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ORIGINAL ARTICLE

Chemical composition and pharmacological properties of the essential oils obtained seasonally from *Lippia thymoides*

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Abstract

Context: *Lippia thymoides* Mart. & Schauer (Verbenaceae) is used in folk medicine to treat wounds, fever, bronchitis, rheumatism, headaches, and weakness.
Objective: This study determinates the chemical composition of essential oils from *L. thymoides*, obtained at during each of the four seasons and correlates with pharmacological properties.
Materials and methods: Essential oils were obtained by hydrodistillation and analyzed by gas chromatography coupled to mass spectroscopy (GC-MS). Antioxidant activity was determined by DPPH free radical scavenging and β-carotene bleaching methods. The antimicrobial assays were performed by minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) methods. Isolated rat aorta and uterus, and guinea-pig trachea were utilized to evaluate relaxant potential in pre-contracted smooth muscle.
Results and discussion: Essential oils from leaves of *L. thymoides* had the sesquiterpene β-caryophyllene (17.22–26.27%) as the major constituent followed by borneol (4.45–7.36%), camphor (3.22–8.61%), camphene (2.64–5.66%), and germacrene D (4.72–6.18%). *In vitro* assays showed that these essential oils do not have antioxidant activity, have antimicrobial selectivity to Gram-positive bacteria *Staphylococcus aureus* (MIC = 0.004 mg/mL and MMC = 0.26–10.19 mg/mL) and *Micrococcus luteus* (MIC = 0.03 mg/mL and MMC = 8.43 mg/mL), relax isolated rat aorta (EC_{50} = 305–544 μg/mL, with endothelium; and EC_{50} = 150–283 μg/mL, without endothelium), and uterus (EC_{50} = 74–257 μg/mL), and minor potency, isolated guinea-pig trachea.
Conclusions: *Lippia thymoides* is a source of natural products of pharmaceutical interest, being necessary additional studies to determine the substances involved in the biological activities.

Keywords

Antimicrobial activity, essential oils, sesquiterpenes, spasmolytic activity, tocolytic activity, vasorelaxation

History

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Introduction

Essential oils or volatile oils are natural, complex, multi-component systems composed mainly of terpenes and phenylpropanoids. Extraction techniques used to obtain essential oils vary according to plant parts, ranging from hydrodistillation to supercritical fluid extraction (Edris, 2007; Lahlou, 2004). The extracted oils and their isolated constituents have been used as ingredients in perfumes, cosmetics, household cleaning, and insecticide, in addition to their large potential use as pharmaceutical products, pharmacological agents, or adjuvants in formulations to improve transdermal drug delivery (Adorjan & Buchbauer, 2010).

Among the plants in which essential oils are abundant, species of the genus *Lippia* (Verbenaceae) are highlighted. This genus contains about 200 species, varying among herbs, shrubs, and small trees, mainly distributed in the tropical and subtropical territories of the America and Africa (Salimena, 2010). The majority of the *Lippia* species are found in Brazil, with about 150 species present especially in the Cerrado and rocky fields (Salimena, 2002). Economical interest have induced studies on the essential oils of these species (Lampasona & Catalan, 2002) that have shown great variation

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in the chemical composition; the components that were found in the highest frequency were limonene, β-caryophyllene, *p*-cymene, camphor, linalool, α-pinene, and thymol (Pascual et al., 2001), as well as biological activities such antihelmintic (Camurça-Vasconcelos et al., 2007, 2008), antimicrobial (Albuquerque et al., 2006; Botelho et al., 2007; Pessoa et al., 2005), antioxidant (Arana-Sánchez et al., 2010; Rocha-Guzmán et al., 2007), antispasmodic (Görnemann et al., 2008), vasorelaxant (Maynard et al., 2011), analgesic, anti-inflammatory (Abena et al., 2003; Mendes et al., 2010), and larvicidal against *Aedes aegypti* (Cavalcanti et al., 2004; Carvalho et al., 2003). Despite the potential demonstrated by this genus, studies are concentrated in few species, for instance *Lippia sidoides* Cham., *Lippia alba* (Mill.) N. E. Brown, *Lippia dulcis* Trev., and *Lippia graveolens* H. B. K.

Lippia thymoides Mart. & Schauer is a shrub of 2 m height, very thin, erect, branched, with little and aromatic leaves, and white or lilac flowers, occurring in the Caatinga vegetation from states of Bahia and Minas Gerais, Brazil (Funch et al., 2004). It is popularly known as ''alecrim-do-mato'' or ''alecrim-do-campo'' and is used in religious rituals and folk medicine to treat wound, fever, bronchitis, rheumatism, headaches, and weakness (Almeida et al., 2010; Funch et al., 2004). Thus, considering the popular uses and the absence of reports in the literature about this plant, the aim of this study was to determinate the chemical composition of the essential oils from *L. thymoides*, harvested during each of the four seasons, and to investigate biological properties that can highlight the potential of this species as a source of pharmaceutical products of interest.

Materials and methods

Drugs and reagents

The following reference chemicals were used in the experiments: butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), resazurin, 2,3,5-triphenyltetrazolium chloride (TTC), acetylcholine chloride (ACh), carbamyl choline chloride (carbachol, CCh), diethylstilbestrol, phenylephrine hydrochloride (Phe), chloramphenicol (CHL), and nystatin (NYS), all provide by Sigma-Aldrich (St. Louis, MO); ascorbic acid (AAc) was purchased of Dinâmica (Diadema, São Paulo, Brazil); linoleic acid was obtained from Vetec (Duque de Caxias, Rio de Janeiro, Brazil); 2,2-diphenyl-1-picrylhydrazyl (DPPH) provided by FLUKA (St. Louis, MO). Stock solutions of these chemicals were prepared with suitable solvent and dilutions were made fresh on the day of experiment. Mueller–Hinton Agar and Mueller–Hinton Broth culture mediums were both purchased of HiMedia (Mumbai, India) and prepared with sterile distilled water. All nutritive solutions used in the experiments with isolated organs were made in distilled water and chemicals used were of the purity grade.

Plant material

Lippia thymoides was collected from Feira de Santana, Bahia, Brazil, always at the same horary and location (12°11′45″ S latitude and 38°58′05″ W longitude), at the end of the first month of each season: April 2009 (autumn), July 2009 (winter), October 2009 (spring), and January 2010 (summer). Voucher specimens were deposited under the number 77554 at the Universidade Estadual de Feira de Santana Herbarium. The specimen was identified by Tânia Regina dos Santos Silva. Leaves of a pool of individuals were separated from the stem and air-dried at room temperature, protected from light, until constant weight, and powdered in the cutting mill. The residual water was measured using 1 g of the dry leaves on a moisture analyzer (Series ID Version 1.8, Marte, São Paulo, SP, Brazil). The essential oils were obtained by hydrodistillation with a Clevenger-type apparatus for 3 h, dried over sodium sulfate anhydrous, and stored at −20 °C protected from light. The essential oils were codified as OAA (autumn), OJW (winter), OOS (spring), and OJS (summer). In the antimicrobial assay, essential oils were diluted in a solution of Tween 80 in distilled water (10% v/v), resulting in a solution at 100 μL/mL which was homogenized on a vortex mixer, and then sterilized by filtration through a cellulose acetate membrane (0.2 μm). In all other experiments, the dilutions were made in Chremophor EL (Sigma-Aldrich, St. Louis, MO) 3% v/v in distilled water.

Animals

Male (250–300 g) and female (200–250 g) Wistar rats and guinea-pigs (400–500 g) of either sex used in the assays were bred and housed at the animal house of Universidade Federal do Vale do São Francisco. All experiments were approved by the Animal and Human Ethic Commission of the Universidade Federal do Vale do São Francisco (protocol no. 18061031). Animals were kept under conditions of controlled temperature (23–25 °C), 12 h light/dark cycle with water and food *ad libitum*. At the moment of experiment, all animals were sacrificed by cervical dislocation.

Chemical analysis

Analyses of the essential oils were performed using a Varian CP-3380 (Palo Alto, CA) gas chromatograph (GC) equipped with a flame ionization detector (FID) and capillary column, Chrompack CP-SIL 5, (30 m × 0.5 mm× 0.25 μm film thickness). The analyses were carried out in the following conditions: injector and detector temperatures, 220 °C and 240 °C, respectively; a carrier gas helium flowing at 1 mL/min; split ratio 1:100; injection volume 0.20 μL; the column temperature was initially 60 °C and was gradually increased in 3 °C/min until 240 °C, and finally held isothermally for 20 min.

Also, essential oils were analyzed by Shimadzu CG-2010 chromatograph (Shimadzu, Tokyo, Japan) coupled to mass spectrometer (MS) CG/MS-QP 2010 equipped with a capillary column, DB-5 ms, (30 m × 0.25 mm × 0.25 μm film thickness). The analysis conditions were the following: injector temperature 220 °C, interface and ionization source temperature 240 °C; the ionization mode was electronic impact at 70 eV; ionization current 0.70 kV; carrier gas helium at a flow 1 mL/min; split ratio 1:30; injection volume 0.20 μL; column temperature was initially 60 °C and was gradually increased at a 3 °C/min until 240 °C, and finally held isothermally for 20 min.

Constituents were identified by comparing its mass spectra with those of the GC/MS and confirmed by comparing the retention indexes relative to C_8–C_{24} *n*-alkanes, with calculation of the Kovats Index (KI) to each peak, and values from the literature. The quantification of relative percentage of the identified components was obtained by the normalization method. The data are reported as mean value of two oils' injections.

Antioxidant assays

The antioxidant activity was evaluated by DPPH free radical scavenging and β-carotene bleaching methods (Mensor et al., 2001; Wannes et al., 2010), each in triplicate and three independent experiments. In the DPPH assay, sample stock solutions (1.0 mg/mL) of the essential oils (OAA, OJW, OOS, and OJS) and positive controls (AAc, BHA, and BHT) were diluted to final concentrations of 243, 81, 27, 9, 3, and 1.0 μg/mL. One mL of a 50 μg/mL DPPH ethanol solution was added to 2.5 mL of sample solutions previously prepared and allowed to react at room temperature. After 30 min, the absorbance values were measured at 518 nm and converted into the percentage antioxidant activity. The essential oils plus diluents were used as blanks and DPPH solution without sample solutions was used as a negative control.

In the bleaching method, β-carotene (2.0 mg) was dissolved in 10 mL chloroform and to 2 mL of this solution, linoleic acid (40 mg) and Tween 40 (400 mg) were added. Chloroform was evaporated under vacuum at 40 °C and 100 mL of distilled water was added, then the emulsion was vigorously shaken during 2 min. The emulsion (3.0 mL) was added to a tube containing 0.12 mL of solutions 1 mg/mL of positive controls (AAc, BHA, and BHT) and sample essential oils (OAA, OJW, OOS, and OJS). The absorbance was immediately measured at 470 nm and the test emulsion was incubated in water bath at 50 °C for 2 h, when the absorbance was measured again. In the negative control, the essential oils were substituted with an equal volume of diluent. The percentage antioxidant activity was evaluated in terms of the bleaching of the β-carotene.

Antimicrobial assays

The antimicrobial assays were performed by minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) methods, according to approved by Clinical and Laboratory Standards Institute (CLSI, 2006, 2008). The microorganism-tested *Escherichia coli* CCMB261 resistant to sulfonamide and sensible to trimethoprim, *Staphylococcus aureus* CCMB262 resistant to streptomycin and dihydrostreptomycin, *Pseudomonas aeruginosa* CCMB268, *Micrococcus luteus* CCMB283, *Salmonella choleraesuis* CCMB281, *Bacillus cereus* CCMB282, and *Candida albicans* CCMB286 resistant to fluconazole and amphotericin B were obtained from Culture Collection of Microorganisms of the Bahia (CCMB). In 96-well plates, 90 μL of the essential oil solution (OAA, OJW, OOS, and OJS) and 90 μL of Müeller–Hinton broth (2 × concentrated) were conditioned in the first well and the serial dilutions were carried out in all subsequent wells. The range of evaluated extract concentration was 22.20–0.005 mg/mL. Cultures of 18 h (bacteria) and 36 h (yeast) were collected to saline solution 0.45% and 10 μL of microorganism suspension at 1.5×10^6 CFU/ml (bacteria) and 5×10^5 CFU/ml (yeast) were added in each well. The MIC of the 10% Tween/water solution and positives controls chloramphenicol (20 mg/mL) and nystatin (10 mg/mL) was also determinate. Controls of the microbial strains viability, sample sterility, and water were also performed. After incubation (24 h at 37 °C to bacteria and 48 h at 28 °C to yeast), 30 μL of resazurin (0.01% w/v) were added in each well. The MIC was defined as the lowest concentrations which did not show any growth of tested organism.

To confirm the activity, all wells of MIC test which not showed any growth of the bacteria fungus after the incubation period were subcultured onto the surface of the freshly prepared Mueller–Hinton Agar plates and incubated at 37 °C for 24 h (bacteria) or 48 h at 28 °C (fungus). The MBC was recorded as the lowest concentration of the essential oil that did not permit any visible bacteria colony growth on appropriate agar plate after the period of incubation.

Preparation of isolated rat thoracic aorta

After sacrifice, the thoracic artery aorta was removed from male rats ($n = 5$) and cleaned from connective tissue and fat. Rings (4–5 mm) were obtained and suspended individually by stainless steel rods knotted to cotton threads in a 10 mL organ bath at 37 °C containing the Krebs solution (NaCl 118.0 mM, KCl 4.55 mM, $MgSO_4 \cdot 7H_2O$ 5.70 mM, $KH_2PO_4 \cdot H_2O$ 1.10 mM, $CaCl_2 \cdot 2H_2O$ 2.52 mM, $NaHCO_3$ 25.0 mM, and glucose 11.00 mM) and aerated with carbogenic mixture (95% O_2 and 5% CO_2). Rings were stabilized with a resting tension of 1.0 g for 1 h, with solution being changed every 15 min. The isometric tension was recorded by a force transducer coupled to a data acquisition system (WinDaq, DATAQ Instruments, Inc., Akron, OH). The preparations were made in the presence or absence of the endothelium, being this removed by gently rubbing the intimal layer with stainless steel rods. The endothelium integrity was verified by relaxation to Ach (1.0 μM) in rings pre-contracted by Phe (1.0 μM). The vascular endothelium was considered intact when aortic rings were equally relaxed or more than 50% of Phe-induced (1.0 μM) pre-contractions (Silva-Filho et al., 2011). After stabilization period, a Phe-induced pre-contraction was elicited in endothelium-intact and endothelium-denuded rings to promote similar magnitude contractions, and essential oils (OAA, OJW, OOS, and OJS) was added cumulatively (1–729 μg/mL), after response to Phe had stabilized, to obtain a concentration–response curve.

Preparation of isolated rat uterus

Virgin female rats ($n = 5$) were pre-treated with diethylstilbestrol (100 μg/kg, subcutaneously) 18–24 h prior to performing the experiments to induce estrous stage. After sacrifice, myometrial tissue was removed and trimmed from surrounding connective tissue. The segment of uterus was cut longitudinally into strips that had about 1 cm in length and 1 mm wide, and were suspended individually in organ bath by cotton threads in a 10 mL organ bath at 32 °C containing Locke Ringer solution (NaCl 154.0 mM, KCl 5.63 mM, $CaCl_2$

2.16 mM, $NaHCO_3$ 5.95 mM, and glucose 11.00 mM) and aerated with carbogenic mixture. Strips were stabilized with a resting tension of 1.0 g for 45 min, with solution being changed every 15 min (Macêdo et al., 2011). The isometric tension was recorded by a force transducer coupled to a data acquisition system (WinDaq, DATAQ Instruments, Inc., Akron, OH). After the stabilization period, tissues were stimulated with KCl (60 mM) to obtain two similar contractile responses and essential oils (OAA, OJW, OOS, and OJS) was added cumulatively (1–729 μg/mL), after response to KCl had stabilized, to obtain a concentration–response curve.

Preparation of isolated guinea-pig trachea

The trachea from guinea-pigs ($n = 5$) sacrificed was cleaned and segmented to each 4–5 cartilaginous rings, being suspended individually by stainless steel rods knotted to cotton threads in a 10 mL organ bath at 37 °C containing the Krebs solution and aerated with carbogenic mixture. Rings were stabilized with a resting tension of 1.0 g for 1 h, with solution being changed every 15 min (Ribeiro et al., 2007). The isometric tension was recorded by a force transducer coupled to a data acquisition system (WinDaq, DATAQ Instruments, Inc., Akron, OH). The rings used in this experiment not were submitted to any mechanical event to retire epithelial layer, being considered as intact; however, the integrity of these epithelium not was experimentally determined. After stabilization period, two similar contractile responses were elicited with CCh (1.0 μM) and essential oils (OAA, OJW, OOS, and OJS) was added cumulatively (1–729 μg/mL), after second response to CCh had stabilized, to obtain a concentration–response curve.

Data analysis and statistics

The essential oil yield was calculated by the formula:

$$Y = V_o/[L_m - (L_m \times H)/100] \times 100$$

where Y is the final yield (%), V_o is the essential oil volume collected, L_m is the vegetal mass of the *L. thymoides* leaves, H is the humidity present in the L_m.

KI was calculated according to the formula:

$$KI = \frac{(100N + 100)\left(\log T'_{R_{(A)}} - \log T'_{R_{(N)}}\right)}{\log T'_{R_{(N+1)}} - \log T'_{R_{(N)}}}$$

where N is the number of carbon atoms of the alkane's pattern (C_8–C_{24}); $T'_{R(A)}$ is the retention time calculated of the peak; $T'_{R(N)}$ is the retention time of the alkane corresponding to the peak calculated; $T'_{R(N+1)}$ is the retention time of the alkane that eluted afterwards to calculated peak.

The antioxidant activity of DPPH assay was calculated using the formula % of activity = [(absorbance of the control − absorbance of the sample)/absorbance of the control] × 100, and concentration that caused 50% of the scavenging (EC_{50}) was calculated by the non-linear curve fitting.

The antioxidant activity of bleaching assay was calculated using the following formula:

$$\%\ \text{activity} = \left[1 - \frac{A_0 - A_t}{A_0^0 - A_t^0}\right] \times 100$$

where A_0 is the initial absorbance, A_t is the final absorbance measured for the test sample, A_0^0 is the initial absorbance, and A_t^0 is the final absorbance measured for the negative control (blank).

All numeric data were expressed as mean ± standard error of the mean (SEM); concentration that caused 50% of the relaxation (EC_{50}) in the assays involving smooth muscle was calculated by the non-linear curve fitting. The Student *t*-test and analysis of variance (ANOVA) were statistical parameters applied following the Tukey post test, being considered as significantly different at $p < 0.05$.

Results

Chemical analysis

To observe if there is variation in the chemical composition of the essential oil from the leaves of *L. thymoides* during the year, the plant was collected in the last week of the first month of each season. Climatological data from the city of Feira de Santana, Bahia, Brazil, where the plant was collected, exhibited characteristics typical of semi-arid climate (Table 1), with higher average temperatures, high insolation, and low rainfall, with irregular rainfall concentrated in a few months. According to the data presented in Table 1, it is observed that there was variation in the content of the oils, with OJS having the lowest value with 2.14% in the period of greatest insolation (420.7 h) and average temperature (28.9 °C), in contrast to OOS that had the highest level with 2.93%, which was obtained in a period that heat stroke was the second highest (382.4 h) and lower rainfall (74.1 mm). With this, it can be noted that rainfall and sunshine, which varied in the months that collections were made, did not influence the oil content of *L. thymoides*.

Chemical analysis of essential oils from *L. thymoides*, of Brazilian Northeast, revealed the presence of 45 terpenoids compounds (Table 2), with predominance of sesquiterpenes. Essential oil obtained in the four seasons had as a major constituent the sesquiterpene β-caryophyllene with concentrations between 17.22% and 26.27%. The highest percentage of β-caryophyllene was found in essential oil collected in the winter season. Other main terpenes that appeared after β-caryophyllene were camphene, camphor, borneol, α-caryophyllene, and germacrene D.

Antioxidant activity

Antioxidant potential of essential oils from *L. thymoides* was determined by scavenging of the DPPH free radical and β-carotene bleaching test, being data presented in Table 3. In both assays, essential oils do not presented efficacy, with values of EC_{50} values more than 236 μg/mL in the DPPH assay and percentual of inhibition minor than 11% in the β-carotene bleaching test.

Antimicrobial activity

Antimicrobial activity of essential oils from *L. thymoides* was tested against Gram-negative and Gram-positive bacteria and fungus, as presented in Table 4. The microorganisms *E. coli*, *P. aeruginosa*, *B. cereus*, and *S. choleraesuis* were resistant to all essential oils. *Staphylococcus aureus* and *C. albicans* were

Table 1. Monthly climatological data and content of essential oils from leaves of *L. thymoides* obtained in the four seasons.

		Essential oil code			
	Information	OAA	OJW	OOS	OJS
Collect	Date of collect	04/28/2009	07/24/2009	10/28/2009	01/28/2010
	Average temperature (°C)	25.5	23.3	26.9	28.9
	Relative humidity (%)	82	84	70	72
	Rainfall (mm)	281.1	107.4	74.1	119.3
	Total insolation (h)	357.1	242.2	382.4	420.7
Content of essential oils	Plant dry mass (g)	166.1	355.0	256.0	133.4
	Residual humidity (%)	10.79	8.72	9.45	8.52
	Oil volume (mL)	4.0	8.2	7.5	2.5
	Oil content (%)	2.41	2.31	2.93	2.14

OAA, essential oil from *L. thymoides* collected in autumn; OJW, essential oil from *L. thymoides* collected in winter; OOS, essential oil from *L. thymoides* collected in spring; and OJS, essential oil from *L. thymoides* collected in summer.

Table 2. Chemical composition of the essential oils from the leaves of *L. thymoides*.

			OAA		OJW		OOS		OJS	
Formula	Constituent	KI_L	KI_C	%	KI_C	%	KI_C	%	KI_C	%
$C_{10}H_{16}$	α-Thujene	930	930	0.11	930	0.09	930	0.27	–	–
$C_{10}H_{16}$	α-Pinene	932	938	1.81	938	0.94	938	2.38	931	1.42
$C_{10}H_{16}$	Camphene	946	953	5.44	953	2.64	954	5.66	943	3.25
$C_{10}H_{16}$	Sabinene	969	976	1.31	978	0.93	976	1.72	962	0.67
$C_{10}H_{16}$	β-Pinene	974	980	1.16	980	0.64	980	1.43	965	1.70
$C_{10}H_{16}$	β-Myrcene	988	991	0.94	991	0.49	991	0.94	–	–
$C_{10}H_{16}$	α-Phellandrene	1002	1007	0.08	1006	0.07	–	–	–	–
$C_{10}H_{16}$	3-Carene	1008	1013	0.27	1013	0.23	1013	0.26	–	–
$C_{10}H_{16}$	*o*-Cimene	1022	1026	0.47	1026	0.91	1027	2.69	1027	1.12
$C_{10}H_{16}$	Limonene	1029	1032	3.63	1032	2.42	1032	3.75	1032	1.67
$C_{10}H_{18}O$	1,8-Cineole	1031	1034	3.04	1034	3.88	1034	4.50	1034	1.86
$C_{10}H_{16}$	γ-Terpinene	1059	1061	0.51	1061	0.65	1062	1.01	1062	0.74
$C_{10}H_{16}$	Terpinolene	1088	1090	0.86	1090	0.47	1090	0.46	–	–
$C_{10}H_{16}O$	Pinocarveol	1139	–	–	1141	0.12	1141	0.22	–	–
$C_{10}H_{16}O$	Camphor	1146	1146	8.61	1146	4.92	1146	3.22	1146	4.52
$C_{10}H_{18}O$	Borneol	1169	1168	5.33	1168	4.45	1168	7.36	1168	6.46
$C_{10}H_{18}O$	4-Terpinenol	1177	1178	0.83	1178	0.59	1179	0.85	–	–
$C_{10}H_{18}O$	α-Terpineol	1188	–	–	1190	0.23	–	–	–	–
$C_{10}H_{14}O$	Verbenone	1205	–	–	1203	0.79	–	–	–	–
$C_{12}H_{20}O_2$	Bornyl acetate	1288	–	–	1286	0.35	–	–	–	–
$C_{10}H_{14}O$	Thymol	1290	–	–	1290	0.12	1291	0.21	1290	0.27
$C_{15}H_{24}$	δ-Elemene	1338	–	–	1340	0.38	–	–	–	–
$C_{15}H_{24}$	α-Cubebene	1348	1352	0.63	1352	0.60	1353	0.77	1353	0.65
$C_{15}H_{24}$	Copaene	1376	1378	2.47	1378	2.55	1379	3.38	1379	2.42
$C_{15}H_{24}$	β-Bourbonene	1388	–	–	1386	0.28	1387	0.40	–	–
$C_{15}H_{24}$	β-Cubebene	1388	–	–	–	–	1392	0.28	–	–
$C_{15}H_{24}$	β-Elemene	1390	–	–	1392	0.24	–	–	–	–
$C_{15}H_{24}$	α-Bergamotene	1412	1406	0.54	1407	0.29	1407	0.40	–	–
$C_{15}H_{24}$	α-Gurjunene	1409	–	–	1411	0.34	1412	0.38	1412	0.82
$C_{15}H_{24}$	α-Cedrene	1411	1411	0.30	1414	0.52	1415	0.82	–	–
$C_{15}H_{24}$	β-Caryophyllene	1419	1422	17.22	1423	26.27	1423	21.20	1423	19.28
$C_{15}H_{24}$	α-Caryophyllene	1454	1457	5.05	1456	3.99	1457	3.06	1457	5.48
$C_{15}H_{24}$	β-Farnesene	1456	–	–	1463	0.44	–	–	–	–
$C_{15}H_{24}$	Alloaromaden-drene	1460	–	–	1469	0.04	–	–	1465	0.57
$C_{15}H_{24}$	Germacrene D	1485	1483	4.80	1483	6.18	1484	4.72	1484	5.39
$C_{15}H_{24}$	α-Zingiberene	1493	–	–	1493	1.43	–	–	–	–
$C_{15}H_{24}$	α-Muurolene	1500	1506	0.57	1498	1.67	1507	1.06	1509	2.00
$C_{15}H_{22}$	Cuparene	1504	–	–	1506	0.78	1510	0.88	–	–
$C_{15}H_{24}$	β-Bisabolene	1505	–	–	1509	0.89	–	–	–	-
$C_{15}H_{24}$	*Z*-α-Bisabolene	1507	1509	1.12	–	–	–	–	–	–
$C_{15}H_{22}$	Calamenene	1529	1525	4.30	1525	3.53	1526	4.75	1526	3.83
$C_{15}H_{24}$	Germacrene B	1561	–	–	–	–	1561	0.88	1561	1.04
$C_{15}H_{26}O$	*trans*-Nerolidol	1561	1564	1.57	1564	3.59	1565	1.52	1565	1.69
$C_{15}H_{24}O$	Spathulenol	1578	–	–	1579	0.43	–	–	1580	1.28
$C_{15}H_{24}O$	Cariophyllene oxide	1582	1585	1.99	1585	2.11	–	–	1586	2.64
Total identified (%)	75.0		82.5		81.4		70.8			
Not identified %	25.0		17.5		18.6		29.2			

OAA, essential oil from *L. thymoides* collected in autumn; OJW, essential oil from *L. thymoides* collected in winter; OOS, essential oil from *L. thymoides* collected in spring; OJS, essential oil from *L. thymoides* collected in summer; KI_L, Kovats index from literature; KI_C, Kovats index calculated; %, relative percentage.

the most sensitive microorganisms (MIC <0.004 mg/mL) being inhibited by OAA, OOS, and OJS samples, as well as *M. luteus*. The essential oil from *L. thymoides* collected in winter (OJW) showed action only against *S. aureus* CCMB263 with lower activity (MIC = 10.19 mg/mL). For most of these essential oils, the values of MIC were not equivalent to MMC (MMC = 0.26 to >8.43 mg/mL).

Vasorelaxant activity in aorta smooth muscle

The essential oils relaxed isolated rat rings aorta with functional endothelium, pre-contracted with Phe 1 μM, in a concentration-dependent manner (1–729 μg/mL), as shown in Figure 1(A). CE_{50} values obtained of OAA (462.8 ± 8.9 μg/mL), OJW (511.1 ± 95.7 μg/mL), OOS (543.9 ± 73.9 μg/mL), and OJS (305.2 ± 126.0 μg/mL) do not differ significantly. None of the essential oils relaxed rings aorta in the endothelium presence to 100%, E_{max} value being obtained by OAA, OJW, OOS, and OJS, in the concentration of 729 μg/mL, 86.8 ± 5.5%, 62.2 ± 7.0%, 64.2 ± 7.1%, and 84.4 ± 10.5%, respectively.

In the endothelium absence, OAA (EC_{50} = 246.9 ± 43.7 μg/mL), OJW (EC_{50} = 282.6 ± 30.8 μg/mL), OOS (EC_{50} = 150.3 ± 26.3 μg/mL), and OJS (EC_{50} = 193.1 ± 57.4 μg/mL) also relaxed rings aorta pre-contracted with Phe 1.0 μM, in a concentration-dependent manner (1–729 μg/mL), as shown in Figure 1(B). There was no difference between CE_{50} values and the maximum relaxations to 729 μg/mL were OAA 90.9 ± 3.2%, OJW 82.8 ± 4.9%, OOS 98.5 ± 1.1%, and OJS 93.9 ± 3.8%.

Comparing the EC_{50} values of rings aorta relaxation pre-contracted with Phe in the presence and absence of functional endothelium (Figure 1C), it was observed that OAA and OOS were more potent into relax smooth muscle in the absence than in the presence of the endothelium. Further, E_{max} values achieved by OJW and OOS to 729 μg/mL were significantly higher in the absence than in the presence of functional endothelium (Figure 1D).

Table 3. Antioxidant activity of the essential oils from the leaves of *L. thymoides*.

Sample	Scavenging of DPPH (EC_{50}, μg/mL)	β-Carotene bleaching test (%AA)
OAA	> 243.0	10.8 ± 0.9
OJW	> 243.0	3.2 ± 0.2
OOS	> 243.0	3.2 ± 1.0
OJS	236.5 ± 3.5	0.3 ± 0.3
AAc	3.8 ± 1.8	15.1 ± 6.7
BHT	18.0 ± 1.0	NT
BHA	1.8 ± 0.2	NT
QUE	NT	45.4 ± 12.7
PYR	NT	2.5 ± 1.3

Data were expressed as mean ± SEM. EC_{50}, concentration that caused 50% of the DPPH scavenging; %AA, percentual of antioxidant activity in the β-carotene bleaching assay; OAA, essential oil from *L. thymoides* collected in autumn; OJW, essential oil from *L. thymoides* collected in winter; OOS, essential oil from *L. thymoides* collected in spring; OJS, essential oil from *L. thymoides* collected in summer; AAc, ascorbic acid; BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; QUE, quercetin; PYR, pyrogallol; NT, non tested.

Tocolytic activity in rat uterus

The essential oils relaxed isolated rat uterus pre-contracted with depolarizing KCl 60 mM, in a concentration-dependent manner (1–729 μg/mL), as shown in Figure 2(A). On one hand, only EC_{50} values of OJW (257.4 ± 26.4 μg/mL) and OOS (74.3 ± 39.2 μg/mL) differed significantly, as shown in Figure 2(B). On the other hand, E_{max} values to 729 μg/mL of OAA (94.4 ± 2.6%), OJW (88.0 ± 2.6%), OOS (94.0 ± 2.6%), and OJS (89.2 ± 2.5%) do not differ significantly.

Relaxant effect in trachea smooth muscle

The essential oils relaxed isolated guinea-pig trachea pre-contracted with CCh 1.0 μM, in a concentration-dependent manner (Figure 3), but E_{max} values of 729 μg/mL were lower than 60% and it was not possible to determine EC_{50}.

Discussion

Lippia thymoides is a native species of the Brazilian Caatinga vegetation, popularly used to treat various illnesses. Thus, due to their ethnopharmacological potential, our group was inspired to study, for the first time, the chemical composition and pharmacological properties of essential oils of this species, attempting to identify a pharmaceutical product of interest.

The essential oils from *L. thymoides* presented chemical composition similar to other species of the genus *Lippia* and

Table 4. Antimicrobial activity of the essential oils from leaves of *L. thymoides*.

	Sample									
	OAA		OJW		OOS		OJS		CHL	NYS
Microorganism	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MIC
E. coli CCMB 261	R		R		R		R		0.675	NT
S. aureus CCMB 262	0.004	0.53	10.19	10.19	<0.004	0.26	<0.004	>8.43	0.003	NT
P. aeruginosa CCMB 268	R		NT		R		R		0.084	NT
S. cholerasuis CCMB 281	8.43	8.43	NT		R		R		0.005	NT
B. cereus CCMB 282	R		NT		R		R		0.005	NT
M. luteus CCMB 283	0.03	8.43	NT		0.07	>8.43	0.03	>8.43	0.003	NT
C. albicans CCMB 286	<0.004	4.21	R		<0.004	4.21	<0.004	8.43	NT	1.25

MIC, minimum inhibitory concentration in mg/mL; MMC, minimum microbicidal concentration in mg/mL; OAA, essential oil from *L. thymoides* collected in autumn; OJW, essential oil from *L. thymoides* collected in winter; OOS, essential oil from *L. thymoides* collected in spring; OJS, essential oil from *L. thymoides* collected in summer; CHL, chloramphenicol; NYS, nystatin; R, resistant; NT, non-tested.

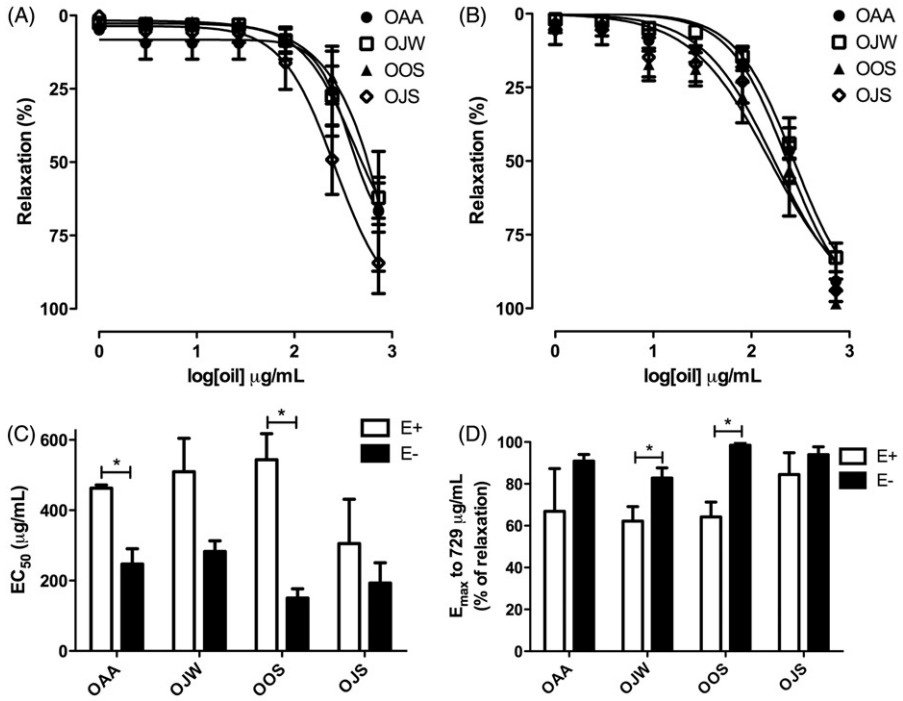

Figure 1. Vasorelaxant effect of essential oils from leaves of *L. thymoides* on tonic contractions induced by phenylephrine (Phe) 1.0 μM in isolated rat rings aorta. (A) Contractions induced in the presence of functional endothelium; (B) contractions induced in the absence of functional endothelium; (C) EC_{50} values in the presence (E+) or absence (E−) of endothelium; (D) E_{max} values obtained in the presence (E+) or absence (E−) of endothelium. Data are presented as mean ± SEM ($n = 5$); *EC_{50} or E_{max} values that were statistically different (*t*-test, $p < 0.05$) when compared with values of the same oil in the presence (E+) or absence (E−) of functional endothelium. OAA, essential oil from *L. thymoides* collected in autumn; OJW, essential oil from *L. thymoides* collected in winter; OOS, essential oil from *L. thymoides* collected in spring; OJS, essential oil from *L. thymoides* collected in summer.

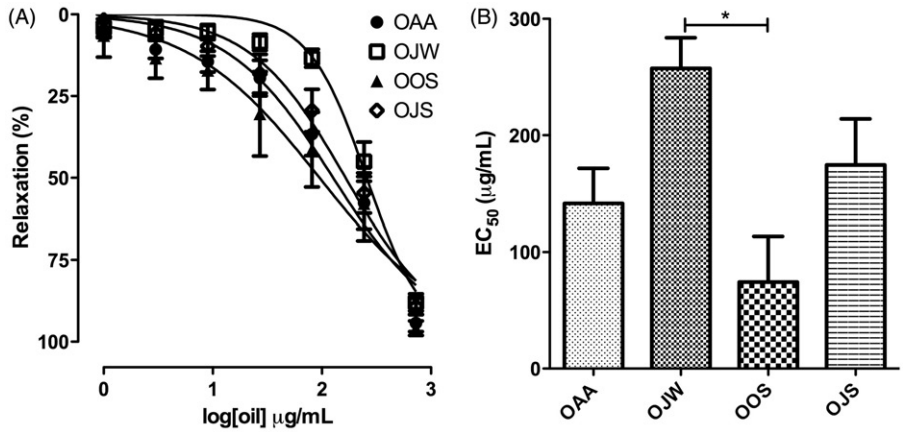

Figure 2. Spasmolytic effect of essential oils from leaves of *L. thymoides* on the tonic contractions induced with KCl 60 mM in isolated rat uterus. (A) Concentration–response curves of essential oils; (B) EC_{50} values of essential oils. Data are presented as mean ± SEM ($n = 5$); *EC_{50} values that were statistically different (ANOVA, $p < 0.05$). OAA, essential oil from *L. thymoides* collected in autumn; OJW, essential oil from *L. thymoides* collected in winter; OOS, essential oil from *L. thymoides* collected in spring; OJS, essential oil from *L. thymoides* collected in summer.

there are not great variations between the seasons, indicating that climatic factors are not associated with the presence of these constituents. Considering that the sesquiterpene β-caryophyllene is the majority in all essential oils, studies have indicated that interrelationship of the plant with various organisms in their environment induce the synthesis this compound, since β-caryophyllene can attract natural enemies of herbivores, as entomopathogenic nematodes below ground and parasitic wasps (Köllner et al., 2008). Furthermore, gene expression of β-caryophyllene synthase, that catalyses the conversion of farnesyl diphosphate to β-caryophyllene, is induced in response to elevated CO_2 and may enhance biosynthesis and phytotoxicity of allelochemicals, as β-caryophyllene (Elakovich & Stevens, 1985; Wang et al., 2010). Pascual et al. (2001) extensively revised the chemistry of *Lippia* and found that the components with the highest frequency in the essential oils of this genus are limonene, β-caryophyllene, *p*-cymene, camphor, linalool, α-pinene, and thymol. Furthermore, the sesquiterpene β-caryophyllene present in *L. thymoides* also appears as the main compound in other species at 11% in *Lippia americana*2 (Bueno et al., 2011), 15–16% in *L. graveolens* (Rivero-Cruz et al., 2011), 13% in *Lippia multiflora* Moldenke (Bassolé et al., 2010), and 18% in *L. dulcis* (Moreno-Murillo et al., 2010). Minor compounds present in the essential oils from *L. thymoides* are also in other species of the same genus, as camphor at 33% in *L. dulcis* (Görnemann et al., 2008), borneol at 9% in *Lippia integrifolia* Griseb. Hieron. (Lima et al., 2011), 1,8-cineole at 40% in *L. multiflora* (Avlessi et al., 2005), and 17% in *L. graveolens* (Rivero-Cruz et al., 2011). Recently, Cruz et al. (2014) reported that the concentration of major constituent from *Lippia gracilis* Schauer showed little variation between seasons, demonstrating the stability of the chemical composition of the essential oils even with different climatic conditions, confirming the findings of this work and indicating that this behavior could possibly be a characteristic of the species *Lippia*.

Despite reports indicate that essential oils also have antioxidant activity (Javanmardi et al., 2003; Terpinc et al., 2012), in this paper we demonstrated that *L. thymoides* was

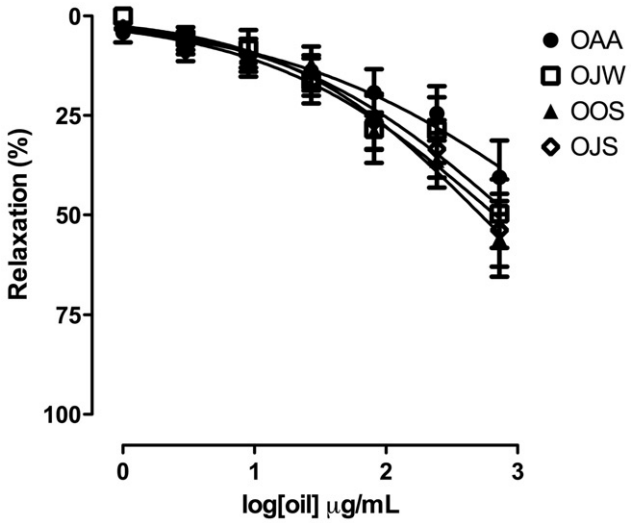

Figure 3. Effect of essential oils from leaves of *L. thymoides* on the tonic contractions induced with carbachol (CCh) 1.0 μM in isolated guinea-pig rings trachea. Data are presented as mean ± SEM ($n = 5$). OAA, essential oil from *L. thymoides* collected in autumn; OJW, essential oil from *L. thymoides* collected in winter; OOS, essential oil from *L. thymoides* collected in spring; OJS, essential oil from *L. thymoides* collected in summer.

not efficient in scavenging DPPH free radical or in inhibiting β-carotene oxidations. The DPPH method involves the use of organic free radical DPPH•, where antioxidants are allowed to react with the stable radical. In its radical form, DPPH• absorbs at 515 nm, but upon reduction by an antioxidant, the absorption disappears, possibly due to the ability of this compound to transfer labile H atoms to radical (Aruoma, 2003; Villaño et al., 2007). The β-carotene bleaching method (coupled oxidation of β-carotene and linoleic acid) estimates the relative ability of antioxidant compounds in essential oils to scavenge the radical of linoleic acid peroxide (LOO•) that oxidizes β-carotene in the emulsion phase (Miguel, 2010). In both the methods, compounds that have the capability of transferring H to DPPH• or LOO• may possibly present antioxidant potential, as observed in essential oils containing phenolic terpenes as a major constituent, as carvacrol, thymol, and eugenol, they are also more responsible for the antioxidant effect than mono- and sesquiterpenes (Adorjan & Buchbauer, 2010; Anthony et al., 2012). Thus, as there is a small amount of phenolic compounds in the essential oils from *L. thymoides*, we observed low potencies in such mixtures to act as an antioxidant. Still, various inflammatory disorders are related to oxidative stress and data of this work suggest that popular uses of this plant to treat fever, rheumatism, headache, and others are not related to essential oils, indicating the necessity to study non-volatile constituents.

In contrast, it was demonstrated that essential oils from *L. thymoides* (OAA, OOS, and OJS) were effective against *S. aureus* and *M. luteus*, both Gram-positive bacteria, as well as against *C. albicans*, but sample collected in winter season (OOW) was not so active. Since sesquiterpene β-caryophyllene is the major constituent in all oils, it is likely that this constituent is not mainly responsible for the antimicrobial effect. Furthermore, essential oils constitute a mixture of various substances and variations on chemical composition can influence the antimicrobial activity, as observed in essential oils containing camphor, borneol, and camphene, which were effective against fungi and Gram-positive and negative bacteria (Alva et al., 2012; Cárdenas et al., 2012; Wang et al., 2006). Studies indicate that monoterpenes and sesquiterpenes induce alteration in the bacterial membrane, promoting disruption and increasing the permeability (Cristani et al., 2007; Kuroda et al., 2007; Trombetta et al., 2005). So the morphology of the microorganisms influences the antimicrobial activity and can explain the selective effect of essential oils from *L. thymoides* against Gram-positive bacteria in comparison with Gram-negative. These data are according to popular use that this species can be used to treat wound and skin problems, since *S. aureus*, *M. luteus* and *C. albicans* are pathogen colonizing of the skin (Miltiadous & Elisaf, 2011; O'Riordan & Lee, 2004) indicating a possible antiseptic effect.

The essential oils obtained from *Lippia* species have demonstrated effective relaxation in different smooth muscles, as *L. alba* in rat mesenteric artery (Maynard et al., 2011) and *L. graveolens* in guinea-pig ileum (Rivero-Cruz et al., 2011). Thus, aiming to identify *L. thymoides* as a source of natural product of pharmacological interest, essential oils were tested in different isolated organs. Our data showed that OAA, OJW, OOS, and OJS relaxed effectively smooth muscle of rat aorta and uterus, but were not capable to relax guinea-pig trachea to a significant degree.

In rat aorta, the relaxation was endothelium independent, indicating that nitric oxide is probably not involved. On one hand, EC_{50} values of the essential oils in aorta preparations with functional endothelium can be related to induction, by some constituent of the mixture, of the production of endothelium-derived contracturant factors, as superoxide anions, endoperoxides, tromboxane A_2, or endothelin-1, which antagonized the spasmolytic effect of other constituent. On the other hand, the relaxation of rat uterus pre-contracted with KCl 60 mM indicates that relaxation induced by essential oils, probably, is related to blockage of Ca^{2+} influx or activation of hyperpolarizing component, as Ca^{2+}-activated K^+ channels (K_{Ca}). The indication of the popularity of *L. thymoides* to treat bronchitis stimulated the investigation on isolated guinea-pig trachea; however, the essential oils were not effective on relaxing airway smooth muscle, suggesting that folk medicine use this plant to treat illness respiratory is not related to essential oils. There are reports about relaxant effect of some constituent of *L. thymoides* essential oil, as β-caryophyllene on rat ileum (Leonhardt et al., 2010) and borneol on rat aorta (Silva-Filho et al., 2011), but their mechanism of action were not elicited.

Thus, this work demonstrated that essential oils obtained from leaves of *L. thymoides*, collected at the four seasons, had as the main compound the sesquiterpene β-caryophyllene with a low variation in other constituents. *In vitro* assays showed that these essential oils do not have antioxidant activity, but relax isolated aorta and uterus rat, and in minor effectiveness, isolated guinea-pig trachea. Antimicrobial selectivity to Gram-positive bacteria and yeast fungus was observed. All these data suggest that *L. thymoides* is a source of natural products of pharmaceutical interest, but additional

studies are necessary to determine the substances involved in the biological activities.

Declaration of interest

The authors report that they have no conflicts of interest. This study was supported by grants from Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB).

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