Essential Oil Composition, Antimicrobial and Pharmacological Activities of *Lippia sidoides* Cham. (Verbenaceae) From São Gonçalo do Abaeté, Minas Gerais, Brazil

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ABSTRACT

Background: *Lippia sidoides* (Verbenaceae) is used in Brazilian folk medicine as an antiseptic, and it is usually applied topically on skin, mucous membranes, mouth, and throat, or used for vaginal washings. **Objectives:** To analyze the chemical composition of the essential oil from *L. sidoides* collected in São Gonçalo do Abaeté, Minas Gerais and grown in Hidrolândia, Goiás; to evaluate the antimicrobial activity of the essential oil, crude ethanol extract, and hexane, dichloromethane, ethyl-acetate, and aqueous fractions (AFs); to study the antinociceptive, anti-inflammatory, and central nervous system activities of the crude ethanol extract. **Materials and methods:** The essential oils were obtained by hydro-distillation using a Clevenger-type apparatus and analyzed by GC/MS. The antimicrobial activity *in vitro* was performed by broth microdilution method. The pharmacological tests were performed using female Swiss albino mice. **Results:** The major components of the essential oil were isoborneol (14.66%), bornyl acetate (11.86%), α-humulene (11.23%), α-fenchene (9.32%), and 1.8-cineole (7.05%), supporting the existence of two chemotypes of this species. The hexane fraction (HF) had good antifungal activity against *Cryptococcus* sp. ATCC D (MIC = 31.25 µg/mL) and *Cryptococcus gatti* L48 (MIC = 62.5 µg/mL). In the pharmacological tests, the crude ethanol extract presented antinociceptive and anti-inflammatory activities. **Conclusion:** Given that the ethanol extract of *L. sidoides* is included in the Formulary of Phytotherapeutic Agents of the Brazilian Pharmacopeia as an anti-inflammatory for oral cavities, the present work provides scientific evidence to back this use and highlight the importance of selecting the appropriate chemotype on the basis of the expected biological response.

Key words: Anti-inflammatory activity, antimicrobial activity, antinociceptive activity, essential oil, *Lippia sidoides*

SUMMARY

The major components of the essential oil of *L. sidoides* were isoborneol bornyl acetate, α-humulene, α-fenchene, and 1.8-cineole.

- The HF had good antifungal activity against *Cryptococcus* sp. ATCC D and *C. gatti* L4.
- The crude ethanol extract of *L. sidoides* presented antinociceptive and anti-inflammatory activities.

- The present work provides scientific evidence of the importance of selecting the appropriate chemotype on the basis of the expected biological response.

Antinociceptive and anti-inflammatory activities
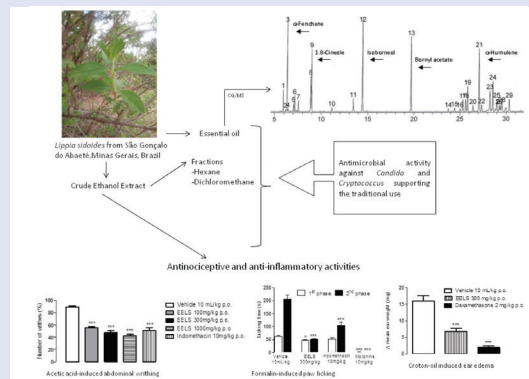
Abbreviations used: UFG: Universidade Federal de Goiás; HF: hexane fraction; DF: dichloromethane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; MeOH: methanol; MIC: minimum inhibitory concentration; ATCC: American Type Culture Collection; MH: Müller Hinton; DMSO: dimethyl sulfoxide; RPMI: Roswell Park Memorial Institute; NaCl: sodium chloride; µL: microliters; mL: milliliters; µg: microgram; kg: kilogram; h: hour; min: minute; cm: centimeter; COBEA: Brazilian College of Animal Experiments; p.o.: oral; i.p.: intraperitoneal; s.c.: subcutaneous; SEM: standard error of the mean; RI: retention indices.

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INTRODUCTION

Lippia sidoides Cham., popularly known in Brazil as "alecrim-pimenta" (pepper-rosemary), is native to the northeastern region of Brazil and north of the state of Minas Gerais. In folk medicine, this aromatic species is used as an antiseptic, and it is usually applied topically on skin, mucous membranes, mouth, and throat, or used for vaginal washings.[1–4] *L. sidoides* has larvicidal activity against *Aedes aegypti*,[5] anthelmintic activity in ovines and caprines,[6] insecticidal activity against *Lutzomyia longipalpis*, a vector of visceral leishmaniasis,[7] trypanocidal activity

against *Trypanosoma cruzi,*[8] and anti-inflammatory activity in mice.[9] *In vitro* studies of *L. sidoides* also revealed antifungal activity against *Candida* spp. and *Cryptococcus neoformans*[10–12] and antibacterial activity against *Escherichia coli, Listeria monocytogenes, Salmonella thyphimurium, Staphylococcus aureus, Streptococcus mutans,* and *Yersinia enterocolitica.*[13–15]

Results from previous studies suggest that the chemical composition of the essential oil of *L. sidoides* is variable. For instance, the major component of the essential oil of *L. sidoides* originated from the Brazilian northeast is thymol,[9,16–18] whereas the major components of *L. sidoides* from Lavras, Minas Gerais and São Gonçalo do Abaeté, Minas Gerais are carvacrol[19] and 1.8-cineole, isoborneol and bornyl acetate,[20] respectively. Therefore, it is likely that chemotypes of this species exist.

Considering the importance of the chemical composition for biological activities, the goals of this study were: to analyze the chemical composition of the essential oil from *L. sidoides* collected in São Gonçalo do Abaeté, Minas Gerais and grown in Hidrolândia, Goiás; to evaluate the antimicrobial activity of the essential oil, crude ethanol extract, and hexane, dichloromethane, ethyl acetate, and aqueous fractions; and to study the antinociceptive, anti-inflammatory, and central nervous system activities of the crude ethanol extract.

MATERIALS AND METHODS

Plant material

Rooted cuttings of *L. sidoides* native to São Gonçalo do Abaeté, Minas Gerais were transplanted and grown in Hidrolândia, Goiás (16°54'1.3" S, 49°15'35.2" W, elevation of 835 m). Voucher specimens were deposited in the herbarium of Universidade Federal de Goiás (record number UFG-47319). Leaves were collected in April 2012, dried at room temperature, and ground in a knife mill.

Extraction, identification, and analysis of essential oil

Powdered leaves were grinded and hydrodistilled in a Clevenger-type apparatus. The essential oil was desiccated with anhydrous sodium sulfate and stored in a freezer at –10°C. The essential oil was subjected to gas chromatography coupled with mass spectrometry (GC/MS) using a Shimadzu QP5050A instrument with a fused silica capillary column (CBP-5; 30 m × 0.25 mm × 0.25 µm). Helium was used as the carrier gas, with a constant flow rate of 1 mL/min. The column was heated with programmed temperatures (60°C for 2 min, 3°C per min up to 240°C, 10°C per min up to 280°C, and 280°C for 10 min). The ionization energy was 70 eV. An injection volume of 1 µL of each sample diluted in dichloromethane (20% w/v) was used, with a split ratio of 1:50.

Essential oil components were identified by comparing mass spectra and retention indices with values found in the literature.[21] Retention indices were calculated by coinjecting a mixture of hydrocarbons (C8–C32) and applying the Van Den Dool and Kratz equation.[22]

Preparation of extracts and fractions

Powdered leaves of *L. sidoides* were macerated three times at room temperature with 95% EtOH (v/v) using a proportion of 1:5 (w/v), then filtered and concentrated in a rotary evaporator below 40°C to obtain the crude ethanol extract (EELS).

Fractions were obtained from the EELS. Fifty grams of EELS were dissolved in 100 mL of MeOH/H$_2$O (7:3) and subjected to liquid–liquid partitioning with solvents of increasing polarity (hexane, dichloromethane, and ethyl acetate).[23] The solvents from each fraction were evaporated in a rotary evaporator. For the final MeOH/H$_2$O fraction, methanol was eliminated in a rotary evaporator and the resulting AF was lyophilized. Four fractions were obtained and designated as follows:

hexane fraction (HF), dichloromethane fraction (DF), ethyl-acetate fraction (EAF), and aqueous fraction (AF).

Antimicrobial activity

Essential oil, crude ethanol extract, and fractions (HF, DF, EAF, and AF) were subjected to a microdilution test in broth to determine the minimum inhibitory concentration (MIC) using sterile 96-well microplates with "U"-shaped wells, as recommended by the Clinical and Laboratory Standards Institute.[24,25] Experiments were carried out in duplicate.

Microorganisms used in the assays were standard strains from the American Type Culture Collection (ATCC) and clinical isolates provided by the Bacteriology and Mycology Laboratory of the Tropical Pathology and Public Health Institute) of Universidade Federal de Goiás (UFG). The following strains were used: fungi: *Candida parapsilosis* (ATCC 22019), *C. parapsilosis* (86U), *C. albicans* (63U), *Cryptococcus* sp. (ATCC D), *C. gatti* (L48), and *C. neoformans* (L3); Gram-positive bacteria: *Bacillus cereus* (ATCC 14579), *B. subtilis* (ATCC 6633), *Micrococcus roseus* (1740), *M. luteus* (ATCC 9341), *Staphylococcus epidermidis* (ATCC 12229), *S. aureus* (6538), and *S. aureus* (ATCC 25923); Gram-negative bacteria: *Enterobacter aerogenes* (ATCC 13048), *E. cloacae* (HMA/FT502), *E. coli* (ATCC 11229), *Pseudomonas aeruginosa* (ATCC 9027), *P. aeruginosa* (SPM1), *Salmonella* spp. (19430), and *Serratia marcenscens* (ATCC 14756).

Fungi were cultivated in Sabourad dextrose agar plates and incubated at room temperature for either 24 h (*Candida* spp.) or 48 h (*Cryptococcus* spp.). The bacteria were incubated in Casoy broth at 35°C for 24 h, transferred to an inclined Casoy agar, and incubated at 35°C for an additional 24 h to reactivate the cultures.

The culture medium used in the antifungal activity test was RPMI 1640 and the medium for the antibacterial activity test was 2× Müller Hinton broth (MH). Samples (essential oil, EELS, HF, DF, EAF, and AF) were solubilized in 10% dimethyl sulfoxide (DMSO) and 0.02% Tween 80® and diluted in either RPMI (antifungal activity) to obtain a concentration of 1000 µg/mL or in MH broth (antibacterial activity) to obtain a concentration of 2000 µg/mL.

For the antifungal assays, we added 100 µL of RPMI to all microplate wells in columns 2–12 and 200 µL of each sample to wells A through G in column 1. Using a multichannel pipette, serial dilutions were performed up to column 11, where 100 µL were discarded. Fungal inoculants were prepared in a sterile saline solution (NaCl 0.85%) to obtain a turbidity equivalent to half of the McFarland 1.0 scale (83.2–85% transmittance, measured with a spectrophotometer at 530 nm). Two dilutions (first 1:50, then 1:20) were prepared in RMPI to obtain cellular concentrations between 1 and 5 × 10⁵ UFC/mL. The final dilution of the inoculants in the wells ranged from 0.50 to 0.25 × 10³ UFC/mL. One hundred µL of each inoculate were added to each well to obtain sample concentrations between 1000 µg/mL and 0.97 µg/mL. No microorganisms were added to wells in line G, since they were used as controls of the sterility of the medium and of the samples tested. The positive control consisted of itraconazole (Sigma) at a concentration of 16 µg/mL. After inoculation, microplates were covered and incubated at room temperature for 48 h for *Candida* species, and 72 h for *Cryptococcus* species. The occurrence of fungal growth was checked visually.

For the antibacterial assays using microplates, 100 µL of MH were added to all wells from columns 2–12 and 200 µL of each sample were added to wells A through G in column 1. A multichannel pipette was used to obtain serial dilutions up to column 11, where the final 100 µL were discarded, for concentrations ranging from 2000 µg/mL to 1.95 µg/mL. Two hundred µL of MH broth containing 10% DMSO and 0.02% Tween 80® were added to line H as a negative control, and pure MH broth was added to column 12 to control for microbial growth. In addition, vancomycin (Sigma) at 32 µg/mL and gentamicin (Sigma) at 128 µg

mL were used as positive controls for Gram-positive and Gram-negative bacteria, respectively.

Bacterial inoculants were prepared in sterile saline solution (NaCl 0.85%) to obtain a turbidity equivalent to half of the McFarland 1.0 scale (79.4–83.2% transmittance, measured with a spectrophotometer at 625 nm). These inoculants were diluted 1:10 in saline to obtain a cell concentration of 10^7 UFC/mL. Five µL of the inoculants were placed in the wells, for a final concentration of 5×10^5 UFC/mL. No microorganisms were added to wells in line G, which were used as controls of the sterility of the medium and the samples tested. After bacterial inoculation, microplates were sealed and incubated at 35°C ± 2°C for 18–24 h. After the incubation period, 20 µL of 0.5% triphenyltetrazolium chloride (TTC) were added to all wells, and the microplates were reincubated for approximately half an hour. The appearance of a reddish color was considered proof of bacterial growth.

The MIC was defined as the lowest concentration of the sample (in µg/mL) fully capable of inhibiting bacterial or fungal growth. The classification proposed by Holetz *et al.*[26] was used to interpret the results of the tests of antimicrobial activity. Using this classification, MIC values below 100 µg/mL indicate good inhibitory activity; values between 100 and 500 µg/mL, moderate inhibitory activity; values between 500 and 1000 µg/mL, weak inhibitory activity; and values above 1000 µg/mL characterize an inactive substance.

Pharmacological tests

Experiments were performed using female Swiss albino mice (25–35 g) from the Central Animal House of Universidade Federal de Goiás (UFG). Animals were kept in plastic cages at 22 ± 2°C and on a 12 h light/dark cycle, with free access to pellet food and water, in compliance with the International Guiding Principles for Biomedical Research Involving Animals. All experimental protocols were developed according to the principles of ethics and animal welfare designated by the Brazilian College of Animal Experiments (COBEA). The experimental protocols were approved by the Ethics Commission of UFG (number: 104/08).

Effect on gross behavior or Irwin test

Experimental groups of mice ($n = 5$ per group) were treated orally (p.o.), intraperitoneally (i.p.), or subcutaneously (s.c.) with increasing doses of 50, 150, 450, and 1350 mg/kg crude ethanol extract solubilized (EELS) in 10% DMSO, whereas control groups received 10 mL/kg of the vehicle (10% DMSO). Animals were observed for 3 min in free ambulation on a flat surface at increasing intervals after treatment (5, 10, 20, 30, and 60 min, 4, 8, 24, and 48 h, and 4 and 7 days). The observed effects were compiled using a standard pharmacological screening approach, adapted from that described by Irwin.[27]

Antinociceptive activity

The antinociceptive effect of the EELS was tested using three different models of analgesia: the acetic acid-induced writhing test, the formalin-induced paw licking test, and the tail flick test.

Acetic acid-induced abdominal writhing test

Acetic acid-induced writhing is commonly used as a screening method for compounds with potential antinociceptive and/or anti-inflammatory activity. Nociception may be caused directly by the stimulation of nerve endings, or indirectly through the release of endogenous mediators involved in pain modulation.[28] The evaluation of abdominal contortions induced by acetic acid was carried out according to Hendershote and Forsaith[29] and Koster *et al.*[30] Groups of mice ($n = 8$) were treated by gavage with 100, 300, or 1000 mg/kg EELS (solubilized in 10% DMSO), vehicle (10% DMSO, 10 mL/kg – control group), or indomethacin (10

mg/kg – positive control for antinociceptive activity) 1 h before the application of an acetic acid solution (1.2% v/v; 10 m/kg, i.p.). The writhing response consisted of contraction of the abdominal wall and pelvic rotation followed by hind limb extension. The number of writhing movements made by each animal after the acetic acid injection was counted for 30 min immediately after acid administration. Results were expressed as mean number of writhes ± standard error of the mean (SEM) in 30 min for each experimental group. The median inhibitory dose was estimated on the basis of the dose-effect observed and used in other models.

Formalin-induced paw-licking test

Groups of mice ($n = 10$) were treated by gavage with 300 mg/kg EELS (solubilized in 10% DMSO), vehicle (10% DMSO, 10 mL/kg – control group), or 10 mg/kg indomethacin (positive control) 60 min before the administration of formalin 3% (formaldehyde 1.2% v/v) in the right hind paw, or s.c. with 5 mg/kg morphine (positive control) 30 min before the formalin application.[31] After the injection of the phlogistic agent, mice were individually placed in a transparent box with a mirror underneath to enable unhindered observation of the formalin-injected paw for 30 min. The duration of the reaction response (paw-licking time) was assessed in two periods. The first phase, from 0 to 5 min, corresponds to neurogenic pain caused by direct stimulation of the nociceptors, whereas in the second phase, between 15 and 30 min, the inflammatory pain is caused by the release of anti-inflammatory mediators.[32,33] Pain reaction was defined as vigorous licking and biting of the formalin-injected paw. The duration of the response was measured in seconds.

Tail flick test

Groups of mice ($n = 8$) were treated p.o. with 300 mg/kg EELS (solubilized in 10% DMSO) or 10 mL/kg vehicle (10% DMSO, 10 mL/kg – control group), or s.c. with 10 mg/kg morphine (positive control). The distal third of the tail of each animal was exposed to thermal stimulation using an analgesymeter. The number of seconds before a tail flick was observed (latency period) was used as an index of nociception. The maximum allowed time of exposure to the stimulus was 20 s. Latency was measured – 30, 0, 30, 60, 120, 150, and 180 min from treatment.[34]

Anti-inflammatory activity
Croton oil-induced ear edema test

Animal groups ($n = 10$) were treated p.o. with 300 mg/kg EELS (solubilized in 10% DMSO), vehicle (10% DMSO, 10 mL/kg – control group), or 2 mg/kg dexamethasone. After 60 min, cutaneous inflammation was induced by applying 20 µL of an acetone solution of 2.5% v/v croton oil to the inner surface of the right ear. The same volume of acetone was applied to the left ear. After 4 h, topical application of croton oil solution, mice were killed by cervical dislocation, and a plug (6 mm in diameter) was taken from both ears with a punch (modified by Carvalho *et al.*)[35] and weighed in an analytical balance. The inflammatory response (edema) was determined by measuring the difference in weight (mg) between the pairs of plugs.[36]

Central nervous system activities
Open-field test

This test was based on the methodology described by Siegel[37] and validated by Archer.[38] The test assesses mice ambulatory behavior and detects anxiolytic- or anxiogenic-like agents. The open field apparatus consisted of a white acrylic arena 40 cm in diameter with a wall height of 30 cm. The floor was marked with eight squares of equal areas. Test groups of mice ($n = 8$) were treated p.o. with 100, 300, or 1000 mg/kg EELS

(solubilized in 10% DMSO), vehicle (10% DMSO, 10 mL/kg – control group), or diazepam (5 mg/kg – positive control for motor impairment). After 60 min of treatment, animals were individually placed in the center of the open field and observed for 5 min. We recorded the exploratory activity of the animals (number of squares invaded in the central region), the amount of time spent at the center and in the periphery, and the intervals of time spent rearing, grooming, or immobile.

Chimney test

Test groups of mice (*n* = 8) were treated p.o. with 100, 300, or 1000 mg/kg EELS (solubilized in 10% DMSO), vehicle (10% DMSO, 10 mL/kg – control group), or 5 mg/kg diazepam (positive control). After 60 min of treatment, animals were individually placed at a mark 10 cm from the end wall of a 25-cm-long glass tube with an inner diameter of 2.5 cm and in a horizontal position. Once the animals reached the opposite end, the tube was moved to a vertical position, and we recorded the amount of time for an animal moving backwards to reach the mark. The motor coordination of animals that did not perform this task within 90 s was considered impaired.[39,40]

Pentobarbital-induced sleeping time

Groups of mice (*n* = 8) were treated p.o. with 100, 300, or 1000 mg/kg EELS (solubilized in 10% DMSO), vehicle (10% DMSO, 10 mL/kg – control), or 5 mg/kg diazepam (positive control for depressant effect). After 60 min, all animals were treated i.p. with 50 mg/kg sodium pentobarbital, a hypnosis inducer. The latency to sleep induction (loss of the righting reflex) and the duration of the hypnosis (time required to recover the righting reflex) were recorded for each animal.[41]

Statistical analysis

All results are expressed as means ± standard error of the mean (SEM). Data were analyzed statistically using either the Student-Newman-Keuls' to compare two means, or one-way analyses of variance (ANOVAs), followed by Tukey tests, when more than two means were involved.

RESULTS

Chemical analysis of the essential oil

The results of the quantitative and qualitative analyses of the essential oil, with volatile components listed in order of elution, are reported in [Table 1]. Total ion chromatograms of the essential oil

can be seen in [Fig. 1].

The essential oil had a yield of 0.8%. Identified compounds were 22 in number (87.99% of the components), including 28.49% oxygenated monoterpenes, 27.10% monoterpene hydrocarbons and 32.40% sesquiterpene hydrocarbons. The major components were isoborneol (14.66%), bornyl acetate (11.86%), α-humulene (11.23%), α-fenchene (9.32%), and 1.8-cineole (7.05%) [Table 1].

Table 1: Chemical compounds (%) and retention indices (RI) of the essential oils of leaves of *Lippia sidoides* Cham. (Verbenaceae) from São Gonçalo do Abaeté, Minas Gerais, Brazil.

Compound	RI[a]	EO
a-Pinene	939	2.08
a-Fenchene	952	9.32
Verbene	967	0.84
β-Pinene	979	1.83
Mircene	990	1.14
Limonene	1029	4.28
1.8-Cineole	1031	7.05
p-Menth-2,4(8)-diene	1088	0.56
Camphor	1146	1.97
Isoborneol	1160	14.66
Bornyl acetate	1288	11.86
a-Copaene	1376	0.52
β-Elemene	1390	0.53
a-Cedrene	1411	2.02
E-Caryophyllene	1419	2.16
cis-Thujopsene	1431	4.19
a-Guaiene	1439	1.01
a-Himachalene	1451	1.36
a-Humulene	1454	11.23
ar-Curcumene	1480	3.51
β-Selinene	1490	4.85
Germacrene a	1509	1.02
Monoterpene hydrocarbons		27.10
Oxygenated monoterpenes		28.49
Sesquiterpenes		32.40
Total identified		87.99
Yield (%, v/p)		0.8

[a]Compounds are listed in order of elution from an HP-5 column using the homologous series of *n*-alkanes.
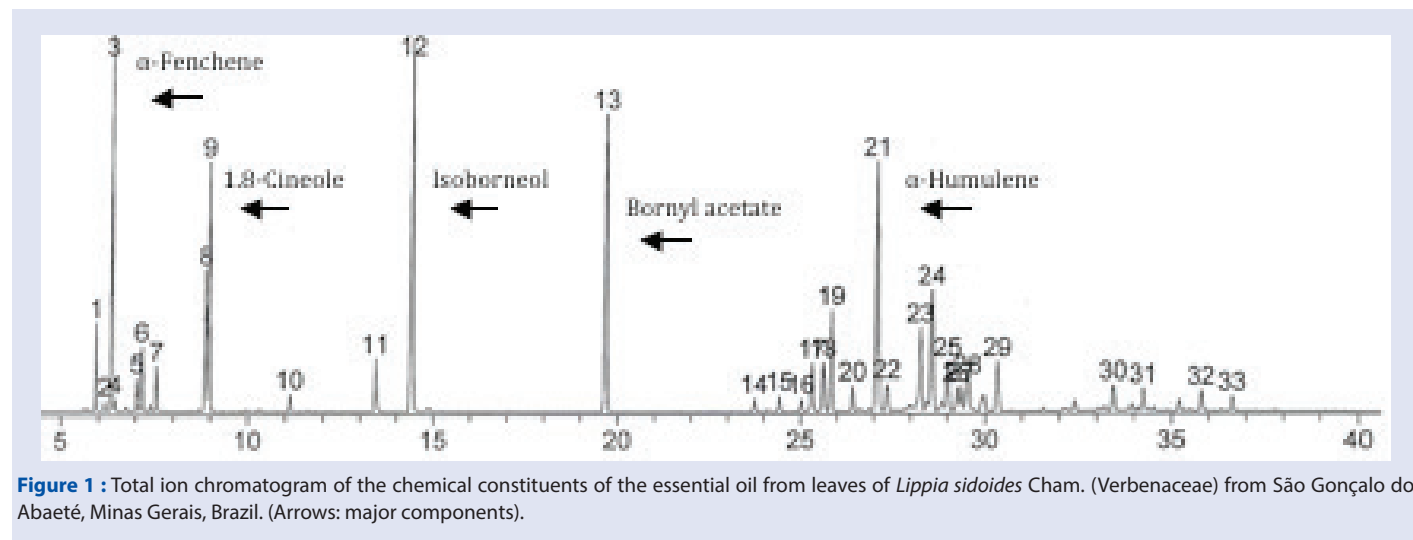

Figure 1 : Total ion chromatogram of the chemical constituents of the essential oil from leaves of *Lippia sidoides* Cham. (Verbenacee) from São Gonçalo do Abaeté, Minas Gerais, Brazil. (Arrows: major components).

Antimicrobial assays

The essential oil showed weak inhibitory activity (MIC = 500 µg/mL) against *S. aureus* 6538, *S. aureus* ATCC 25923, *C. parapsilosis* ATCC 22019, *C. parapsilosis* 86U, *Cryptococcus* sp. ATCC D and *C. gatti* L48, and was inactive toward the remaining microorganisms [Table 2].

Moderate inhibitory activity (MIC = 125–250 µg/mL) was observed for EELS against *Cryptococcus* sp. ATCC D, *C. gatti* L48, *C. neoformans* L3 and *C. parapsilosis* 86U, and weak inhibitory activity against *C. parapsilosis* ATCC 22019 and *C. albicans* 63U (MIC = 250 µg/

mL). Moderate antibacterial activity was observed against *M. luteus* ATCC9341 and *S. aureus* 6538, and the activity was weak or nonexistent for the other bacteria [Table 2].

The HF had good antifungal activity against *Cryptococcus* sp. ATCC D (MIC = 31.25 µg/mL) and *C. gatti* L48 (MIC = 62.5 µg/mL), and moderate inhibitory activity (MIC = 250 µg/mL) against *C. neoformans* L3, *C. parapsilosis* 86U, and *C. parapsilosis* ATCC 22019. This fraction had moderate inhibitory activity (MIC = 250 µg/mL) against *Bacillus cereus* ATCC 14579, *S. aureus* 6538, and *S. aureus* ATCC 25923, and

Table 2: Minimum inhibitory concentration (µg/mL) of the essential oil, extracts and fractions from leaves of **Lippia sidoides** Cham. (Verbenaceae) from São Gonçalo do Abaeté, Minas Gerais, Brazil.

Microorganisms	EO	EELS	HF	DF	EAF	AF	Vanc.	Gent.	Itrac.
Gram (+) bacteria									
Bacillus cereus ATCC 14579	1000	1000	250	1000	2000	2000	2	NC	NC
Bacillus subtilis ATCC 6633	1000	1000	2000	250	2000	2000	2	NC	NC
Micrococcus roseus 1740	1000	500	500	250	2000	2000	0.5	NC	NC
Micrococcus luteus ATCC 9341	1000	125	500	125	2000	2000	0.25	NC	NC
Staphylococcus epidermidis ATCC 12229	1000	1000	500	1000	1000	2000	1	NC	NC
Staphylococcus aureus 6538	500	250	250	500	2000	2000	2	NC	NC
Staphylococcus aureus ATCC 25923	500	1000	250	500	1000	2000	1	NC	NC
Gram (-) bacteria									
Enterobacter aerogenes ATCC 13048	2000	1000	500	250	2000	2000	NC	0.125	NC
Enterobacter cloacae HMA FTA502	2000	2000	500	500	1000	2000	NC	4	NC
Escherichia coli ATCC 11229	2000	1000	500	500	2000	2000	NC	2	NC
Pseudomonas aeruginosa ATCC 9027	2000	1000	500	2000	2000	2000	NC	4	NC
Pseudomonas aeruginosa SPM1	1000	1000	1000	2000	2000	2000	NC	4	NC
Salmonella spp. 19430	1000	2000	1000	500	2000	2000	NC	2	NC
Serratia marcenscens ATCC 14756	1000	2000	2000	2000	2000	2000	NC	4	NC
Fungi									
Candida parapsilosis ATCC 22019	500	1000	250	500	1000	1000	NC	NC	1
Candida albicans 63U	500	1000	1000	500	1000	1000	NC	NC	1
Candida parapsilosis 86U	1000	250	250	500	1000	1000	NC	NC	1
Cryptococcus sp ATCC D	500	250	31.25	125	1000	1000	NC	NC	2
Cryptococcus gatti L48	500	250	62.5	250	1000	1000	NC	NC	2
Cryptococcus neoformans L3	1000	250	250	250	1000	1000	NC	NC	2

EO, essential oil; EELS: crude ethanol extract; HF, hexane fraction; DF, dichloromethane fraction; EAF, ethyl acetate fraction; AF, aqueous fraction; Controls: Vanc., vancomycin (32 µg/mL); Gent., gentamicin (128 µg/mL); Itrac., itraconazole (16 µg/mL); NC, not carried out.

weak inhibitory activity (MIC = 500 μg/mL) toward *M. roseus 1740, M. luteus* ATCC 9341, *S. epidermidis* ATCC 12229, *E. aerogenes* 13048, *E. cloacae* AMA FTA502, *E. coli* ATCC 11229, and *P. aeruginosa* ATCC 9027 [Table 2].

The DF had moderate inhibitory activity (MIC = 125–250 μg/mL) for all strains of *Cryptococcus* and weak activity (MIC = 500 μg/mL) against *Candida*. It had moderate inhibitory activity (MIC = 125–250 μg/mL) against *Bacillus subtilis* ATCC 6633, *Micrococcus roseus* 1740, *M. luteus* ATCC 9341, and the Gram-negative bacterium *Enterobacter aerogenes* ATCC 13048, and weak inhibitory activity (MIC = 500 μg/mL) toward *S. aureus 6538, S. aureus* ATCC 25923, *E. cloacae* AMA FTA502, and *E. coli* ATCC 11229. The EAF and AF were inactive against all bacterial and fungal strains [Table 2].

Pharmacological activities of the crude ethanol extract

Effect on gross behavior

Animals treated subcutaneously (s.c.) with EELS at the 450 mg/kg dose erected their tails after 60 min. The intraperitoneal (i.p.) application of the same dose reduced their spontaneous ambulation between 5 and 20 min. When mice were treated p.o and s.c. at a dose of 1350 mg/kg, tail erection occurred within 10 to 20 min. No other effects, including palpebral ptosis, alienation, catatonia, defecation, urination, and death, were observed.

Analgesic activity

For the acetic-acid-induced abdominal writhing test, the number of abdominal writhes in the groups treated with EELS at doses of 100, 300, or 1000 mg/kg (55.55 ± 6.28, 47.28 ± 9.89, and 42.25 ± 7.12, respectively) were significantly lower than for the control group (88.85 ± 5.90), but there was no significant difference in relation to indomethacin (51.00 ± 13.06) [Fig. 2].

In the first phase of the formalin test, the animals treated with vehicle had a paw-licking of 62.90 ± 3.97 s. EELS (300 mg/kg) or morphine (5 mg/kg) led a significant reduction of time to reactivity to pain to 46.77 ± 2.78 s and 1.16 ± 0.79 s, respectively, whereas indomethacin (10 mg/kg) did not alter the reaction time (52.45 ± 5.14 s). During the second phase of the test, paw-licking time was decreased by EELS from 205.20 ± 15.67 s (control group) to 50.66 ± 4.08 s, by indomethacin to 105.16 ± 11.69 s, and by morphine to 0 ± 0.00 s [Fig. 3].

There was no significant difference in the latency to thermal stimulus in the tail of animals treated with EELS 300 mg/kg and those belonging to the control group. Treatment with 10 mg/kg of morphine increased latency to 30 min after the treatment [Fig. 4].

Evaluation of anti-inflammatory activity

In the croton oil-induced ear edema test, 300 mg/kg EELS or 2 mg/kg dexamethasone reduced the formation of ear edema from 15.99 ± 1.56 to 6.85 ± 0.85 and 2.02 ± 0.53, respectively [Fig. 5].

Central nervous system activities

In open field test, treatment with EELS (100, 300, or 1000 mg/kg) did not affect the number of squares crossed, squares invaded in the central region, time spent in the peripheral and central region, and rearing, parameters that were modified by diazepam at a dose of 5 mg/kg [Table 3].

In the chimney test, treatment with EELS (100, 300, or 1000 mg/kg) did not increase time spent by animals moving backwards to reach the chimney mark when compared to the control group. Treatment with diazepam (5 mg/kg) increased this time significantly in relation to the control group [Table 4].
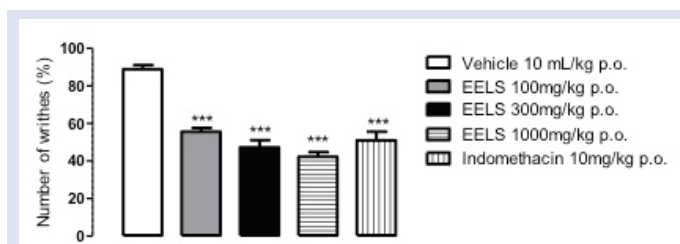

Figure 2 : Analgesic effect of the crude ethanolic extract from leaves of *Lippia sidoides* (EELS), as indicated by the number of acetic-acid-induced abdominal writhes in mice (*n* = 8). Indomethacin was used as a positive control. Bar heights and error bars express mean ± SEM of the number of writhing movements (%) in 30 min for each experimental group. ***$P \leq 0.001$ versus the control group (vehicle).
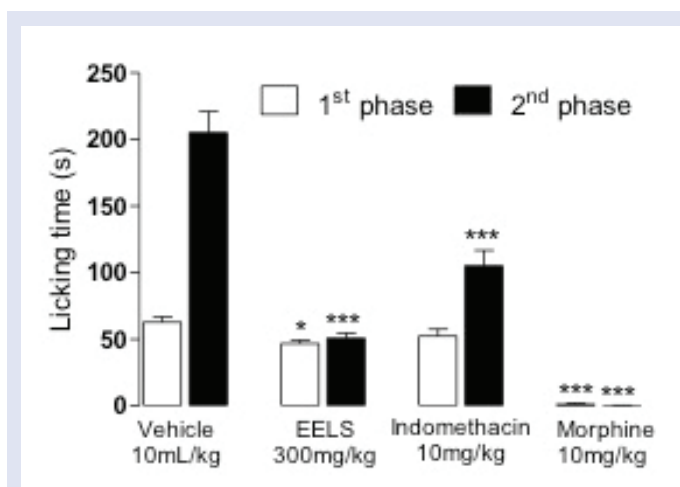

Figure 3 : Effect of the ethanolic extract from leaves of *Lippia sidoides* (EELS) on formalin-induced paw-licking test in mice (*n* = 8) during the neurogenic (1st phase; 0–5 min) and inflammatory phases (2nd phase; 15-30 min). Bar heights and error bars express means ± SEM of paw-licking time, in seconds. *$P \leq 0.05$ and ***$P \leq 0.001$ versus the control group (vehicle).
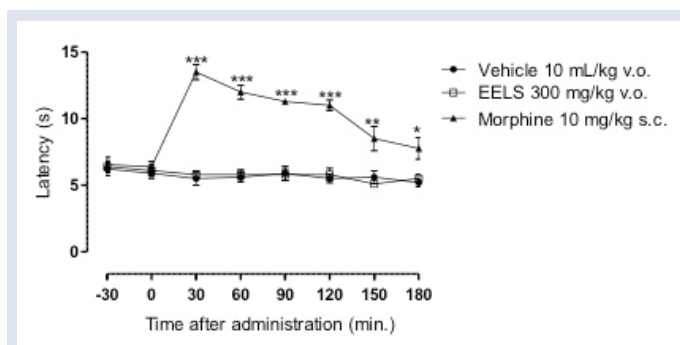

Figure 4: Effect of the ethanolic extract from leaves of *Lippia sidoides* (EELS) on pain reaction time in mice (*n* = 8). Symbols and error bars indicate means and SEM, respectively, expressed in seconds relative to the control group. Morphine (10 mg/kg) was used as a positive control. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ versus the control group (vehicle).

In the pentobarbital-induced sleeping time test, treatment with EELS (100, 300, or 1000 mg/kg) did not alter sleep latency or sleeping time induced by pentobarbital. Animals treated with diazepam (5 mg/kg), used as a positive control for this test, had decreased sleep latency and increased sleep time [Table 4].

DISCUSSION

The major constituents of the essential oil from leaves of *L. sidoides* from São Gonçalo do Abaeté, Minas Gerais were isoborneol (14.66%), bornyl acetate (11.86%), α-humulene (11.23%), α-fenchene (9.32%), and 1.8-cineole (7.05%). These results confirm the existence of chemical polymorphism in this species, since thymol was the major component in most studies that examined the essential oil of *L. sidoides* in northeastern Brazil, using individuals from Ceará (Horizonte, 59.65%, Fontenelle *et al.*[10] and 83.24%, Lima *et al.*;[42] Juazeiro do Norte, 84.90%, Marco *et al.*;[18] Crato, 84.87%, Mota *et al.*[43] and 84.90%, Veras *et al.*[9]), Rio Grande do Norte (Mossoró, 68.81% and Limoeiro do Norte, 70.36%, Cavalcanti *et al.*[17]) and Sergipe (Poço Redondo, 38.70%, Farias-Junior *et al.*;[44] São Cristovão, 43.33%, Carvalho *et al.*[16]) In contrast, Lima *et al.*[19] found that the major constituents in *L. sidoides* from Lavras, Minas Gerais were carvacrol (31.68%), ρ-cymene (19.58%) and 1.8-cineole (9.26%). Thymol was the major constituent of *L. sidoides* collected in Ceará and grown in Hidrolândia under the same conditions as described for this work (61.59%, preliminary results from the Research Laboratory of Natural Products/UFG), indicating that differences among populations in the chemical composition of the essential oil cannot be attributed to edaphoclimatic factors. The occurrence of chemical polymorphism was described for the genus *Lippia* by Tavares *et al.*[45] who identified three chemotypes of *Lippia alba*, and Jannuzzi *et al.*,[46] who found seven chemotypes in the same species.

Morais *et al.*[20] described 1.8 cineole as the major constituent (26.67%) of the essential oil of *L. sidoides* collected in São Gonçalo do Abaeté, Minas Gerais and grown in Hidrolândia, Goiás (August 2010), followed by isoborneol, bornyl acetate and α-humulene (14.60%, 10.77% and 5.66%, respectively). Although *L. sidoides* was collected and grown under the same conditions described in this work, differences between major constituents might be caused by the seasonality of the savanna biome in central Brazil, since in Morais *et al.*[20] leaves were collected during the dry season, but in the present work they were collected during the rainy season.

Regarding the antimicrobial assays, moderate activities of the ethanol extract and the HF were observed against *Candida albicans*. These results support the traditional use of *L. sidoides* for vaginal washings to treat candidiasis. Silva *et al.*[12] reported activity of the ethanol extract of *L. sidoides* collected in northeastern Brazil against *Candida albicans, C. krusei, and C. tropicalis*, and Fontenelle *et al.*[10] verified that the essential oil of *L. sidoides* from Horizonte, Ceará inhibited the growth of *Candida* spp. isolated from dogs and cats.

The HF had good inhibitory activity against *Cryptococcus,* the causal agent of cryptococcosis, a common infection in immunocompromised patients.[47] Activity against *C. neoformans* was related by Funari *et al.*[11] for the crude ethanol extract of *L. sidoides* collected in Iaras, São Paulo.

Antibacterial activity assays revealed moderate activity of the ethanol extract against *M. luteus* ATCC 9341 and *S. aureus* 6538, and activity of the DF against *B. subtilis* ATCC 6633, *M. roseus* 1740, *M. luteus* ATCC 9341, and *E. aerogenes* ATCC 13048. Bara and Vanetti[13] registered good inhibitory activity against *E. coli* for the crude ethanol extract of *L. sidoides* collected in Minas Gerais. In addition, the essential oil of *L. sidoides* from northeastern Brazil had good inhibitory activity against *S. aureus*,[15] *P. aeruginosa,* and *E. coli.*[1]
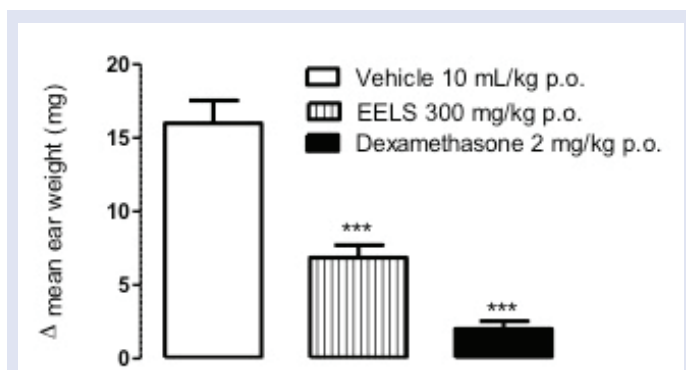

Figure 5: Effect of the ethanol extract from leaves of *Lippia sidoides* (EELS) on croton oil-induced ear edema in mice (*n* = 10). Bar heights and error bars show means ± SEM of differences in weight between right and left ear plugs. Dexamethasone (2 mg/kg) was used as a positive control. ***P ≤ 0.001 versus the control group (vehicle).

Table 3: Effects of the ethanol extract from leaves of *Lippia sidoides* (EELS) in the open field test with mice (*n* = 8). Diazepam was used as a positive control.

	Squares crossed (number)	Squares crossed in central region (number)	Squares crossed in peripheral region(number)	Time spent in central region (s)	Time spent in peripheral region (s)	Rearing (number)	Grooming (s)	Immobility time (s)	Defecation (number)
Vehicle (10 mL/kg)	107.2 ± 9.96	51.5 ± 3.37	41.5 ± 6.98	128.9 ± 16.94	172.4 ± 15.86	55.8 ± 2.91	6.1 ± 1.08	25.7 ± 2.65	1.3 ± 0.47
EELS (100 mg/kg)	105.0 ± 4.38	49.5 ± 3.41	57.8 ± 2.60	122.4 ± 3.94	177.3 ± 5.91	49.4 ± 2.24	5.4 ± 0.53	21.1 ± 2.98	1.3 ± 0.26
EELS (300 mg/kg)	118.3 ± 6.12	51.0 ± 3.14	53.3 ± 3.47	122.8 ± 6.69	171.3 ± 8.27	55.5 ± 2.29	3.9 ± 0.93	16.0 ± 3.83	1.5 ± 0.42
EELS (1000 mg/ kg)	90.8 ± 3.05	38.3 ± 4.93	51. 1 ± 4.74	120.9 ± 8.41	189.6 ± 9.21	47.7 ± 2.62	4.0 ± 0.44	39.8 ± 9.18	2.0 ± 0.57
Diazepam (5 mg/kg)	64.5± 7.30***	13.6 ± 2.89***	60.3 ± 5.24	34.8 ± 4.87***	273.6 ± 5.94***	22.8 ± 5.68***	7.2 ± 0.79	126.9 ± 19.38***	0.1 ± 0.14

Values are given as the mean ± SEM; ***P ≤ 0,001 relative to significance level of the differences between treated and control groups; according to ANOVA followed by Tukey test.

Table 4: Effects of treatment with the ethanol extract of leaves of *Lippia sidoides* (EELS) on the chimney test and on the pentobarbital-induced sleeping time test using mice (*n* = 8). Diazepam was used as a positive control.

	Chimney (s)	Sleep latency (s)	Sleeping time (min)
Vehicle (10 mL/kg)	4.77 ± 0.36	145.3 ± 7.01	70.8 ± 6.13
EELS (100 mg/kg)	3.7 ± 0.42	130.3 ± 4.64	69.6 ± 6.86
EELS (300 mg/kg)	4.4 ± 0.42	124.2 ± 3.97	69.9 ± 6.16
EELS (1000 mg/kg)	4.7 ± 0.42	128.1 ± 5.08	63.6 ± 9.32
Diazepam (5 mg/kg)	58.2 ± 1.75***	86.6 ± 5.01***	136.0 ± 10.84***

Values are given as means ± SEM; ***$P \leq 0.001$ (significance level of the differences between groups, based on ANOVAs followed by Tukey tests).

In the pharmacological tests, EELS presented antinociceptive activity in acetic acid-induced abdominal writhing test in all doses used (100, 300, and 1000 mg/kg, p.o.), and at the dose of 300 mg/kg in both first and second phases of formalin-induced pain test. However, EELS did not produce any analgesia in tail flick test, suggesting that this extract has no central analgesic properties. For the essential oil of *L. sidoides*, Alves *et al.*[48] observed antinociceptive activity through a reduction in the number of abdominal writhes, and Marçal *et al.*[49] found antinociceptive effects conforming to both thermal and chemical models.

The results obtained here showed that EELS inhibited the inflammation response induced by croton oil as well as dexamethasone, the positive control. Costa *et al.*[5] registered anti-inflammatory activity of the crude ethanol extract in an experimental model using zymosan-induced arthritis. Other published studies only investigated the anti-inflammatory activity of the essential oil of this species.[9,50–52] This result suggests that the antinociceptive activity of EELS in the in acetic acid-induced abdominal writhing test and the second phase of formalin-induced pain test may be associated with an anti-inflammatory effect.

Given that the ethanol extract of *L. sidoides* is included in the Formulary of Phytotherapeutic Agents of the Brazilian Pharmacopeia[53] as an anti-inflammatory for oral cavities, the present work provides scientific evidence to back the anti-inflammatory use of the crude ethanol extract of this species.

CONCLUSION

The major constituents of the essential oil of *L. sidoides* from São Gonçalo do Abaeté were isoborneol, bornyl acetate, α-humulene, α-fenchene, and 1.8 cineole, supporting the existence of two chemotypes of this species. Moderate activity of the HF was observed against *Candida*. The HF had good activity against *Cryptococcus*. Moderate activity was registered for the EELS against *M. luteus* and *S. aureus*, and for the DF against *B. subtilis*, *M. roseus*, *M. luteus*, and *E. aerogenes*. It was observed anti-inflammatory and antinociceptive activities of EELS, being that the antinociceptive activity may be associated with an anti-inflammatory effect. These results support the traditional use of this species, and highlight the importance of selecting the appropriate chemotype on the basis of the expected biological response.

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Conflict of interest
There is no conflict of interest.

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