Journal of Antimicrobial Chemotherapy (2007) **59**, 934–940
doi:10.1093/jac/dkm066
Advance Access publication 21 March 2007

JAC

Chemical composition, toxicological aspects and antifungal activity of essential oil from *Lippia sidoides* Cham.

R. O. S. Fontenelle[1], S. M. Morais[1,2], E. H. S. Brito[1], M. R. Kerntopf[1], R. S. N. Brilhante[3], R. A. Cordeiro[3], A. R. Tomé[1], M. G. R. Queiroz[4], N. R. F. Nascimento[1], J. J. C. Sidrim[3] and M. F. G. Rocha[1,3]*

[1]Post-Graduation Program in Veterinary Sciences, Veterinary Faculty, State University of Ceará, Fortaleza, Ceará, Brazil; [2]Department of Chemistry, State University of Ceará, Fortaleza, Ceará, Brazil; [3]Medical Mycology Specialized Center, Faculty of Medicine, Department of Pathology and Legal Medicine, Federal University of Ceará, Fortaleza, Ceará, Brazil; [4]Department of Clinical Biochemistry, School of Pharmacy, Federal University of Ceará, Fortaleza, Ceará, Brazil

Received 10 November 2006; returned 11 December 2006; revised 26 January 2007; accepted 8 February 2007

***Objectives*: The aims of this study were to test the essential oil from *Lippia sidoides* Cham. for antifungal activity, *in vitro*, against *Candida* spp. and *Microsporum canis*, to evaluate its acute and subchronic toxicological effects, *in vivo*, and to determine its chemical constituents.**

***Methods*: The antifungal activity, *in vitro*, was initially evaluated by the agar-well diffusion technique, and the MIC and minimum fungicidal concentration (MFC) were determined by the broth microdilution method. The acute and subchronic toxicological effects were determined in mice and rats, respectively. The chemical composition of the essential oil was determined by gas chromatography coupled to mass spectroscopy.**

***Results*: The essential oil obtained from *L. sidoides* was effective against all tested strains by the agar-well diffusion method. The MICs of *L. sidoides* essential oil for strains of *M. canis* ranged from 4 to 70 mg/L and the MFCs ranged from 9 to 150 mg/L. The MICs for strains of *Candida* spp. ranged from 620 to 2500 mg/L and the MFCs ranged from 1250 to 5000 mg/L. The main constituents of *L. sidoides* essential oil were thymol (59.65%), E-caryophyllene (10.60%) and *p*-cymene (9.08%). The acute administration of the essential oil up to 3 g/kg by the oral route to mice was devoid of overt toxicity. The 30 day oral administration of *L. sidoides* oil (117.95 mg/kg/day) to rats did not induce any significant histopathological, haematological or serum biochemical alterations.**

***Conclusions*: The essential oil from *L. sidoides* may be a promising source in the search for new antifungal drugs due to its efficacy and low toxicity.**

Keywords: *L. sidoides*, dermatophytes, yeasts, antifungal activity

Introduction

Mycosis constitutes a common health problem, especially in tropical and subtropical developing countries; dermatophytes, *Malassezia* spp. and *Candida* spp. being the most frequent pathogens in humans and animals.[1–7] In recent years, there has been an increasing search for new antifungal compounds due to the lack of efficacy, side effects and or resistance associated with some of the existing drugs.[8–11] Much attention has been paid to plant-derived antifungal compounds,[12] based on the knowledge that plants have their own defence systems against fungal pathogens.[13]

Natural products obtained from many plants have been attracting scientific interest.[9–16] More recently, the antifungal properties of allicin and ajoene isolated from garlic (*Allium sativum*) were demonstrated.[14,15] In traditional medicine, many essential oils have been claimed to be effective against fungal pathogens, although most of them are not clinically available.

*Corresponding author. Tel: +55-85-3295-1736; Fax: +55-85-3295-1736. E-mail: rocha@rapix.com.br

Many authors have reported that essential oils are one of the most promising groups of natural compounds from which a new prototype of antifungal agents may be developed.[11,14–21] Therefore, research in this field may lead to the development of effective drugs against pathogenic fungi.[11,14]

Widely spread in North-East Brazilian flora, *Lippia* species are known to be a natural topical antiseptic. Previous studies have reported that the essential oil of *Lippia sidoides* Cham. shows antimicrobial activity *in vitro* as well as larvicidal effect against *Aedes aegyptii*.[22,23]

The aims of this study were to test the essential oil from *L. sidoides* Cham. for *in vitro* antifungal activity against *Candida* spp. and *Microsporum canis*, to evaluate its acute and subchronic toxicological effects, *in vivo*, and to determine its chemical constituents.

Materials and methods

Plant material and essential oil extraction

Plant samples were collected in Horizonte city (3°33′46″ latitude S, 41°05′42″ longitude W), North-East Brazil. Taxonomic identification was confirmed by experts at the Prisco Bezerra Herbarium (Federal University of Ceará, Brazil), where a voucher sample was deposited with a reference number 25 149. *L. sidoides* essential oil was extracted from the leaves by the steam distillation method in a Clevenger apparatus, as described by Craveiro *et al*.[24]

Gas chromatography/mass spectral analysis

The chemical composition of the essential oil was determined by gas chromatography coupled to mass spectroscopy performed on a Hewlett–Packard 5971 GC/MS instrument in a polydimethylsiloxano-DB-5 (30 mm × 0.25 μm film thickness)-fused silica capillary column; the carrier gas was helium (1 mL/min). The column temperature ranged from 35°C to 180°C at 4°C/min, then from 180°C to 280°C at 20°C/min; mass spectra were obtained by electronic impact at 70 V. The identification of the constituents was performed by a computer-based library search, with retention indices and visual interpretation of the mass spectra.[23]

Fungal strains

The strains were obtained from the fungal collection of the Medical Mycology Specialized Centre (CEMM, Federal University of Ceará, Brazil), where they were maintained in saline (0.9% NaCl) at 28°C. At the time of the analysis, an aliquot of each suspension was taken and inoculated onto potato dextrose agar (Difco, Detroit, MI, USA), and then incubated at 28°C for 2–10 days. A total of 10 strains of *M. canis*, 5 strains of *Candida albicans* and 3 strains of *Candida tropicalis* were included in this study. Both *M. canis* and *Candida* spp. strains were isolated from dogs and cats. In addition, *Candida parapsilosis* (ATCC 22 019) and *Candida krusei* (ATCC 6528) strains were used for quality control.

Inoculum preparation for antifungal susceptibility tests

For the agar-well diffusion method, based on Tepe *et al*.[25] and Gurgel *et al*.,[12] stock inocula were prepared on day 2 and day 10 for *Candida* spp. and *M. canis*, respectively, grown on potato dextrose agar (Difco) at 28°C. Potato dextrose agar was added to the agar slant and the cultures were gently swabbed to dislodge the conidia. The suspension of conidia with blastoconidia of *Candida* spp. or that of hyphal fragments of *M. canis* was transferred to a sterile tube and adjusted by turbidimetry to obtain an inoculum of $\sim 10^6$ or 10^5 cfu/mL, respectively. The optical densities of the suspensions were spectrophotometrically determined at 530 nm.

For the broth microdilution method, the standardized inocula for *Candida* spp. ($2.5–5 \times 10^3$ cfu/mL) and *M. canis* (5×10^4 cfu/mL) were also prepared by turbidimetry. Stock inocula were prepared on day 2 and day 10 for *Candida* spp. and *M. canis*, respectively, grown on potato dextrose agar at 28°C. Sterile saline solution (0.9%) was added to the agar slant and the cultures were gently swabbed to dislodge the conidia from the hyphal mat and from the blastoconidia for *M. canis*[26] and *Candida* spp.,[4] respectively. The suspension of conidia with hyphal fragments of *M. canis* and the blastoconidia suspension of *Candida* spp. were transferred to sterile tubes and the volume of both suspensions adjusted to 4 mL with sterile saline solution. The resulting suspension was allowed to settle for 5 min, at 28°C, and its density was read at 530 nm and then adjusted to 95% transmittance. The suspensions were diluted to 1 : 2000 for *Candida* spp. and 1 : 500 for *M. canis* [both with RPMI 1640 medium with L-glutamine and without sodium bicarbonate (Sigma Chemical Co., St Louis, MO, USA), buffered at pH 7.0 with 0.165 M MOPS (Sigma Chemical Co.)] to obtain inocula of $\sim 2.5–5 \times 10^3$ and 5×10^4 cfu/mL, respectively.

Agar-well diffusion susceptibility test

The antifungal activity of essential oils from *L. sidoides* was evaluated against *C. albicans* ($n = 5$), *C. tropicalis* ($n = 3$) and *M. canis* ($n = 10$) by the agar-well diffusion method.[12,25] Petri dishes with a diameter of 15 cm were prepared with potato dextrose agar (Difco). The wells (6 mm in diameter) were then cut from the agar and 0.100 mL of essential oil or drugs was delivered to them. The oil was weighed and dissolved in mineral oil to obtain the test concentrations of 25, 50, 75 and 100 mg/mL. Stock solutions of griseofulvin (1 mg/mL; Sigma Chemical Co.) and amphotericin B (5 mg/L; Sigma Chemical Co.) were prepared in distilled water and tested as positive controls for *M. canis* and *Candida* spp., respectively. Each fungal suspension was inoculated onto the surface of the agar. After incubation, for 3–5 days for *Candida* spp. and 5–8 days for *M. canis*, at 28–35°C, all dishes were examined for zones of growth inhibition and the diameters of these zones were measured in millimetres. Each experiment was repeated at least twice.

Broth microdilution method

The MIC and minimum fungicidal concentration (MFC) for *Candida* spp. were determined by the broth microdilution method, in accordance with the CLSI (formerly NCCLS) guidelines (M27-A2).[27] The broth microdilution assay for *M. canis* was performed as described previously,[26,28,29] based on the M38-A document,[30] in accordance with the CLSI.

The essential oil of *L. sidoides* was prepared in 100% mineral oil. Amphotericin B (Sigma Chemical Co.) and griseofulvin (Sigma Chemical Co.) were prepared in distilled water. For the susceptibility analysis, the essential oil was diluted in mineral oil and tested in a concentration range between 0.002 and 5 mg/mL.

The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for each isolate tested. The microplates were incubated at 37°C and read visually after 2 days for *Candida* spp. and 5 days for *M. canis*. All isolates were run in duplicate and repeated at least twice. The MIC was defined as the lowest oil concentration that caused 80%

inhibition of visible fungal growth. The results were read visually as recommended by the CLSI. The MFC was determined by subculturing 100 μL of solution from wells without turbidity, on potato dextrose, at 28°C. The MFCs were determined as the lowest concentration resulting in no growth on the subculture after 2 days for *Candida* spp. and 5 days for *M. canis*.

Animals

Wistar rats (*Rattus norvegicus*; 180–200 g) and Swiss mice (*Mus musculus*; 25–30 g), of both sexes, were housed in temperature-controlled rooms and were given food and water *ad libitum* until used. All the protocols that included animals were approved by the Ethics Committee in research of the State University of Ceará, Fortaleza, Ceará, Brazil. The animals were used as recommended by the guide for the care and use of laboratory animals from the National Academy Press (USA; 1996), which fulfils the principles for animal use in Brazil.

Acute and subchronic toxicity

For the acute toxicity analysis, the essential oil was administered to the mice ($n = 10$ mice per group) orally or intraperitoneally (ip) at doses ranging from 100 to 3000 mg/kg. The results obtained were compared with those for the control animals [3% (v/v) Tween 80 in saline]. The LD_{50} was calculated by the probit method by using SPSS 7.0 for Windows. The animals were observed for an additional period of 1 h and the general effects were noted in a table modified from Malone and Robichaud.[31]

To investigate the subchronic toxicity of the essential oil of *L. sidoides*, after 30 days of oral administration to rats, the following parameters were evaluated: haematological, histopathological and serum biochemistry. The rats were separated into two groups ($n = 10$ per group) and treated with *L. sidoides* essential oil (117.95 mg/kg/day) or 3% (v/v) Tween 80 in saline by oral gavage. Blood samples were collected by puncture in the infraorbital plexus on day 0 (1 day before starting essential oil or vehicle administration) and then on day 15 and day 30. The serum concentrations of urea, creatinine, glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were determined by using commercial kits (Labtest, Lagoa Santa, MG, Brazil). The blood samples collected on day 0 and day 30 were used for determining red cell and leucocyte counts and for haemoglobin, haematocrit and biochemical parameter analysis. The values obtained were compared within and between the groups. Additionally, at the end of the experimental period (30 days), histopathological analysis of heart, lungs, liver, kidneys and spleen was performed by optical microscopy.

Statistical analysis

The antifungal activity evaluated by the agar-well diffusion method was expressed as mean ± SD of the diameter of the growth inhibition zones (mm). The antifungal activity of the essential oils was analysed by linear correlation for individual analysis and the two-tailed paired Student's *t*-test was used to evaluate differences between the data of essential oils and the controls. The LD_{50} was calculated at 95% confidence intervals, using SPSS 7.0 for Windows. The data obtained from subchronic toxicological studies were expressed as mean ±95% confidence intervals and data range. The differences within and between the groups were evaluated by the analysis of variance method, followed by the correction of Tukey–Kramer with the significance level set at 5%.

Results

The chemical analysis of the *L. sidoides* is shown in Table 1. The major constituents of the essential oil of *L. sidoides* were thymol (59.65%), E-caryophyllene (10.60%) and *p*-cymene (9.08%).

The essential oil from *L. sidoides* was effective against all tested strains in the agar-well diffusion susceptibility tests (Table 2). The *L. sidoides* oil induced a significant growth inhibition zone (36.8 ± 12.4 mm) at the lower concentration (25 mg/mL) against *M. canis* ($n = 10$). At concentrations ≥50 mg/mL, this essential oil totally inhibited *M. canis* ($n = 10$) grown in culture. For *Candida* strains ($n = 8$), the maximal inhibition of fungal growth induced by *L. sidoides* oil was 23.3 ± 1.8 mm, at the higher dose used (100 mg/mL). The positive control, griseofulvin, induced a significant growth inhibition zone (51.6 ± 6.7 mm) against *M. canis* ($n = 10$) and amphotericin B induced a significant growth inhibition zone (10.8 ± 1.5 mm) against *Candida* spp. ($n = 8$).

By the broth microdilution method, it was seen that MICs for *M. canis* strains ($n = 6$) ranged from 4 to 70 mg/L and MFCs ranged from 9 to 150 mg/L. The MICs for *Candida* spp. strains ($n = 6$) ranged from 620 to 2500 mg/L and the MFCs ranged from 1250 to 5000 mg/L (Table 3).

The oral administration of the essential oil at doses ranging from 100 to 3000 mg/kg did not induce any remarkable alterations in the behaviour pattern of mice. The calculated LD_{50} (ip) for essential oil of *L. sidoides* was 117.95 (110.61–125.29) mg/kg.

The subchronic oral administration of *L. sidoides* essential oil (117.95 mg/kg/day) was devoid of overt toxicity. The body weight, which was not affected by the treatment, was

Table 1. Chemical composition of *Lippia sidoides* Cham. essential oil

Retention index	Components	Composition (%)
931	α-thujene	1.48
939	α-pinene	0.51
991	myrcene	5.43
1018	α-terpinene	1.43
1026	***p*-cymene**	**9.08**
1031	limonene	1.01
1050	E-β-ocimene	0.27
1062	γ-terpinene	3.83
1098	linalool	0.28
1171	umbellulone	0.46
1235	methyl thymylether	1.79
1290	**thymol**	**59.65**
1376	α-copaene	0.66
1419	**E-caryophyllene**	**10.60**
1439	aromadendrene	0.53
1455	α-humulene	0.56
1459	dehydroaromadendrane	0.91
1524	δ-cadinene	0.35
1581	caryophyllene oxide	0.72

The identified constituents are listed in their order of elution from a non-polar column.

Table 2. Antifungal activity of the essential oil from *Lippia sidoides* Cham. against *Microsporum canis* and *Candida* spp. by the agar-well diffusion assay

Strains	Growth inhibition zones (mm)					
	L. sidoides essential oil (mg/mL)				griseofulvin (1 mg/mL)	amphotericin B (5 mg/L)
	25	50	75	100		
M. canis						
CEMM 01-3-188	48	TI	TI	TI	55	—
CEMM 01-5-190	35	TI	TI	TI	60	—
CEMM 01-4-104	29	TI	TI	TI	55	—
CEMM 01-3-165	30	TI	TI	TI	60	—
CEMM 01-2-133	20	TI	TI	TI	58	—
CEMM 01-4-097	34	TI	TI	TI	48	—
CEMM 01-3-173	35	TI	TI	TI	47	—
CEMM 01-4-086	35	TI	TI	TI	46	—
CEMM 01-3-004	60	TI	TI	TI	45	—
CEMM 01-4-102	42	TI	TI	TI	42	—
Mean ± SD	36.8 ± 12.4a	TI	TI	TI	51.6 ± 6.7	—
Candida spp.						
CEMM 01-3-075 (*C. albicans*)	10	16	24	25	—	14
CEMM 01-3-069 (*C. albicans*)	10	18	24	24	—	10
CEMM 01-3-077 (*C. albicans*)	11	17	23	23	—	12
CEMM 01-3-074 (*C. albicans*)	10	16	22	24	—	11
CEMM 01-3-068 (*C. albicans*)	10	19	25	25	—	10
CEMM 01-2-063 (*C. tropicalis*)	10	17	24	24	—	10
CEMM 01-2-078 (*C. tropicalis*)	08	12	13	20	—	10
CEMM 01-2-081 (*C. tropicalis*)	09	16	17	21	—	09
Mean ± SD	9.8 ± 0.9a	16.4 ± 2.1b	21.5 ± 4.2b	23.3 ± 1.8c	—	10.8 ± 1.5

TI, total inhibition of fungal growth. Letters mean significant differences in the columns at $P < 0.05$. Each experiment was repeated at least twice.

322.9 ± 18.96 g on day 1 and 328.3 ± 22.67 g on day 30 when compared with 331.1 ± 24.0 g versus 357.2 ± 21.2 g in vehicle-treated animals. Moreover, the serum biochemical parameters observed, i.e. creatinine, urea, GOT and GPT, were not significantly affected (Table 4). The histopathological evaluation of liver, kidneys, lungs, heart and spleen did not reveal any structural alterations in those organs obtained from *L. sidoides* essential oil-treated animals or in vehicle-treated animals. Similarly, the evaluations of red and white blood cells did not reveal any remarkable sign of haematological toxicity induced by *L. sidoides* essential oil (Table 5).

Discussion

Plant essential oils are a potentially useful source of antimicrobial compounds.[11,14] It is often quite difficult to compare the results obtained from different studies, because the compositions of the essential oils can vary greatly depending upon the geographical region, the variety, the age of the plant, the method of drying and the method of extraction of the oil.

In spite of the above-mentioned difficulties, essential oils from medicinal plants are excellent candidates for the development of remedies for many infectious diseases, including mycosis, due to the increasing development of antimicrobial resistance as well as the appearance of undesirable effects of some antifungal agents.[11]

Early reports on *L. sidoides* essential oil revealed its antimicrobial action. Lemos *et al.*[22] reported the highest and broadest activity against bacteria and fungi, including yeasts, dermatophytes and non-dermatophyte fungi. The present study shows that the essential oil from *L. sidoides* is quite effective against *M. canis*, the most common species of dermatophytes that cause superficial fungal infection in cats and dogs worldwide.[2,26] It induced a significant growth inhibition zone at the lower concentration (25 mg/mL), and at concentrations ≥50 mg/mL, this essential oil totally inhibited *M. canis* grown in culture. The positive control, griseofulvin, induced inhibition zones of 51.6 ± 6.7 mm in the agar-well diffusion method.

Concerning *Candida* spp., which are important yeasts involved in human and animal mycoses,[4,6,10] *L. sidoides* essential oil induced significant growth inhibition zones varying from 9.8 ± 0.9 to 23.3 ± 1.8 mm. Amphotericin B induced inhibition zones of 10.8 ± 1.5 mm in the agar-well diffusion method.

Previous research has suggested that several essential oils show important *in vitro* antifungal activity, with varied MIC and MFC values, against dermatophytes, yeasts and other fungi.[4,16,17,19,33,34] In this study, the MICs for *M. canis* strains ranged from 4 to 70 mg/L and the MFCs ranged from 9 to 150 mg/L. The MICs for *C. albicans* and *C. tropicalis* ranged

Table 3. MIC and MFC of *Lippia sidoides* Cham. essential oil against *Microsporum canis* and *Candida* spp. in the broth microdilution method

Strains	*L. sidoides* essential oil	
	MIC (mg/L)	MFC (mg/L)
Candida albicans		
CEMM 01-3-075	1250	2500
CEMM 01-3-069	1250	2500
CEMM 01-3-077	620	1250
CEMM 01-3-074	1250	2500
Candida tropicalis		
CEMM 01-2-078	2500	5000
CEMM 01-2-063	1250	2500
Geometric mean	1240	2500
M. canis		
CEMM 01-3-188	10	30
CEMM 01-5-190	30	70
CEMM 01-4-104	70	150
CEMM 01-3-165	9	10
CEMM 01-2-133	9	10
CEMM 01-4-097	4	9
Geometric mean	13.7	25.6

CEMM, Specialized Centre of Medical Mycology.

from 620 to 2500 mg/L and the MFCs ranged from 1250 to 5000 mg/L. *Candida* spp. and *M. canis* strains used by Brito *et al*.[4] and Brilhante *et al*.,[26] respectively, as well as *C. parapsilosis* ATCC 22 019 and *C. krusei* ATCC 6528, were used as controls in MIC determinations and the results were within the recommended limits (*Candida* spp. MIC $\leq$1 mg/L for amphotericin B and *M. canis* MIC $\leq$1 mg/L for griseofulvin).

By the agar-well diffusion and broth microdilution methods, this study shows that the essential oil of *L. sidoides* causes fungicidal activity. As there is a good correlation between the MICs, MFCs and the agar-well diffusion values of the essential oil of *L. sidoides*, it may be concluded that the antifungal activity of essential oils could be preliminarily investigated by the agar-well diffusion test for rapid screening.

The antimicrobial activity of essential oils from *Achillea setacea*,[17] *Pimpinella anisum*,[21] *Sesuvium portulacastrum*,[18] *Melaleuca alternifolia*,[11,19,20] *Juniperus* spp.,[16] *Allium* spp.[14,15] and *Thymus* spp.[32,33] is well known. The results obtained in the present research were very important to include the *L. sidoides* Cham. in this list of plants with antifungal activity.

Concerning *Malassezia pachydermatis*, which is the most common yeast in dermatitis and otitis externa in dogs,[5] although it was not the aim of this research, the essential oil from *L. sidoides* Cham. was also tested for antifungal activity against this yeast, *in vitro*, by the agar-well diffusion method. Our preliminary data showed that essential oil from *L. sidoides* was effective in a dose-related way, being, at the lower dose used (25 mg/mL), as efficient (growth inhibition zone of

Table 4. Serum biochemical parameters during subchronic oral administration of *Lippia sidoides* Cham. essential oil

Groups	Urea (mg/dL)	Creatinine (mg/dL)	GOT (U/mL)	GPT (U/mL)
L. sidoides				
day 0	88.71 $\pm$ 19.85 ($n = 10$)	0.87 $\pm$ 0.09 ($n = 10$)	100.2 $\pm$ 15.89 ($n = 10$)	52.1 $\pm$ 9.98 ($n = 10$)
day 15	55.96 $\pm$ 4.87 ($n = 9$)	0.87 $\pm$ 0.16 ($n = 8$)	125.7 $\pm$ 10.33 ($n = 9$)	55.54 $\pm$ 9.15 ($n = 9$)
day 30	46.56 $\pm$ 8.65 ($n = 9$)	0.84 $\pm$ 0.06 ($n = 9$)	135.3 $\pm$ 12.94 ($n = 9$)	41.86 $\pm$ 10.52 ($n = 9$)
Vehicle				
day 0	51.8 $\pm$ 18.65 ($n = 10$)	1.04 $\pm$ 0.05 ($n = 10$)	138.3 $\pm$ 15.89 ($n = 10$)	37.02 $\pm$ 13.68 ($n = 10$)
day 15	64.4 $\pm$ 9.53 ($n = 10$)	0.89 $\pm$ 0.13 ($n = 10$)	120.3 $\pm$ 10.33 ($n = 10$)	66.36 $\pm$ 13.77 ($n = 10$)
day 30	48.4 $\pm$ 7.68 ($n = 10$)	0.97 $\pm$ 0.15 ($n = 10$)	122.2 $\pm$ 12.94 ($n = 10$)	50.25 $\pm$ 14.25 ($n = 10$)

Data are expressed as mean $\pm$ SD. No statistical differences were noted.

Table 5. Haematological parameters during subchronic oral administration of *Lippia sidoides* Cham. essential oil

Parameters	Day 0		Day 30	
	mean $\pm$ SD	CI (95%)	mean $\pm$ SD	CI (95%)
Red cells ($\times 10^6$/mm^3)	3.6 $\pm$ 1.75	$3.5 \times 10^6 - 3.7 \times 10^6$	$3.5 \times 10^6 \pm 0.18 \times 10^6$	$3.3 \times 10^6 - 3.6 \times 10^6$
Haemoglobin (g/dL)	8.9 $\pm$ 1.3	8.08 $-$ 9.69	8.4 $\pm$ 1.28	7.47 $-$ 9.32
Haematocrit (%)	28.7 $\pm$ 1.2	27.84 $-$ 29.62	28.45 $\pm$ 1.17	27.61 $-$ 29.29
Leucocytes ($\times 10^3$/mm^3)	6820 $\pm$ 877	6121 $-$ 7519	6590 $\pm$ 967.8	5898 $-$ 7282

CI, confidence interval. No statistical differences were noted.

30.0 ± 10.0 mm; $n = 10$; data not shown) as the positive control itraconazole (29.7 ± 9.0 mm; $n = 10$; data not shown). Therefore, these data reinforce the potential antifungal activity of this essential oil.

To identify the composition of the oil from *L. sidoides* Cham., the oil derived from steam distillation was analysed by gas chromatography/mass spectroscopy. The main component was thymol (59.65%). The main constituents of essential oils, which show important antifungal activity, are phenolic compounds (terpenoids and phenylpropanoids), such as thymol, carvacrol or eugenol, of which antimicrobial activity is well documented.[16] Therefore, the activity of the essential oil from *L. sidoides* against *Candida* spp., *M. canis* and *M. pachydermatis* may partly be explained by the high amounts of thymol, which was previously reported to be effective as an antifungal.[32,33]

Regarding pharmacokinetic studies with the essential oil from *L. sidoides*, this research was limited in this field. However, owing to its high liposolubility, it would appear that the absorption of this essential oil after oral or ip administration would not be impaired, as can be confirmed by the LD_{50} experimental protocol ($LD_{50} = 117.95$ mg/kg). Corroborating the methodology used for the evaluation of the acute and subchronic toxicity performed in this research, other studies have used a similar strategy for the toxicological study of essential oils.[34,35] In addition, a study using thymol has shown that thymol sulphate and thymol glucuronide can be detected, after a single oral administration, for 24 h in urine and 41 h in plasma.[36]

The use of the essential oil from *L. sidoides* did not induce any significant acute toxicological alterations in the mice. The subchronic daily administration of *L. sidoides* essential oil for 30 days (oral administration) did not induce any remarkable alterations in the biochemical or haematological parameters analysed and nor was there any increase in the weight or structural pattern of the main organs, as revealed by the histopathological analysis. Although additional tests, such as reproductive toxicity analysis and cytotoxic and mutagenesis evaluation, must be performed, the present results show that *L. sidoides* essential oil is probably safe for acute use *in vivo*.

In this preclinical phase, the crude essential oil from *L. sidoides* Cham. administered by oral or ip route was evaluated to determine whether it induces any toxicity (physical, behavioural, biochemical, haematological or histopathological changes) after acute or subchronic experiments. Thus, the results obtained in this stage will certainly be helpful in future clinical studies, where specific tests will be performed to establish the safe profile of this essential oil for clinical use.

Owing to its broad spectrum of antifungal effect, *in vitro*, and low toxicity, the essential oil of *L. sidoides* Cham. is a promising source in the search for new antifungal drugs. However, specific pharmacological approaches will be needed in future clinical trials to validate its use as a phytotherapeutic product.

Acknowledgements

We are thankful for the financial support of FUNCAP (Ceará State Research Funding) and CNPq (National Council for Technological and Scientific Development, Brazil, Proc. CNPq: 478906/2004-8).

Transparency declarations

None to declare.

References

1. Gueho E, Boekhout T, Ashbee HR *et al*. The role of *Malassezia* species in the ecology of human skin and as pathogens. *Med Mycol* 1998; **36** Suppl 1: 220–9.

2. Brilhante RSN, Cavalcante CSP, Soares-Júnior FA *et al*. High rate of *Microsporum canis* feline and canine dermatophytoses in Northeast Brazil: epidemiological and diagnostic features. *Mycopathology* 2003; **156**: 303–8.

3. Prado MR, Brito EHS, Girão MD *et al*. Higher incidence of *Malassezia pachydermatis* in the eyes of dogs with corneal ulcer than in healthy dogs. *Vet Microbiol* 2004; **100**: 115–20.

4. Brito EHS, Fontenelle ROS, Brilhante RSN *et al*. Phenotypic characterization and *in vitro* antifungal sensitivity of *Candida* spp. and *M. pachydermatis* strains from dogs. *Vet J* 2006; doi: 10.1016/j.trjl.2006.05.021.

5. Girão MD, Prado MR, Brilhante RSN *et al*. *Malassezia pachydermatis* isolated from normal and diseased external ear canals in dogs: a comparative analysis. *Vet J* 2006; **172**: 544–8.

6. Colombo AL, Nucci M, Park BJ *et al*. Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical centers. *J Clin Microbiol* 2006; **44**: 2816–23.

7. Nardin ME, Pelegri DG, Manias VG *et al*. Etiological agents of dermatomycoses isolated in a hospital of Santa Fe City, Argentina. *Rev Argent Microbiol* 2006; **38**: 25–7.

8. Barker KS, Rogers PD. Recent insights into the mechanisms of antifungal resistance. *Curr Infect Dis Rep* 2006; **8**: 449–56.

9. Klepser ME. *Candida* resistance its clinical relevance. *Pharmacotherapy* 2006; **26**: 68S–75S.

10. Aperis G, Myriounis N, Spanakis EK *et al*. Developments in the treatment of candidiasis: more choices and new challenges. *Expert Opin Investig Drugs* 2006; **15**: 1319–36.

11. Mondello F, De Bernardis F, Girolamo A *et al*. *In vivo* activity of terpinen-4-ol, the main bioactive component of *Melaleuca alternifolia* Cheel (tea tree) oil against azole-susceptible and -resistant human pathogenic *Candida* species. *BMC Infect Dis* 2006; **6**: 158.

12. Gurgel LA, Sidrim JJC, Martins DT *et al*. *In vitro* antifungal activity of dragon's blood from *Croton urucurana* against dermatophytes. *J Ethnopharmacol* 2005; **97**: 409–12.

13. Wojtaszek P. Oxidative burst an early plant response to pathogen infection. *Biochem J* 1997; **322**: 681–92.

14. Pyun MS, Shin S. Antifungal effects of the volatile oils from *Allium* plants against *Trichophyton* species and synergism of the oils with ketoconazole. *Phytomedicine* 2006; **13**: 394–400.

15. Ledezma E, Apitz-Castro R. Ajoene the main active compound of garlic (*Allium sativum*): a new antifungal agent. *Rev Iberoam Micol* 2006; **23**: 75–80.

16. Cavaleiro C, Pinto E, Gonçalves MJ *et al*. Antifungal activity of *Juniperus* essential oils against dermatophyte, *Aspergillus* and *Candida* strains. *J Appl Microbiol* 2006; **100**: 1333–8.

17. Unlu M, Daferera D, Donmez E *et al*. Compositions and the *in vitro* antimicrobial activities of the essential oils of *Achillea setacea* and *Achillea teretifolia* (Compositae). *J Ethnopharmacol* 2002; **83**: 117–21.

18. Magwa ML, Gundidza M, Gweru N *et al*. Chemical composition and biological activities of essential oil from the leaves of *Sesuvium portulacastrum*. *J Ethnopharmacol* 2006; **103**: 85–9.

19. Hammer KA, Carson CF, Riley TV *et al*. *In vitro* activity of *Melaleuca alternifolia* (tea tree) oil against dermatophytes other filamentous fungi. *J Antimicrobial Chemother* 2002; **50**: 195–9.

20. Hammer KA, Carson CF, Riley TV *et al*. Antifungal effects of *Melaleuca alternifolia* (tea tree) oil and its components on *Candida albicans, Candida glabrata* and *Saccharomyces cerevisiae. J Antimicrob Chemother* 2004; **53**: 1081–5.

21. Kosalec I, Pepeljnjak S, Kustrak D *et al*. Antifungal activity of fluid extract and essential oil from anise fruits (*Pimpinella anisum* L., Apiaceae). *Acta Pharm* 2005; **55**: 377–85.

22. Lemos TLG, Matos FJA, Alencar JW *et al*. Antimicrobial activity of essential oils of Brazilian plants. *Phytother Res* 1990; **4**: 82–4.

23. Carvalho AFU, Melo VMM, Craveiro AA *et al*. Larvicidal activity of the essential oil from *Lippia sidoides* Cham. against *Aedes aegypti* linn. *Mem Inst Oswaldo Cruz* 2003; **98**: 569–71.

24. Craveiro AA, Matos FJA, Alencar JW *et al*. A simple and inexpensive steam generator for essential oils extraction. *J Chem Edu* 1976; **53**: 652.

25. Tepe B, Daferera D, Sokmen A *et al*. Antimicrobial and antioxidant activities of the essential oil and various extracts of *Salvia tomentosa* Miller (Lamiaceae). *Food Chem* 2005; **90**: 333–40.

26. Brilhante RSN, Cordeiro RA, Medrano DJ *et al*. Antifungal susceptibility and genotypical pattern of *Microsporum canis* strains. *Can J Microbiol* 2005; **51**: 507–10.

27. National Committee for Clinical Laboratory Standards. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts—Second Edition: Approved Standard M27-A2*. Clinical and Laboratory Standards Institute, NCCLS, Villanova, PA, USA, 2002.

28. Jessup CJ, Warner J, Isham I *et al*. Antifungal susceptibility testing of dermatophytes: establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical solates. *J Clin Microbiol* 2000; **38**: 341–4.

29. Fernandez-Torres B, Cabanes FJ, Carrillo-Munoz AJ *et al*. Collaborative evaluation of optimal antifungal susceptibility testing conditions for dermatophytes. *J Clin Microbiol* 2002; **40**: 3999–4003.

30. National Committee for Clinical Laboratory Standards. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi: Approved Standard M38-A*. Clinical and Laboratory Standards Institute, NCCLS, Villanova, PA, USA, 2002.

31. Malone MH, Robichaud RC. A Hippocratic screening for pure or crude drug material. *Lloydia* 1962; **25**: 320–32.

32. Pina-Vaz C, Gonçalves Rodrigues A, Pinto E *et al*. Antifungal activity of *Thymus* oils and their major compounds. *J Eur Acad Dermatol Venereol* 2004; **18**: 73–8.

33. Pinto E, Pina-Vaz C, Salgueiro L *et al*. Antifungal activity of the essential oil of *Thymus pulegioides* on *Candida, Aspergillus* and dermatophyte species. *J Med Microbiol* 2006; **55**: 1367–73.

34. Viana GS, Vale TG, Silva CM *et al*. Anticonvulsant activity of essential oils and active principles from chemotypes of *Lippia alba* (Mill.) N.E. Brown. *Biol Pharm Bull* 2000; **23**: 1314–7.

35. Orafidiya LO, Agbani EO, Iwalewa EO *et al*. Studies on the acute and sub-chronic toxicity of the essential oil of *Ocimum gratissimum* L. leaf. *Phytomedicine* 2004; **11**: 71–6.

36. Kohlert C, Schindler G, Marz RW *et al*. Systemic availability and pharmacokinetics of thymol in humans. *J Clin Pharmacol* 2002; **42**: 731–7.