µg/ml), which was used as standard antioxidant. EC_{50} values lower than 30 µg/ml indicates high potential for radical scavenging (Ramos et al., 2003). This means that the *L. gracilis* oil showed a significant antioxidant potential for radical free scavenging (Figure 2).

Antifungal activity

The fungicide activity resulted from evaluation of direct bioautography using TLC, after the nebulization of fungal spores (Figure 3). The leaf oil of *L. gracilis*, tested against the *Cladosporium sphaerospermum* and *C. cladosporioides* fungi, showed a minimum inhibitory concentration (MIC) of 5.0 µg/ml. Miconazole, at the maximum concentration of 0.5 µg/ml, was used as positive control, meaning that the leaf oil showed antifungal activity comparable to standard compound.

Conclusion

The essential oil of *L. gracilis* collected in the locality of

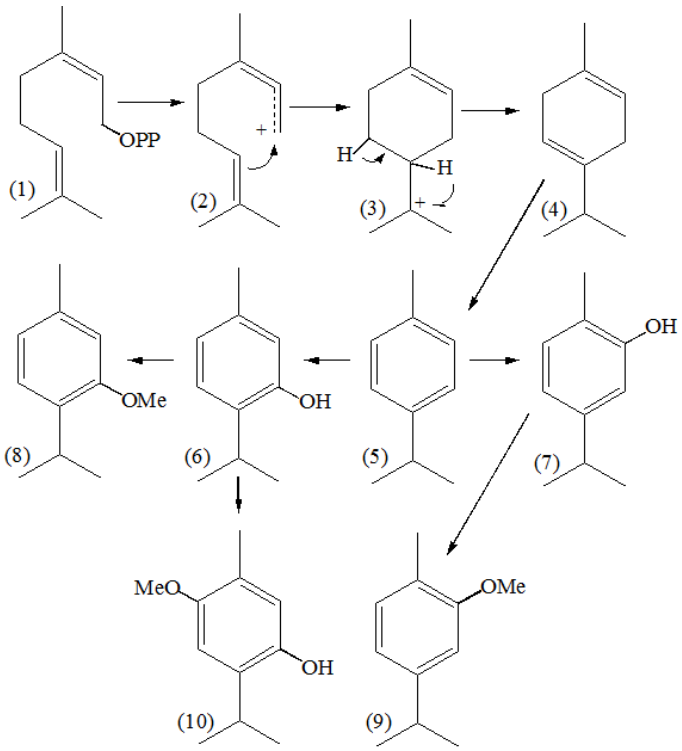
Table 1. Constituents identified in the oils of *Lippia gracilis*.

Constituent	RI	Leave (%)	Thin stem (%)
α-Pinene	934	0.4	
Myrcene	990	1.7	0.6
α-Terpinene	1014	0.4	0.2
p-Cymene	1025	9.2	3.8
1,8-Cineole	1032	0.4	0.2
γ-Terpinene	1056	1.0	0.8
cis-Sabinene hydrate	1067		0.1
p-Cymenene	1090	0.2	0.3
Linalool	1095	0.2	0.5
allo-Ocimene	1128		0.1
cis-Limonene oxide	1132		0.1
Borneol	1165		0.5
Umbellulone	1169	0.3	0.1
Terpinen-4-ol	1174	0.6	0.8
p-Cymen-8-ol	1181		0.2
α-Terpineol	1187	0.2	0.4
Methyl salicylate	1192		0.3
Shisofuran	1198		0.2
Thymol methyl ether	1233	5.4	4.4
Thymol	1290	73.5	70.1
p-Cymen-7-ol	1291		0.1
Carvacrol	1297		1.5
Eugenol	1357		0.1
α-Copaene	1376	0.3	0.8
β-Elemene	1390		0.1
(*E*)-Caryophyllene	1416	0.9	2.1
2,5-Dimethoxy-*p*-cymene	1425		0.1
trans-α-Bergamotene	1433	0.1	0.2
α-Guaiene	1439		0.1
α-Humulene	1455	1.4	2.4
allo-Aromadendrene	1461		0.1
cis-Cadina-1(6),4-diene	1462		0.2
Dodecanol	1470		0.1
p-Methoxythymol	1475	2.7	4.0
α-Selinene	1498	0.1	0.1
α-Muurolene	1501	0.1	0.2
β-Bisabolene	1506		0.1
γ-Cadinene	1513		0.1
δ-Cadinene	1523	0.3	0.8
α-Calacorene	1546		0.1
(*E*)-Nerolidol	1563		0.1
Spathulenol	1577		0.2
Caryophyllene oxide	1582	0.2	0.6
Globulol	1584		0.1
Humulene epoxide II	1609		0.6
Dillapiole	1621		0.3
1-*epi*-Cubenol	1629		0.1
epi-α-Cadinol	1639	0.2	0.1
α-Cadinol	1654		0.1

Table 1. Contd.

Aromatic monoterpenes	91.0	84.4
Aliphatic monoterpenes	5.2	4.6
Sesquiterpenes (hydrocarbons and oxigenated)	3.6	9.4
Other		0.8
Total	99.8	99.2

RI = Retention time on DB-5ms column



(1) neryl pyrophosphate
(2), (3) intermediate carbocations
(4) y-terpinene
(5) *p*- cynene
(6) thymol
(7) carvacrol
(8) thymol methyl ether
(9) carvacrol methyl ether
(10) *p*-methoxythymol

Figure 1. Proposed biosynthetic pathway for the aromatic monoterpenes occurring in the oil of *Lippia gracilis*.

São Félix de Balsas, Maranhão state, Brazil, showed a composition where the aromatic monoterpenes, thymol, *p*-cymene, thymol methyl ether and *p*-methoxythymol were the main constituents. It was characterized as the chemical type thymol + *p*-cymene. The values obtained for the antioxidant capacity assay (DPPH inhibition), and antifungal test (direct bioautography) showed significant biological properties for the oil at the concentrations tested in this experiment.

Conflict of Interests

The author(s) have not declared any conflict of interests.

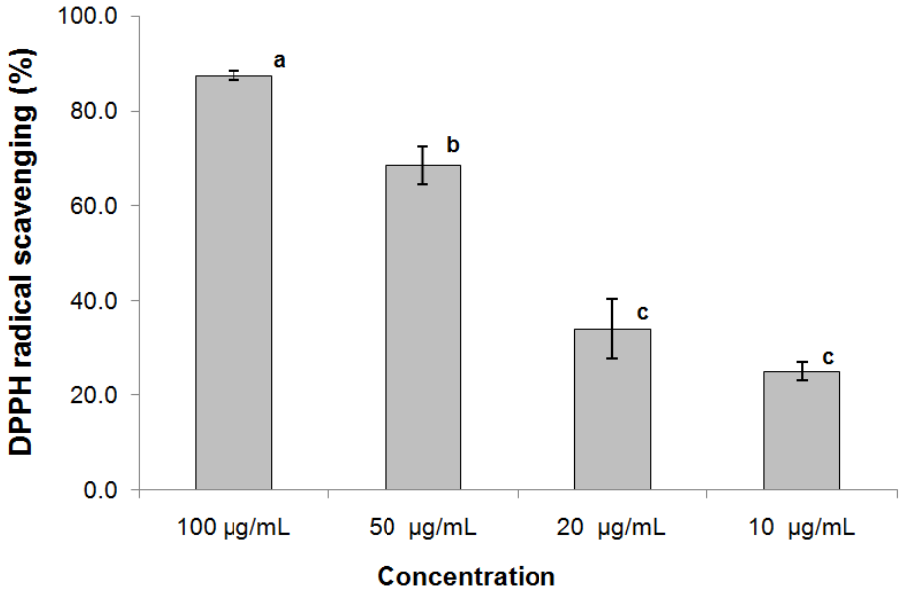


Figure 2. DPPH radical scavenging activity results for essential oil from *L. gracilic*. Error bars show the variation of three determinations in terms of SD. [abc]Values with the same letter are not statistically different at the $p < 0.05$ level (Tukey's test).

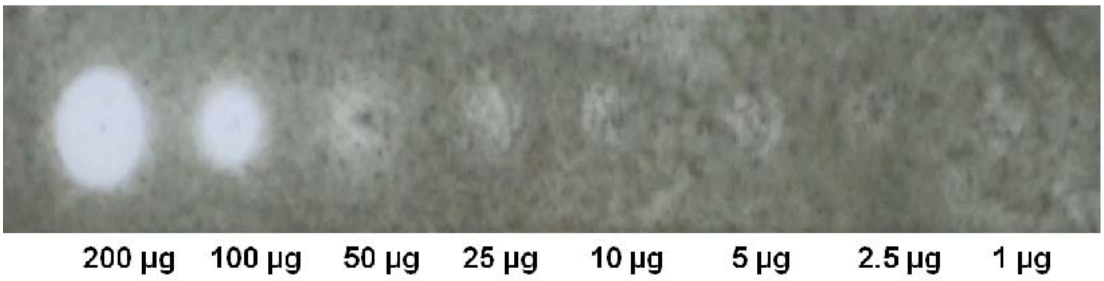


Figure 3. Bioautogram of *L. gracilic* essential oil. TLC plates sprayed with *Cladosporium sphareospermum* and *Cladosporium cladosporioides* culture (identical results). White areas indicate inhibition fungal growth.

ACKNOWLEDGEMENTS

The authors are grateful to CNPq/BIONORTE Program and FAPESPA/PA for their financial support.

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