© 2010 Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas, 9 (3), 206-211
BLACPMA ISSN 0717 7917

Artículo Original | Original Article

Chemical and Biological Investigations of *Pilocarpus spicatus* essential oils

[Estudios químicos y biológicos del aceite esencial de *Pilocarpus spicatus*]

Adriana P. OLIVEIRA[1]*, Rodrigo A. S. CRUZ[1], Gisele S. BOTAS[1], Marcelo S. GONZALEZ[3], Marcelo G. SANTOS[4], Lenise A. TEIXEIRA[2], Leandro M. ROCHA[1].

1. Laboratório de Tecnologia de Produtos Naturais, Faculdade de Farmácia, Universidade Federal Fluminense, Rua Mário Viana 523, Santa Rosa, CEP 24241-000, Niterói, RJ, Brazil. 2. Laboratório de Controle Microbiológico, Faculdade de Farmácia, Universidade Federal Fluminense, Rua Mário Viana 523, CEP 24241-000, Niterói, RJ, Brazil. 3. Departamento de Biologia Geral, Universidade Federal Fluminense, Morro do Valonguinho S/N, Centro, CEP 24020-140, Niterói, RJ, Brazil. 4. Departamento de Ciências, Faculdade de Formação de Professores, Universidade Estadual do Rio de Janeiro, Dr. Francisco Portela 794, CEP 24435-000, São Gonçalo, RJ, Brazil.

Abstract

*E*ssential oil was obtained by steam distillation of aerial parts of *Pilocarpus spicatus* Saint-Hilaire (Rutaceae) from the northern coast of Rio de Janeiro State and examined by GC–MS. A total of 17 components were identified accounting for 96,06% of the oil composition. The major components were limonene (41,87%), 2-undecanone (11,0%) and sabinene (10,78%). *P. spicatus* essential oil had inhibitory effects on the growth of bacteria (*Escherichia coli* and *Staphylococcus aureus*) and showed anticholinesterase activity in TLC assay. In addition, the volatile oil was toxic to larvae of the brine shrimp.

Keywords: *Pilocarpus spicatus*; antibacterial activity; anticholinesterase activity; cytotoxic activity; essential oil

Resumen

El aceite esencial fue extraido por arraste de vapor de las partes aéreas de *Pilocarpus spicatus* Saint-Hilaire (Rutaceae) de la costa Norte del estado de Rio de Janeiro y examinado por GC-MS. Fueron identificados 17 componentes que corresponden al 96,06% de la composición quìmica del aceite. Los componentes mayoritarios fueron el limoneno (41,87%), 2 undecanona (11,0%) y sabineno (10,78%). El aceite esencial de *P. spicatus* tiene efecto inhibitorio sobre el crecimiento bacteriano (*Escherichia coli* y *Staphylococcus aureus*) y presentando también actividad anticolinesterasa en ensayo de TLC. Adicionalmente el aceite volátil ha demostrado ser tóxico para las larvas de *Artemia salina*.

Palabras Clave: *Pilocarpus spicatus*; actividad antibacteriana; actividad anticolinesterasa; actividad citotóxica; aceite esencial

List of abbreviations: CG-MS - Gas chromatography-mass spectrometry, TLC – thin layer chromatography

Recibido | Received: June 25, 2009 .
Aceptado en versión corregida | Accepted in revised form: April 28, 2010. .
Publicado en línea | Published online: May 25, 2010. .
Declaración de intereses | Declaration of interests: the authors have no competing interests. .
Financiación | Funding: none declared.
This article must be cited as: Adriana P Oliveira, Rodrigo AS Cruz, Gisele S Botas, Marcelo S Gonzalez, Marcelo G Santos, Lenise A Teixeira, Leandro M Rocha. 2010. Chemical and Biological Investigations of *Pilocarpus spicatus* essential oils. Bol Latinoamer Caribe Plant Med Aromat 9(3): 206-211. {EPub May 25, 2010}.

***Contactos | Contacts:** passosoliv@hotmail.com

INTRODUCTION

Pilocarpus spicatus Saint-Hilaire (Rutaceae) is one of the various *Pilocarpus spp*. called 'jaborandi'. Nowadays, the great importance of *Pilocarpus spp.* is due to the extraction of the alkaloid pilocarpine from the leaves (Pinheiro, 2002). The Rutaceae has secretory cavities lysigenous and schizo-lysigenous containing essential oils (Simões and Spitzer, 1999). The leaves of *Pilocarpus microphyllus* Stapf ex Wardleworth and *Pilocarpus pennatifolius* Lem produce 0.25-0.50% of essential oil (Lorenzi and Mattos, 2002).

In this way, a lot of essential oils from different species of the *Pilocarpus* genus have been analyzed and chemical substances as terpenoids, alcohols, aldehydes, hydrocarbons aliphatic and aliphatic ketones have been described (Craveiro et al., 1979; Santos et al., 1997; Andrade-Neto et al., 2000, 2002; Santos et al., 2004). In addition, chalepin is a coumarin extracted from *P. spicatus* essential oil. Experiment *in vitro* showed that binding of chalepin to glycosomal glyceraldehyde-3-phosphate dehydrogenase of *T. cruzi*, a protozoan exclusively transmitted by hematophagous triatomines (*Rhodnius prolixus*), disrupted flagellate development (Mafezoli et al., 2000; Pavão et al., 2002). Moreover, we observed a variety of effects of *P. spicatus* essential oil on *Rhodnius prolixus* -a vector of Chagas disease -which indicates their secondary metabolites, nowadays under investigation in our laboratory, as good candidates for the study of insect physiology, vector control population and perhaps, blockage of protozoan development in triatomine hosts (Melo et al., 2007).

It has well established that *P. spicatus* essential oil collected in the state of Ceará (Brasil) displays *in vitro* antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Santos et al., 1997). However, the chemical composition of *P. spicatus* essential oil showed qualitative and quantitative variation by the influence of local environmental conditions of soil and seasonal period of collections (Taveira et al., 2003).

A variety of plants has been reported to show acetylcholinesterase (AChE) inhibitory activity and so may be relevant to the treatment of neurodegenerative disorders such as Alzheimer's disease (AD). (Mukherjee et al., 2007). Numerous essential oils and their monoterpene constituents have been investigated for their effects on AChE, and have shown inhibitory activity. Although, several extracts of plants and essential oils already have been investigated, this is the first account in the *Pilocarpus* genus.

The brine shrimp lethality test (BST) is a simple and efficient method used to predict compounds or extracts as cytotoxic agents and that may have anticancer activity (Meyer et al., 1982). Although several essential oils have been tested against brine shrimp, *P. spicatus* essential oil cytotoxicity has not been investigated yet. In this work, studies were carried out to elucidate the chemical composition and analyses antibacterial, anticholinesterase and cytotoxicity activities *in vitro* of *P.spicatus* essential oil from Rio de Janeiro State (Brazil).

MATERIALS AND METHODS

Plant material

Aerial parts of *P. spicatus* Saint-Hilaire (Rutaceae) was collected in Sandy Coastal Plains (Restinga de Jurubatiba National Park) located on the northern coast of Rio de Janeiro State, Brazil (October 2004) and was identified by Dr. Marcelo Guerra Santos. The dried specimens were deposited in the herbarium of the Faculdade de Formação de Professores, UERJ (M. Guerra Santos 1.824) and of the Museu Nacional, UFRJ (M. Guerra Santos 1.406).

Extraction of the essential oil

The essential oil was obtained by steam distillation (1.37 kg of fresh plant) during 4 h in a Cleavenger-type apparatus (yield 0,42% v/w), and stored at 4°C until tested and analyzed.

Gas chromatography/mass spectrometry analysis

Essential oil was analyzed by a GCMS-QP5000 (SHIMADZU) gas chromatograph equipped with a mass spectrometer using electron ionization. The gas-chromatographic (GC) conditions were as follows: injector temperature, 260°C; FID temperature, 280°C; carrier gas (Helium), flow rate 1 mL/min and split injection with split ratio 1:40. Oven temperature was initially 60°C and then raised to 240°C at a rate of 3°C/min. One microliter of each sample, dissolved in CH_2Cl_2 (1:100 mg/μL), was injected at ZB5MS column (i.d. = 0.25 mm, length 30 m, film thickness = 0.25 mm) column was used. The mass spectrometry (MS) conditions were ionization

voltage, 70 eV and scan rate; 1 scan/s. The percentage composition of the oils was computed by the normalization method from the GC peak areas. The identification of compounds was performed by comparison of Kovat's index (KI), determined relatively to the retention times of a series of n-alkanes, with corresponding reference data (Adams, 1995) and MS fragmentation pattern was checked with NIST mass spectra libraries.

Microbial strain

Staphylococcus aureus ATCC25923 and *Escherichia coli* ATCC36298 obtained from the culture collections of the Laboratório de Controle Microbiológico, Faculdade de Farmácia, Universidade Federal Fluminense, were used for the antibacterial activity experiments. Overnight cultures were prepared by inoculating approximately 2 mL Tryptic soy broth (TSB; Difco) with 2-3 colonies of each organism. Bacterial strains were cultured overnight at 37ºC. Inocula were prepared by diluting overnight cultures in saline to approximately 10^8 CFU/mL.

Antibacterial activity

Antimicrobial tests were carried out by disk diffusion method (CLSI, 2006). Briefly, a suspension of microorganism (10^8 UFC/mL) was spread on the solid media plates of Tryptic soy agar (TSA; Oxoid). The disks (6 mm in diameter) were impregnated with the essential oil until saturation was reached and placed on the inoculated agar. Vancomycin (30 µg) and ampicillin (30 µg) were used as positive reference standards of the test. The inoculated plates were incubated at 37ºC for 24 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Each experiment was repeated three times.

TLC assay for acetylcholinesterase inhibitors

TLC assay for acetylcholinesterase inhibitors was done as described by Marston et al. (2002) with modifications. Sample of essential oil was dissolved in toluene (1:1) and 1µL was applied on the silica gel Alugram® SIL G UV254 for TLC (MACHEREY - NAGEL). Physostigmine (Sigma, 1µL at 5mM) was used as positive control. After the chromatographic run of the plate with toluene-ethyl acetate mixture (93:7), it was sprayed with DTNB/ATCI (Sigma) reagent (1:1 solution of 0.4 mM 5,5'-dithiobis(2-nitrobenzoic acid) in phosphate buffer (pH 7.4) and 2.0 mM acetylthiocholine iodide in water) until saturation of the silica. The plate was allowed to dry for 5 min, and then 1.5 U/mL of enzyme suspension of the rat brain was sprayed. After a while, a yellow background appeared, with white spots showing the inhibitory compounds. To confirm the result, it was done another plate under the same conditions (Rhee et al, 2003). It was used 1µL of p-anisaldehyde 10% as false inhibitor reference compound. Finally, DTNB solution was applied and after thiocholine solution obtained from enzymatic hydrolysis.

Acetylcholinesterase origin

Acetylcholinesterase enzyme suspension of the rat brain was obtained as it described to Cunha Bastos et al (1991) and Lima et al (1996) with modifications of Moura (1998).

Brine shrimp lethality test

The protocol established by McLaughlin and Rogers (1998) was employed with modifications. Essential oil dilutions at 50, 10, 1 and 0.1 mg/mL were prepared in DMSO and 50µL were transferred to vials. Seawater (5 mL) was added to each vial, resulting in final concentrations of 1000, 500, 100, 10 and 1 µg/mL, respectively. Second instar larvae of *A. salina* (ten per vial) were added. After 24 h contact, the survivors were counted and the LC50 calculated using the Trimmed Spearman-Karber method (Hamilton et al., 1977). Positive control test was done using sodium lauryl sulfate. All these experiments were performed in triplicate.

Statistical analysis

All experiments were performed in triplicate. The mean, standard deviation and coefficient of variation (CV) of the three experiments were determined. The CV values of 15.0 or height were considered statistically significant. The VC values were calculated using the Microsoft Excel program.

RESULTS

The chemical composition of *P. spicatus* essential oil was analysed by GC/MS and 17 components were identified, as shown in Table 1. The major components were limonene (41.87%), 2-undecanone (11.0%) and sabinene (10.78%).

Table 1. Chemical composition of *Pilocarpus spicatus* essential oil

	Compounds [a]	RI(min.) [b]	KI [c]	%
1	α-Pinene	6.613	935	5.08
2	Sabinene	7.983	974	10.78
3	β-Pinene	8.162	979	1.43
4	β-Myrcene	8.570	989	4.95
5	α-Terpinene	9.679	1017	2.04
6	Limonene	10.275	1031	41.87
7	(Z)-β-Ocimene	10.472	1037	1.05
8	(E)-β-Ocimene	10.915	1047	0.97
9	γ-Terpinene	11.432	1059	2.70
10	Terpinolene	12.626	1083	1.84
11	Terpinen-4-ol	17.086	1180	7.90
12	Undecanone-2	22.384	1292	11.00
13	Germacrene D	30.650	1477	1.34
14	Viridiflorene	31.261	1491	1.13
15	γ-Cadinene	32.230	1514	0.67
16	δ-Cadinene	32.412	1519	0.60
17	Elemol	33.493	1545	0.71
	Total			96.06

[a] Compounds listed in order of elution from a ZB-5 MS column.
[b] Retention time (minutes).
[c] Kovats Index on ZB-5 MS column in reference to n-alkanes (Adams,1995).

The *in vitro* antibacterial activity of *P. spicatus* essential oil in comparison with the reference standard included in the study, are shown in Table 2. All the tested strains were sensitive to the essential oil. The differences in inhibition zones diameters of *S. aureus* (CV = 5.0%) and *E. coli* (CV = 3.9%) were not statistically significant.

Table 2. Antibacterial activity of *Pilocarpus spicatus* essential oil

Microorganisms	Essential oil	Antibiotics	
	DD[a]	Vancomycin[b]	Ampicillin[c]
Staphylococcus aureus ATCC25923	12	20	nt
Escherichia coli ATCC36298	14	nt	32

[a] Zone of inhibition (mm).
nt - not tested.
[b] Vancomicin 30 (μg/disc), [c]Ampicilin 10 (μg/disc).

In the chromatographic assay for acetylcholinesterase inhibitors, two white spots were seen, one on the base (false positive) and other on the middle of the plate (a true inhibitory result).

P. spicatus essential oil showed toxicity against brine shrimp nauplii (*Artemia salina* L.) with a lethal concentration 50% (the concentration of test compound that kills 50% of *A. salina*) value of 3.98 μg/mL. Positive control test was done using sodium lauryl sulfate whose LC_{50} is approximately 22 μg/mL and the blank test (50 μL DMSO) no lethality of brine shrimp was observed.

DISCUSSION

P. spicatus essential oil from Rio de Janeiro State presents monoterpenoids, sesquiterpenoids and aliphatic ketones. Regarding the previously reported chemical composition of many essential oils of *Pilocarpus* genus has been described (Craveiro et al., 1979; Santos et al., 1997; Andrade-Neto et al., 2000, 2002). However, here we note that (Z)-β-ocimene and viridiflorene were detected for first time in *P. spicatus*. About this, it is necessary to point out that environmental factors strongly influence the chemical composition of essential oil (Kaastra, 1982)

P. spicatus essential oil collected in the state of Ceará (Brasil) displays *in vitro* antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* but no activity against *E. coli* was reported (Santos et al., 1997). It is not clear whether the antibacterial effect may be caused mainly by a single active constituent or by the combined action of the many active constituents found in essential oil. Terpenoids are active against bacteria but the mechanism of action of terpenes is not fully understood although is speculates to involve membrane disruption by lipophilic compounds (Cowan, 1999). It has been reported by Sacchetti et al. (2005) that essential oil with high terpenoids percentages was probably more effective, as a consequence of higher specificity of the assay for lipophilic compounds.

Several monoterpenes and other terpenoids are known to inhibit AChE (Houghton et al, 2006). The principal monoterpene identified in *P. spicatus* essential oil is limonene (41.87%), which is known to be an inhibitor of AChE (Miyazawa et al, 1997). In this oil, there are others monoterpenes that have anticolinesterase activity as α-Pinene, β-Pinene (Miyazawa and Yamafuji, 2005), α-Terpinene

(Miyazawa et al, 1997), γ-Terpinene (Perry et al, 2002) and sesquiterpene alcohol elemol (Miyazawa et al, 1997).

P. spicatus essential oil showed LC_{50} = 3.98 µg/mL that is a value less than 1000 µL/mL, which suggests good cytotoxic activity potential and indicating that they may possess a significant anti-tumor activity since McLaughlin et al (1998). The cytotoxic activity showed the *P. spicatus* essential oil should be more studied as regards a possible antitumoral activity.

CONCLUSION

The present study is the first report of (Z)-β-ocimene and viridiflorene for *P. spicatus* essential oil. In addition, showed antibacterial activity against Gram-negative (*E. coli)* and Gram-positive (*S. aureus*), anticholinesterase activity and cytotoxic activity in the BST. So, *P. spicatus* essential oil might represent a valuable source for pharmaceutical applications.

REFERENCES

Adams RP. 2001. Identification of essential oil component by chromatography/quadrupole mass spectroscopy. Allured Publishing Corporation. Carol Stream. USA.

Andrade-Neto M, Cunha UA, Mafezoli J, Silveira ER. 2000. Volatile constituents of *Pilocarpus trachyllophus* Holmes and *Pilocarpus jaborandi* Holmes (Rutaceae) from Northeast of Brazil. J Essent Oil Res 12:769-774.

Andrade-Neto M, Cunha UA, Mafezoli J, Silveira ER. 2002. Volatile constituents of different populations of *Pilocarpus spicatus* Saint Hill. (Rutaceae) from the Northeast of Brazil . J Essent. Oil Res 14:319-324.

CLSI - Clinical and Laboratory Standards Institute - (Formerly NCCLS). 2006. Performance standards for antimicrobial disk susceptibility tests; Approved Standard, 7th ed.

Cowan MM. 1999. Plant Products as Antimicrobial Agents. Clin Microbiol Rev 12:564-582.

Craveiro AA, Andrade CHS, Matos FJA, Alencar JW. 1979. Essential oils from Brazilian Rutaceae. I. genus Pilocarpus. J Nat Prod 42:669-671.

Cunha Bastos VLF, Cunha Bastos JF, Lima JS. 1991. Brain acethylcholinesterase as an "in vitro" detector of organophosphorus and carbamate insectides in the water. Water Research 25:835-840.

Hamilton MA, Russo RC, Thurston RV. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ Sci Technol 11:714-719.

Houghton PJ, Ren Y, Jayne M. 2006. Howes: Acetylcholinesterase inhibitors from plants and fungi. Nat Prod Rep 23:181-199.

Kaastra RC. 1982. A monograph of the Pilocarpine (Rutaceae). Flora Neotropica 33:198

Lima JS, Cunha Bastos J, Cunha Bastos VLF. 1996. Methyl parathion activation by a partially purified rat brain fraction. Toxicol Lett 87:53-60.

Lorenzi H, Matos FJA. 2002. Plantas medicinais no Brasil: Nativas e Exóticas. Instituto Plantarum, Nova Odessa, São Paulo. pp 424-425.

Mafezoli J, Vieira PC, Fernandes JB, Silva MFGF, Albuquerque S. 2000. In vitro activity of Rutaceae species against the trypomastigote form of *Trypanosoma cruzi*. J Ethnopharmacol 73:335-340.

McLaughlin TL, Rogers LL. 1998. The use of Biological Assays to Evaluate Botanicals. Drug Inf J 32:513-524.

Melo CB, Gonzalez MS, Kelecom A, Oliveira AP, Rocha LM 2007. Effects of the essential oil obtained from Pilocarpus spicatus Saint-Hilaire (Rutaceae) on the development of *Rhodnius prolixus nympha*e. Rev Bras Farmacogn 17:514-520.

Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. 1982. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med 45:31-34.

Miyazawa M, Watanabe H, Kameoka H. 1997. Inhibition of Acetylcholinesterase Activity by Monoterpenoids with a p-Menthane Skeleton. J Agric Food Chem 45:677-679.

Miyazawa M, Yamafuji C. 2005. Inhibition of Acetylcholinesterase Activity by Bicyclic Monoterpenoids. J Agric Food Chem 53:1765-1768.

Moura CM. 1998. Metodologia enzimática para a detecção de pesticidas organofosforados e carbamatos em água e frutas. Dissertação (Mestrado em Biologia), Universidade do Estado do Rio de Janeiro, Instituto de Biologia Roberto Alcantara Gomes. pp 20-21.

Mukherjee PK, Kumar V, Mal M, Houghton PJ. 2007. Acetylcholinesterase inhibitors from plants. Phytomedicine 14:289-300.

Pavão F, Castilho MS, Pupo MT, Dias RLA, Correa AG, Fernandes JB, Silva MFGF, Mafezoli J, Vieira PC, Oliva G. 2002. Structure of *Trypanosoma cruzi* glycosomal glyceraldehyde-3-phosphate dehydrogenase complexed with chalepin, a natural product inhibitor, at 1.95 Å resolution. FEBS Lett 520:13-17.

Perry NSL, Houghton PJ, Jenner P, Keith A, Perry EK. 2002. *Salvia lavandulaefolia* essential oil inhibits cholinesterase in vivo. Phytomedicine 9:48-51.

Pinheiro CUB. 2002. Extrativismo, cultivo e privatização do jaborandi (Pilocarpus microphyllus Stapf ex Holm.; Rutaceae) no Maranhão. Acta Bot Bras 16:141-150.

Marston A, Kissling J, Hostettmann K. 2002. A Rapid TLC Bioautographic Method for the Detection of Acetylcholinesterase and Butyrylcholinesterase Inhibitors in Plants Phytochemical Anal 13:51-54.

Rhee IK, Rijin RMV, Veerporte R 2003. Qualitative Determination of False-positive Effects in Acetylcholinesterase Assay using Thin Layer Chromatography. Phytochem Anal 14:127-131.

Sacchetti G, Maietti S, Muzzoli M, Scaglianti M, Manfreni S, Radice M, Bruni R 2005. Qualitative Comparative evaluation of 11 essential oil of different origin as funcional antioxidantes, antiradicais and antimicrobials in foods. Food Chem 91:621-632.

Santos FA, Cunha GMA, Viana GSB, Rao VSN, Manoel AN, Silveira ER 1997. Antibacterial Activity of Essential Oils from Psidium and Pilocarpus species of plants. Phytother Res 11:67-69.

Santos AP, Lopes MC, Limberger RP, Apel MA, Henriques AT, Moreno PRH 2004. Analysis of the volatile oil from *Pilocarpus pennatifolius* Lemmaire (Rutaceae) leaves by GC-MS. Flavour Fragr J 19:325-326.

Simões CMO, Spitzer V. 1999. Óleos voláteis. In SIMÕES CMON (Porto Alegre: UFRGS) Farmacognosia da planta ao medicamento, pp 403.

Taveira FSN, Andrade EHA, Lima WN, Maia JGS 2003. Seasonal variation in the essential oil of *Pilocarpus microphyllus* Stapf. An Acad Bras Cienc 75:27-31.