NPC Natural Product Communications

2014
Vol. 9
No. 6
737 - 739

A New Source of (*R*)-Limonene and Rotundifolone from Leaves of *Lippia pedunculosa* (Verbenaceae) and their Trypanocidal Properties

Leociley Rocha Alencar Menezes[a], Nilmara Nunes Santos[a], Cássio Santana Meira[b], Jamyle Andrade Ferreira dos Santos[b,c], Elisalva Teixeira Guimarães[b,c], Milena Botelho Pereira Soares[b,d], Angelita Nepel[e], Andersson Barison[e] and Emmanoel Vilaça Costa[f,*]

[a]*Departamento de Química, Universidade Federal de Sergipe, São Cristóvão, Sergipe, Brazil, 49100-000*
[b]*Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia, Brazil, 40296-710*
[c]*Departamento de Ciências da Vida, Universidade do Estado da Bahia, Salvador, Bahia, Brazil, 41150-000*
[d]*Centro de Biotecnologia e Terapia Celular, Hospital São Rafael, Salvador, Bahia, Brazil, 41253-190*
[e]*Departamento de Química, Universidade Federal do Paraná, Curitiba, Paraná, Brazil, 81531-990*
[f]*Departamento de Química, Universidade Federal de Sergipe, Itabaiana, Sergipe Brazil, 49500-000*

emmanoelvc@gmail.com

Received: April 2nd, 2014; Accepted: April 19th, 2014

Investigation by GC-FID and GC-MS of the essential oil (LPOE) from the leaves of *Lippia pedunculosa* revealed, as the major compounds, the monoterpenes rotundifolone (71.7%) and (*R*)-limonene (21.8%). These two compounds and the minor constituent piperitenone (1.2%) were also isolated from the leaves and identified by spectrometric analysis. LPOE and isolated compounds were evaluated for their trypanocidal activity against epimastigote and trypomastigote forms of *Trypanosoma cruzi*. Significant results with IC_{50} values lower than 34.0 μg.mL^{-1} were observed against these forms of *T. cruzi* for LPOE and isolated compounds. Rotundifolone was the most active compound with an IC_{50} lower than 10.0 μg.mL^{-1} for both forms of *T. cruzi*. The effects of LPOE and isolated compounds were also evaluated in cultures of macrophages infected with *T. cruzi*. Treatment with (*R*)-limonene and rotundifolone caused a moderate reduction in the percentage of macrophages infected by *T. cruzi* and in the number of intracellular parasites at concentrations non-toxic to macrophages.

Keywords: *Lippia pedunculosa*, Essential oil, Trypanocidal activity, Monoterpenes, Limonene, Rotundifolone.

The genus *Lippia* L. is widely distributed throughout South and Central America, and Tropical Africa. It belongs to the family Verbenaceae and comprises approximately 254 species of herbs, shrubs and small trees [1a,1b]. In Brazil, this genus is represented by nearly 120 species [1b], some of which are used in folk medicines as an alternative to current drugs. Among their interesting properties, it is possible to list analgesic, anti-inflammatory, antipyretic, sedative, antifungal, antihypertensive, diuretic, larvicidal, antimicrobial, antioxidant, antiviral, molluscicidal, antimalarial, antispasmodic, anticonvulsant, fumigant, trypanocidal, and stimulant activities [1a,1b,2a-2j,3].

Species of Verbenaceae in general are known to produce monoterpenes and sesquiterpenes, although other compounds, such as alkaloids, carotenoids, flavonoids, iridoids, phenolic acids, saponins, sterols, sugars, tannins and triterpenes have been also identified [1b]. Limonene, (*E*)-caryophyllene, *p*-cymene, camphor, linalool, α-pinene and thymol were frequently found in the essential oil of *Lippia* species.

However, despite several medicinal properties, only very few *Lippia* species have been chemically and pharmacologically investigated [1b,2a-2j,3]. To the best of our knowledge, there have been no scientific investigations of *L. pedunculosa* Hayek. This species is a shrub, 0.7 – 1.5 m high, found in the northeast (Alagoas and Sergipe) and southeast (São Paulo) regions of Brazil. In the northeast, it occurs in areas of "Caatinga" and is considered rare [1a]. In our continuing search for bioactive natural products from Sergipe plants with trypanocidal properties, herein we report, for the first time, the phytochemical and pharmacological investigation of the essential oil from the leaves of *L. pedunculosa*.

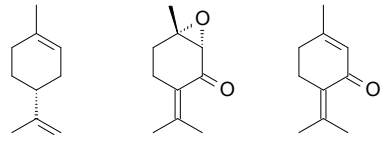

Figure 1: Isolated compounds of the essential oil from the leaves of *Lippia pedunculosa*.

Hydrodistillation of the leaves of *L. pedunculosa* resulted in 2.1±0.1% of essential oil (light yellow) in relation to the dry weight of plant material. CG-FID and GC-MS analysis allowed the identification of ten compounds in the essential oil and revealed (*R*)-limonene (**1**), rotundifolone (**2**) and piperitenone (**3**) as major constituents (Table 1, Figure 1); their identities were confirmed by isolation using preparative TLC, and structure elucidation from spectroscopic and spectrometric data, and by comparison with literature information [4a-4c].

The predominant presence of rotundifolone in *L. pedunculosa* is uncommon for this genus. Although a minor constituent of the essential oil of other *Lippia* species, like *L. turbinata* Griseb and *L. junelliana* (Mold.) Tronc., this compound is considered as a chemotaxonomic marker of species of *Mentha* (Labiatae/ Lamiaceae) [6a,6b]. However, high levels of limonene are typical of *Lippia* species [1b,2a-2j, 3,6a,6b].

In *in vitro* assays, the essential oil (LPOE) and the isolated compounds rotundifolone and (*R*)-limonene showed promising trypanocidal activity against epimastigote and trypomastigote forms of *T. cruzi*, with IC_{50} values lower than 34.0 μg.mL^{-1}

Table 1: Essential oil composition of *Lippia pedunculosa* leaves.

	Compound	RI[a]	RI[b]	Peak area %
1	α-Pinene	927	932	1.0±0.0
2	Myrcene	987	988	0.1±0.0
3	Limonene	1028	1024	21.8±1.4
4	Methylchavicol	1197	1195	0.4±0.1
5	Thymol	1295	1289	0.3±0.0
6	Piperitenone	1338	1340	1.2±0.1
7	Rotundifolone	1363	1366	71.7±2.3
8	α-Copaene	1377	1374	0.2±0.0
9	(*E*)-Caryophyllene	1421	1417	0.7±0.1
10	Bicyclogermacrene	1494	1500	0.1±0.0
	Monoterpenes			96.5
	Sesquiterpenes			1.0
	Total Identified			97.5

Data are expressed as mean ± SD of three analyses. RI (retention indices): [a]calculated on RTx®-5MS column according to Van Den Dool and Kratz [7a] based on a homologous series of normal alkanes; [b]according to Adams [7b].

Table 2: IC_{50} values for trypanocidal activity.

Samples	IC_{50} μg.mL^{-1}	
	Epimastigote forms	Trypomastigote forms
Essential Oil (LPOE)	15.1±2.4	11.3 ±0.3
(*R*)-Limonene	33.7±0.6	14.1 ±2.1
Rotundifolone	9.2±1.8	9.3 ±1.5
Benznidazole[a]	2.7±0.7	2.7 ±0.5

Data are expressed as mean ±SD of three independent experiments. [a] Reference drug (positive control).

(Table 2). The most significant activity was observed against trypomastigote forms (infecting forms), with IC_{50} values lower than 10.0 μg.mL^{-1}, reaching 9.3 μg.mL^{-1} for rotundifolone, the most effective compound. Hence, the trypanocidal activity against both forms of *T. cruzi* observed for the essential oil could be attributed to the presence of rotundifolone. However, the presence of (*R*)-limonene in the LPOE diminishes the trypanocidal activity against both forms.

The effects of the LPOE and isolated compounds on the intracellular form of the parasite were also evaluated in cultures of macrophages infected with *T. cruzi* (amastigote forms) at a concentration of 5.0 μg.mL^{-1} (Figure 2). The treatment with (*R*)- limonene and rotundifolone caused a moderate reduction in the percentage of macrophages infected by *T. cruzi* and in the number of intracellular parasites at concentrations non-toxic to macrophages (5.0 μg.mL^{-1}) (Figure 2). Nevertheless, they were less effective than the positive control benznidazole (drug used in the treatment of Chaga's disease). It is important to highlight, however, that this medicine is highly toxic to mammalian cells and that its cure rate is approximately 70–80% in the acute phase and a mere 10–20% in the chronic phase [8]. When compared with the essential oils of other *Lippia* species, the essential oil of *L. pedunculosa* was demonstrated to be the most active [3].

This is the first report of the volatile composition of the leaves of *L. pedunculosa* and its trypanocidal properties. Rotundifolone and (*R*)-limonene were found to be the major compounds. The trypanocidal activities suggest that this species is a rich source of biologically active compounds, such as rotundifolone. In addition, this work demonstrates the importance of chemical and biological investigations of the essential oils of Verbenaceae.

Experimental

General experimental procedures: IR spectra were measured in KBr pellets on a Shimadzu IR Prestige-21, and optical rotations were recorded in $CHCl_3$ on Jasco P-2000 spectrophotometers. MS were acquired on a Shimadzu QP2010 GC/MS system equipped with an AOC-20i auto-injector. 1D and 2D NMR data were acquired at 298 K in $CDCl_3$ on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.4 Tesla, observing ^{1}H and ^{13}C at 400 and 100 MHz, respectively. Silica gel 60 F_{254} was used for analytical (0.25 mm), and preparative (1.00 mm) TLC. Compounds were visualized by exposure under $UV_{254/365}$ light and spraying with *p*-anisaldeyde reagent, followed by heating on a hot plate.

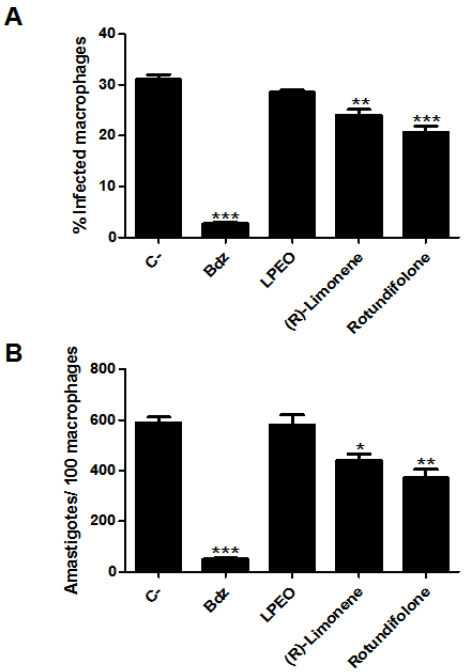

Figure 2: *In vitro* trypanocidal activity of the LPEO and isolated compounds of *Lippia pedunculosa* against amastigotes proliferation in macrophages at the concentration of 5.0 μg.mL^{-1}. (A) Percentage of infected macrophages. (B) Relative number of amastigotes per 100 macrophages. Benznidazole (Bdz) was the positive control and (C-) negative control. Values represent the mean±SEM of triplicates. * p<0.05; ** p<0.01 *** p<0.001 compared with the control group.

Plant material: The leaves of *L. pedunculosa* were collected in October 2013 at the 'Povoado Cajueiro', city of Poço Redondo [coordinates: S 09^o 40' 46" W 37^o 39' 41"] in Sergipe State, Brazil. The identity of the plant was confirmed by Dr Ana Paula do Nascimento Prata of the Department of Biology from Sergipe Federal University (UFS), Brazil, and a voucher specimen (23159) was deposited in the Herbarium of UFS (ASE/UFS).

Hydrodistillation, GC-FID and CG-MS analysis: The essential oil from dried leaves (for 24 h) of *L. pedunculosa* (100 g) was obtained by hydrodistillation for 3 h using a Clevenger-type apparatus. The essential oil was dried over anhydrous sodium sulfate and the content percentage was calculated in relation to the dry weight of plant material used. It was then stored in a freezer until analysis. The hydrodistillation was performed in triplicate. GC-FID and GC-MS analysis were performed according to Silva *et al*. [5]. Essential oil components were identified by comparing the retention times of the GC peaks with standard compounds ran under identical conditions, as well as by comparison of retention indices [7a] and MS [7b]. GC-FID and GC-MS analyses were performed on a Shimadzu GC-2010 Plus GCMS-QP2010 Ultra GC-FID, equipped with a Shimadzu AOC-20i auto-injector. The separation of the compounds was achieved on a RTx®-5MS fused capillary chromatography column (30 m x 0.25 mm x 0.25 μm film thickness) coated with 5%-diphenyl-95%-dimethylpolysiloxane.

Isolation of limonene, rotundifolone and piperitenone: Part of the essential oil (250 mg) was subjected to a preparative TLC eluted

with *n*-hexane 100%, giving 34.3 mg of (*R*)-limonene (**1**). The other part (250 mg) was subjected to preparative TLC, eluting with a mixture of *n*-hexane-ethyl acetate (95:05, v/v), yielding rotundifolone (**2**) and piperitenone (**3**). This process was repeated 4 times (4 x 250 mg), affording 687.5 mg and 14.0 mg of rotundifolone (**2**) and piperitenone (**3**), respectively.

(*R*)-Limonene (**1**)**:** Colorless oil. $[\alpha]^{25}_{D}$: +100.7 (*c* 1.0 g/100 mL, $CHCl_3$). MS, ^1H and ^{13}C NMR data compared with authentic sample.

Rotundifolone (**2**)**:** Light yellow oil; $[\alpha]^{25}_{D}$: +168.4 (*c* 1.94 g/100 mL, $CHCl_3$). IR, MS, ^1H and ^{13}C NMR data according to [4a,4b].

Piperitenone (**3**)**:** Light yellow oil. IR, MS, ^1H and ^{13}C NMR data according to [4c].

Assessment of trypanocidal activity of essential oil and isolated compounds: The trypanocidal assay on epimastigote and trypomastigote forms of *T. cruzi* were carried out according to Silva *et al*. [5]. For *in vitro* infection, peritoneal macrophages (2 x 10^5 cells.well^{-1}) obtained from BALB/c mice were cultured in a 24 well-plate with rounded coverslips on the bottom in RPMI supplemented with 10% FBS and incubated for 24 h. Cells were then infected with trypomastigotes (1:10) for 2 h. Free trypomastigotes forms were removed by successive washes using saline solution and the cells were incubated for 24 h. Next, cultures were incubated in complete medium alone or with the compounds (5 μg.mL^{-1}) for 72 h. Cells were fixed in absolute alcohol and the percentage of infected macrophages and the number of amastigotes forms per 100 macrophages was determined by manual counting (100 cells per slide) after hematoxylin and eosin staining in an optical microscope. The one-way ANOVA and Bonferroni for multiple comparisons were used to determine the statistical significance of the group comparisons.

Acknowledgments - The authors are grateful to FAPITEC/SE (Editais No 07/2009 and 10/2009), FAPESB, CNPq, FINEP, UFPR and CAPES for financial support and fellowships, as well as to Professor Dr A. P. N. Prata of the DBI/UFS for the botanical identification.

References

[1] (a) Santos JS, Melo, JIM, Abreu, MC, Sales MF. (**2009**) Verbenaceae *sensu stricto* na região de Xingó: Alagoas e Sergipe, Brasil. *Rodriguésia*, ***60***, 985-998; (b) Pascual ME, Slowing K, Carretero E, Mata DS, Villar A. (**2001**) *Lippia*: traditional uses, chemistry and pharmacology: a review. *Journal of Ethnopharmacology*, ***76***, 201-214.

[2] (a) Leitão SG, Damasceno JPL, Martini MG, Miranda SN, Neufeld PM, Salimena FR, Bizzo HR. (**2011**) Analysis of the chemical composition and antimicrobial activity of the essential oil from *Lippia triplinervis* Gardner (Verbenaceae). *Journal of Essential Oil Research*, ***23***, 20-24; (b) Oliveira OR, Terao D, Carvalho ACPP, Innecco R, Albuquerque CC. (**2008**) Efeito de óleos essenciais de plantas do gênero *Lippia* sobre fungos contaminantes encontrados na micropropagação de plantas. *Revista Ciência Agronômica*, ***39***, 94-100; (c) Almeida MCS, Alves LA, Souza LGS, Machado LL, Matos MC, Oliveira MCF, Lemos TLG, Braz-Filho R. (**2010**) Flavonoides e outras substâncias de *Lippia sidoides* e suas atividades antioxidantes. *Química Nova*, ***33***, 1877-1881; (d) Stashenko EE, Ruiz C, Muñoz A, Castañeda M, Martínez J. (**2008**) Composition and antioxidant activity of essential oils of *Lippia origanoides* H.B.K. grown in Colombia. *Natural Product Communications*, ***3***, 563-566; (e) Silva NA, Silva JK, Andrade EH, Carreira LM, Sousa PJ, Maia JG. (**2009**) Essential oil composition and antioxidant capacity of *Lippia schomburgkiana*. *Natural Product Communications*, ***4***, 1281-1286; (f) Lima RK, Cardoso MG, Moraes JC, Carvalho SM, Rodrigues VG, Guimaraes LGL. (**2011**) Chemical composition and fumigant effect of essential oil of *Lippia sidoides* Cham. and monoterpenes against *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae). *Ciência e Agrotecnologia*, ***35***, 664-671; (g) Cavalcanti ESB, Morais SM, Lima MAA, Santana EWP. (**2004**) Larvicidal activity of essential oils from Brazilian plants against *Aedes aegypti* L. *Memórias do Instituto Oswaldo Cruz*, ***99***, 541-544; (h) Santiago, GMP, Lemos TLG, Pessoa ODL, Arriaga AMC, Matos, FJA, Lima MAS, Santos HS, Lima MCL, Barbosa FG, Luciano JHS, Silveira ER, Menezes GHA. (**2006**) Larvicidal activity against *Aedes aegypti* L. (Diptera: Culicidae) of essential oils of *Lippia* species from Brazil. *Natural Product Communications*, ***1***, 573-576; (i) Mendes SS, Bomfim RR, Jesus HCR, Alves PB, Blank AF, Estevam CS, Antoniolli AR, Thomazzi SM. (**2010**) Evaluation of the analgesic and anti-inflamatory effects of the essential oil of *Lippia gracilis* leaves. *Journal of Ethnopharmacology*, ***129***, 391-397; (j) Gómez LA, Stashenko E, Ocazionez RE. (**2013**) Comparative study on *in vitro* activities of citral, limonene and essential oils from *Lippia citriodora* and *L. alba* on yellow fever virus. *Natural Product Communications*, ***8***, 249-252.

[3] Escobar P, Leal SM, Herrera LV, Martinez JR, Stashenko EE. (**2010**) Chemical composition and antiprotozoal activities of Colombian *Lippia* spp. essential oils and their major components. *Memórias do Instituto Oswaldo Cruz*, ***105***, 184-190.

[4] (a) Guedes DN, Silva DF, Barbosa-Filho JM, Medeiros IA. (**2004**) Calcium antagonism and the vasorelaxation of the rat aorta induced by rotundifolone. *Brazilian Journal of Medical and Biological Research*, ***37***, 1881-1887; (b) Trach LN, Nhung TH, My VTN, Tran H-A. (**2013**) The new rich source of rotundifolone: *Mentha aquatica* Linn. var. *crispa* oil from microwave-assisted hydrodistillation. *Journal of Essential Oil Research*, ***25***, 39-43; (c) Lee IK, Kim MA, Lee SY, Hong JK, Lee JH, Lee KR. (**2008**) Phytochemical constituents of *Schizonepeta tenuifolia* Briquet. *Natural Product Sciences*, ***14***, 100-106.

[5] Silva TB, Menezes LRA, Sampaio MFC, Meira CS, Guimarães ET, Soares MBP, Prata APN, Nogueira PCL, Costa EV. (**2013**) Chemical composition and anti-*Trypanosoma cruzi* activity of essential oils obtained from leaves of *Xylopia frutescens* and *X. laevigata* (Annonaceae). *Natural Product Communications*, ***8***, 403-406;

[6] (a) Quiroga PR, Grosso NR, Lante A, Lomolino G, Zygadlo JA, Nepote V. (**2013**) Chemical composition, antioxidant activity and anti-lipase activity of *Origanum vulgare* and *Lippia turbinata* essential oils. *International Journal of Food Science and Technology*, ***48***, 642-649; (b) Juliani HR, Koroch AR, Zygadlo JA, Trippi VS. (**2011**) Evaluation of micropropagation for the introduction into cultivation and conservation of *Lippia junelliana*, an endemic aromatic plant from Argentina. *Industrial Crops and Products*, ***34***, 1353-1357.

[7] (a) van Den Dool H, Kratz PD. (**1963**) A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography*, ***11***, 463-471; (b) Adams RP. (**2007**) *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*. 4th ed., Allured Publ. Corp., Carol Stream, IL.

[8] Izumi E, Ueda-Nakamura T, Dias Filho BP, Veiga Júnior VF, Nakamura CV. (**2011**) Natural products and Chagas' disease: a review of plant compounds studied for activity against *Trypanosoma cruzi*. *Natural Product Reports*, ***28***, 809-823.