Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
20(2): 261-266, Abr./Mai. 2010
Received 15 October 2008; Accepted 7 February 2009

Artigo

The essential oil from *Lippia gracilis* Schauer, Verbenaceae, in diabetic rats

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RESUMO: "O oleo essencial de *Lippia gracilis* Schauer, Verbenaceae, em ratos diabeticos". O óleo essencial das folhas de *Lippia gracilis* Schauer, Verbenaceae, foi examinado por CG e CG-MS. Quinze constituintes foram identificados, onde o carvacrol, *p*-cimeno e o γ-terpineno foram as substâncias majoritárias. No estudo *in vitro*, a solução a 5% do óleo de *L. gracilis* Schauer apresentou atividade antibacteriana para *Staphylococcus aureus* isolado de úlcera infectada de paciente diabético. O estudo avaliou a atividade antibacteriana da solução a 5% do óleo de *L. gracilis* Schauer em modelo experimental em diabetes utilizando ratos albinos Wistar machos com membro pélvico infectado com cepa de *Staphylococcus aureus*. No experimento foram utilizados 28 ratos Wistar distribuídos em quatro grupos (G1-branco, G2-controle negativo, G3-controle positivo, G4-teste) de sete ratos. Quando comparado o grupo G4 com G3, observou-se que a solução a 5% promoveu uma redução nas CFU/mL após 24h da administração do inóculo (*S.aureus* sem *L. gracilis* Schauer $10^8 \pm 313$ versus *S.aureus* com *L. gracilis* Schauer 13,28 ± 4,03). Os resultados foram expressos através do cálculo da média±EPM) e análise de variança (ANOVA). A diferença entre a concentração inibitória mínima no estudo *in vitro* foi determinada pelo teste Turkey (p<0.05). O teste Newman-Keuls com nível de significância (p<0.05) foi utilizado para o cálculo dos resultados obtidos no experimento *in vivo*. A solução a 5% do óleo essencial de *Lippia gracilis* Schauer apresentou boa atividade antibacteriana tanto no estudo *in vivo* como no *in vitro*.

Unitermos: *Lippia gracilis* Schauer, Verbenaceae, alecrim-de-tabuleiro, monoterpenos, carvacrol, *p*-cimeno, γ-terpineneo.

ABSTRACT: The essential oil from *Lippia gracilis* Schauer (Verbenaceae) leaves was examined by GC and GC-MS. Fifteen constituents were identified. Carvacrol, *p*-cymene and γ-terpinene were found to be the major components. In the in vitro study, 5% solution of the *Lippia gracilis* Schauer oil presented antibacterial activity against *Staphylococcus aureus* isolated from diabetic patients with infected ulcers. The study evaluated the antibacterial activity of the 5% solution of the *Lippia gracilis* Schauer oil on the experimental model of diabetic adult male albino Wistar rats with leaft pelvic limb infected by *Staphylococcus aureus* strain. In this experiment, 28 diabetic Wistar rats were used, randomly distributed in four different groups of seven rats, (G1-white; G2-negative control; G3-positive control and G4-test). When comparing group G4 with G3, it was observed that the 5% solution presented a reduced CFU/mL level showing the antibacterial effect of the oil 24 hours after the administration of the inoculum (*S .aureus* without *Lippia gracilis* Schauer 108 ±313 versus *S.aureus* with *Lippia gracilis* Schauer 13.28±4.03). The results were expressed as mean±S.E.M. One-way analysis of the variance (ANOVA) was used. The differences between the minimum inhibitory concentration *in vitro* test were determined by the Tukey test ($p<0.05$). The Newman-Keuls test with level of significance ($p<0.05$) was used to measure the results *in vivo*. The findings have shown that 5% solution of the *Lippia gracilis* Schauer oil presented antibacterial activity *in vitro* and *in vivo*.

Keywords: *: Lippia gracilis* Schauer, Verbenaceae, alecrim-de-tabuleiro, monoterpenes, carvacrol, *p*-cymene, γ-terpinene.

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ISSN 0102-695X

INTRODUÇÃO

Diabetes Mellitus (DM) is one of the world's most serious health problems (Motta et al., 2003, Vischer al., 2009). In Brazil, there are about 5 million diabetics; 7.6% of Brazilians between the ages of 30 and 65 suffer from diabetes. The four important factors that make diabetic subjects more prone to complications are: susceptibility to infections, hyperglycemia,vascular disease and nerve damage (Levin, 1995). Diabetic foot infection represents one of the most serious chronic complications of DM (Goldstein et al., 1996). In this country, as in other developing countries, there is a widespread and uncontrolled use of antibiotics, and patients often do not take a full course treatment because they are unable to afford it. Thus, it is important to find inexpensive antimicrobial agents (Guzmán-Blanco et al., 2000). Essential oils are rich sources of biologically active compounds (Lemos et al., 1990, Aguiar & Jaciana, 2008) Recently, great interest has been shown in the antimicrobial properties of extracts from aromatic plants, particularly essential oils (Matos et al., 2004). Many oils and extracts from different plants have been investigated because of their antimicrobial properties against bacteria and yeasts (Salgueiro et al., 2003). Essential oils have been found to be antibacterial, antifungal and therapeutic in cancer treatment and can present other pharmacological properties. Thus, the use of natural antimicrobial compounds seems to be important not only in the preservation of food, but also for the control of human and plant diseases of microbial origin (Rasooli & Irmostafa, 2002, Barbosa et al., 2005). The genus *Lippia* has been credited with a long list of pharmacological properties. *Lippia* spp oils present high antimicrobial effects on various microorganisms (Bassole al., 2002). The variability of the oils in the *Lippia genus* has been the subject of several studies reviewed by Matos (Matos et al., 2004). The aims of this work were to study the chemical composition and antimicrobial activity of the 5% solution of the *Lippia gracilis* Schauer oil in diabetic adult male Wistar rats subjected to *Staphylococcus aureus* pelvic limb infection *in vivo* and *in vitro* experiments.

MATERIAL AND METHODS

Plant material and essential oil extraction

Lippia gracilis Schauer fresh leaves were collected in August 2005 at the Medicinal Plant Nursery - Natural Products Laboratory of the Federal University of Ceará, Brazil. The plant was identified by botanists of the Biology Department of the Federal University of Ceará, where a voucher specimen was deposited at Herbarium Prisco Bezerra under number 23427. The leaves were placed in a glass container and the essential oil (EO) was extracted by steam distillation in a Clevenger type apparatus. The extraction was performed for 2 h in 500 mL of water. The oils were stored in glass bottles in a freezer until they were used.

Analysis of *Lippia gracilis* EO

The EO was analyzed using a Hewlett-Packard 5971 GC/MS instrument under the following conditions column: dimethyl-polysiloxane DB-1 fused silica capillary column (30m x 0.25 mm); carrier gas: He (1 ml/min); injector temperature: 250 °C; detector temperature: 200 °C; column temperature: 35-180 °C at 4 °C/min then 180-25 °C at 10 °C/min; mass spectrum: electronic impact 70eV. The constituents were identified by computer-based library search, retention indices and visual interpretation of the mass spectra (Alencar et al., 1984; Adams et al., 1989).

Animals

Twenty-eight adult male Wistar rats (*Rattus norvegicus* - albinos), aged 60 days and weighing 180±25 g, bred in the Central Animal House of the Federal University of Ceará, were used. The animals were housed in polycarbonate cages in a room with a 12 h light/dark cycle, temperature of 22±2 °C, and humidity of 45-64% during the whole experimental period. They were fed with a balanced commercial diet (Nuvital, Curitiba, PR, Brazil) and water *ad libitum*. All the animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council (CMV) and the Brazilian College of Animal Experiments (COBEA). The Ethics Committee of The Federal University of Ceará approved the protocols employed (Reg. N°. 46/04 dated 11 October 2005).

Induction of experimental diabetes mellitus

After an overnight fasting, the rats were induced by intraperitoneal injection (150 mg/kg) of alloxan monohydrate (Acros Organics Research Laboratories, Inc., New Jersey, USA) dissolved in 0.1 M sodium citrate buffer (pH 3.0) (Zarrow et al., 1964). After two weeks, rats with marked hyperglycemia (fasting serum glucose > 250 mg/dL) were selected and used for study. The animals had free access to food and water after the alloxan injection.

Determination of the serum glucose concentration

Blood samples from the tail vein of the anesthetized (Ketamine cloridrate 90 mg/kg/im) rat were collected and (100 μL) centrifuged, and the serum was used to determine the glycemia by the glucose oxidase method (Calore et al., 2007).

Antibacterial assay

Bacterial strain

Staphylococcus aureus strain isolated from diabetic patients exhibiting infected ulcers was used in the study.

Antibacterial activity (in vitro)

The Minimal Inhibitory Concentration (MIC) was estimated by the broth dilution method (CLSI, 2008). The microorganism (*Staphylococcus aureus*) was grown overnight at 37 °C in 10 mL of Mueller Hinton Broth (v/v) (Bioxon). The culture was adjusted with sterile DMSO (dymethylsulfoxide; Sigma) solution 2% (v/v). The density of the suspension of the respective microorganism was adjusted to 0.5 Mc Farland turbidity standards. The suspension was then inoculated onto the agar plate (15 x 100 mm) containing Mueller Hinton Agar (Bioxon). Concentrations of 10 to 1.25 % (v/v) of each essential oil were prepared. Discs of filter paper (Whatman no 5) of 5 mm diameter were impregnated with 5 µL of the essential oil placed on the agar surface. Discs impregnated with DMSO solution 2% (v/v) was used as negative control, and oxacillin (DIFCO) (1 µg) was used as positive control. The plate was incubated overnight at 37 oC and the diameter of any resulting zones of inhibition (mm) of growth was measured. Each experiment *in vitro* was performed three times.

Antibacterial activity (in vivo)

Twenty-eight adult male Wistar (*Rattus norvegicus-albinus*) diabetic rats were used in the experiment, distributed in four groups of seven rats. The rats were inoculated intracutaneously (left pelvic limb) with different solutions. Group 1 (negative control-white): the rats were inoculated with 0.1 mL of 2% DMSO solution (v/v). Group 2 (negative control-oil): the rats were inoculated with 0.1 mL of the 5% *Lippia gracilis* Schauer solution (v/v) preparation with 2% DMSO solution (v/v). Group 3 (positive control): the rats were inoculated with 0.1 ml of 2% DMSO solution (v/v) containing 10^5 CFU(colony-Forming Units)/mL of *Staphylococcus aureus*. Group 4 (test): the rats were inoculated with 0.05 mL of 2% DMSO solution (v/v) containing 10^5 CFU/mL of *Staphylococcus aureus*; after 24 h, they were inoculated with 0.05 mL of Lippia gracilis Schauer solution (v/v) preparation with 2% DMSO solution (v/v). Upon 48 h inicial experiment the rats were sacrificed by decapitation, using Ketamine Cloridate anesthesia (90 mg/kg/*i.m*) (Calore et al., 2007).

Bacterial culture

Pelvic limbs from 28 rats were used for bacterial analysis. After disinfecting them with 70% alcohol, left pelvic limb samples corresponding to the injection sites were deposited in 20 x 150 mm sterilized plastic tubes, homogenized, and suspended in 2.0 mL of BHI (Brian Heart Infusion) (DIFCO). Aliquots of 0.1 mL of the suspension from each of three dilutions (10^{-1}, 10^{-2} e 10^{-3}) were added to the center of the 15x100mm Petri containing agar 5 % sheep blood (v/v) and incubated at 37 °C for 24 h. The number of Colony-Forming Units (CFU/mL) per pelvic limb surface on the plates was determined by the method described by Serguei et al., (2009), and bacterial colonies were tested for coagulase and catalase activity. Quality control was done by the *Staphylococcus aureus* ATCC (American Type Culture Collection) 29213. The antimicrobial agent tested was oxacillin (CLSI, 2008).

Statistical analysis

The results were expressed as mean±S.E.M. One-way analysis of variance (ANOVA) was used. The differences between the minimum inhibitory concentrations *in vitro* test were determined by Tukey test ($p<0.05$). A Newman-Keuls test (mean±S.E.M) with level of significance ($p<0.05$) was conducted to analyze the results.

RESULTS

Chemical analysis

The chemical analysis of *Lippia gracilis* Schauer EO is displayed in Table 1 according to the order of elution from a non-polar column. The main constituent as previously reported was carvacrol. The percentage yield in this oil was 50.13%.

Antibacterial activity (*in vitro*)

The results show that the concentration of *Lippia gracilis* Schauer EO displays a moderate antibacterial activity against *Staphylococcus aureus*. *L. gracilis* Schauer EO inhibited the growth of the bacteria producing a zone diameter of 26±0.88 and 25±0.57 mm in the oil concentrations of 10 and 5% (v/v) respectively.

Antibacterial activity (*in vivo*)

The concentration of *Lippia gracilis* Schauer EO 5% (v/v), was injected in the left pelvic limb, and after 24 h, the solution containing 10^5CFU/mL of *Staphylococcus aureus* was administered. A moderate antibacterial activity was displayed. When comparing Group 4 with 3, it was observed that the 5% solution presented an antibacterial effect, and reduced the number of CFU/mL.

DISCUSSION

Diabetic foot infections are generally polymicrobial. *S. aureus* are among the most frequently isolated microorganisms from the lesion (Motta et al., 2005).

The essential oil of *Lippia gracilis* Schauer is mostly constituted by monoterpenes and sesquiterpenes. The major components are: carvacrol (50.13%), p-cymene (10.73%) and β-caryophyllene (5.96%) (Table 1). The 5% solution of the *L. gracilis* oil presented antibacterial activity in vitro and in vivo against the tested strain of *Staphylococcus aureus* isolated from diabetic patients exhibiting infected ulcers (Tables 2 and 3). The antimicrobial activity of different essential oils is well documented, particularly those which contain relatively high concentrations of the antibacterial phenols. *Lippia* sp oils are potential candidates for emulsion formulation as a topical product (Oladimeji et al., 2000; Oliveira et al.,2007). While essential oils are noted for their antimicrobial activities (Hammer et al., 1999; Smith-Palmer et al., 1998), as demonstrated by *Lippia* oil in this study, such activities vary, depending on their composition. The composition of *Lippia* oil has already been reported (Oladimeji et al., 2001). Among its components, some that have been confirmed to have antimicrobial activities are linalol, geraniol, (Onawunmi et al., 1984), thymol (Mwangi et al., 1994), α-pinene and terpineol (Weston et al., 1997). Such antimicrobial activities could form the basis to apply the oil in food preservation and in natural therapy (Albuquerque et al., 2007). The antimicrobial activity of carvacrol is well established (Kunle et al., 2003) and this study has shown that it is a major component of *Lippia gracilis* Schauer. One of the important cell types that are affected by diabetes is polymorphonuclear neutrophils (PMN). These PMN granulocytes represent the host's defense barrier against bacterial agents (Mandrup et al., 1987). In diabetic patients, these cells show functional alterations in chemotaxis, adhesion, phagocytosis and intracellular killing of the microorganism. PMN dysfunction has been attributed to an increased glucose concentration in PMN, which could lead to protein glycosylation (Marhoffer et al., 1992), depletion of NADPH (Wilson et al., 1987) and increased concentration of advanced glycosylation and products in macrophages, which in turn induce the release of cytokines such as interleukin-1 and tumor necrosis factor (Radoff et al., 1997). Diabetes has also been known to affect cell and tissue metabolism in different ways and numerous research studies have demonstrated these effects in other chronic diseases, despite the fact that the precise mechanism of such alterations remains unclear. One of the studies, conducted by Lebovici et al., 1996), showed that a crucial risk factor for the evolution of an infectious process in the foot is the length of time the person has been a diabetic. The antibacterial activity shown by the essential oil from *Lippia gracilis* Schauer may be attributed to the presence of some components such as carvacrol, terpineol acetate, cymene, tymol, pinene, cineole and linalool, which are already known to exhibit antibacterial activity (Knobloch et al., 1985; Juven et al., 1994; Harborne & Williams, 1995; Kim et al., 1995; Cimanga et al., 2002; Albuquerque et al., 2007; Aguiar&Jaciana et al., 2008). In conclusion, the 5% solution of the *Lippia gracilis* Schauer oil significantly reduced the colony count when compared with the control group, revealing its potential antibacterial qualities.

Table 1. Percentage composition of leaf essential oil of *Lippia gracilis* Schauer.

Constituents	RIa	Yield (%)
α-Thujene	930	0.60
β-Myrcene	991	2.08
α-Terpinene	1017	1.46
p-Cymene	1025	10.73
1,8-Cineole	1031	2.74
γ-Terpinene	1060	8.04
Borneol	1169	0.68
4-Terpineol	1177	0.71
Methyl thymol ether	1245	4.95
Thymol	1290	4.92
Carvacrol	1299	50.13
β-Caryophyllene	1419	5.96
Aromadendrene	1441	0.78
Biciclogermacrene	1500	3.34
Spathulenol	15778	1.77

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

Table 2. Antibacterial activity of the different concentrations of the essential oil (EO) of *Lippia gracilis* Schauer leaves.

EO doses	Concentrations (% of v/v)	Zone of inhibition of *Staphylococcus aureus* a (mm)	Control (mm) Oxacillin (1μg)
I	10	26±0.88	-
II	5	25±0.57	18±0.57
III	2.5	0±0	-
IV	1.25	0±0	-

The results were expressed as mean+S.E.M. a5μL of concentration EO. Tukey test; (p<0.05) when compared to control.

Table 3. Number of Colony Forming Units (CFU/mL) isolated from pelvic limb culture per group.

Groups	Colony Forming Units (CFU/mL)
I	00±00 (7)
II	00±00 (7)
III	108±3.13 (7)
IV	13.28±4.03 (7)[a,b,c]

Values are means+S.E.M. of the number of colony forming units. In parenthesis is the number of animals per group; a p<0.001 when compared with 1, as compared to controls (ANOVA and test t-Student-Newman-Kewls as the *post hoc* test); b p<0.001 when compared with 2, as compared to controls (ANOVA and test t-Student-Newman-Kewls as the post hoc test); c p<0.001

when compared with 3, as compared to controls (ANOVA and test t-Student-Newman-Kewls as the *post hoc* test).

ACKNOWLEDGEMENTS

The authors wish to thank CAPES for supporting this work and Terezinha de Jesus dos Santos Rodrigues for technical assistance.

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