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Antimicrobial activity of essential oil of *Pimenta racemosa* var. *racemosa* (Myrtaceae) leaves

[Actividad antimicrobiana del aceite esencial de las hojas de *Pimenta racemosa* var. *racemosa* (Myrtaceae)]

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Abstract

Context: Essential oils represent a therapeutic alternative in natural products against pathogenic bacteria that have become resistant to antibiotics and threaten public health and individual health of patients.

Aims: To determine the antimicrobial activity of two essential oils of different densities, obtained by hydrodistillation of *Pimenta racemosa* var. *racemosa* fresh leaves collected from Táchira, Venezuela against different multirresistant bacterial strains of nosocomial origin.

Methods: Disc diffusion agar method was carried out against seven reference strains: *Candida albicans* (CDC-B385), *Candida krusei* (ATCC 6258), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 23357), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and three different bacterial strains of nosocomial origin: Methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Enterobacter cloacae*.

Results: The essential oils of *Pimenta racemosa* var. *racemosa* inhibited the development of all microorganisms tested with minimum inhibitory concentration (MIC) values ranging from 20 to 400 µL/mL.

Conclusions: This is the first report concerning antimicrobial activity of essential oils obtained from *Pimenta racemosa* var. *racemosa* collected from Táchira, Venezuela with different densities. Furthermore, results showed the essential oils of this species might be an alternative as antimicrobial agent for the pharmaceutical industry.

Keywords: antimicrobial activity; *C. krusei*; essential oil; MRSA; *Pimenta racemosa*.

Resumen

Contexto: Los aceites esenciales representan una alternativa terapéutica en productos naturales contra bacterias patógenas que se han hecho resistentes a los antibióticos y que amenazan la salud pública y la salud individual de los pacientes.

Objetivos: Evaluar la actividad antimicrobiana de dos aceites esenciales de diferentes densidades, obtenidos por hidrodestilación de las hojas frescas de *Pimenta racemosa* var. *racemosa* recolectadas en Táchira, Venezuela frente a diferentes cepas bacterianas multiresistentes de origen nosocomial.

Métodos: Se usó el método de difusión en agar con disco para evaluar la actividad antimicrobiana de los aceites esenciales frente a siete cepas de referencia internacional: *Candida albicans* (CDC-B385), *Candida krusei* (ATCC 6258), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 23357), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923); y tres cepas bacterianas de origen nosocomial *Staphylococcus aureus* resistente a meticilina (SARM), *Escherichia coli* y *Enterobacter cloacae* productoras de β-lactamasa de espectro extenso (BLEE).

Resultados: Los aceites esenciales de *Pimenta racemosa* var. *racemosa* evaluados inhibieron el desarrollo de todos los microorganismos ensayados con valores de concentración inhibitoria mínima (CIM) que oscilaron entre 20 y 400 µL/mL.

Conclusiones: Este es el primer reporte sobre actividad antimicrobiana de los aceites de diferentes densidades de esta especie colectada en Táchira, Venezuela. Además, los resultados revelaron que los aceites esenciales de esta especie pueden ser una alternativa como agente antimicrobiano para la industria farmacéutica.

Palabras Clave: aceite esencial; actividad antimicrobiana; *C. krusei*; *Pimenta racemosa*; SARM.

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INTRODUCTION

Pimenta racemosa var. *racemosa* (Mill.) J.W. Moore is an aromatic arboreal plant belonging to the genus *Pimenta* (Myrtaceae), native to the Caribbean and northwestern South America. However, it has been introduced successfully in the southeastern United States, Sri Lanka, East and West Africa and Indonesia (Dupont et al., 1954; Weiss, 2002; Contreras-Moreno et al., 2014a). In Venezuela, this genus is only represented by *P. racemosa* (Mill.) J.W. Moore (*P. acris* Kostel), and is distributed in Capital District, Falcón, Lara, Mérida, Nueva Esparta, Sucre, Táchira and Zulia states (Hokche et al., 2008). This species is cultivated as an ornamental, used in folk medicine, and commonly known as: Bay rum, Malagueta, Pepita and pepper species (Aristeguieta, 1973; Contreras-Moreno et al., 2014a; 2014b). Furthermore, it has been widely used due to the content of volatile essences, once distilled, are used in cosmetics, especially in formulations such as aftershave lotions, soaps, perfumes and hair treatments (Weiss, 2002; Boning, 2010; Contreras-Moreno et al.,. 2014a; 2014b). Regarding biological properties, the essential oil of this species has been studied for antioxidant (Jirovetz et al., 2007; Alitonou et al., 2012), insecticide (Leyva et al., 2007), antibacterial (Tajkarimi et al., 2010) and antifungal (Kim et al., 2008) activities.

On the other hand, resistance to antibiotics has increased rapidly in recent years, causing a lot of concern, since it is becoming a major threat for patients, as the alternatives against infections caused by resistant pathogens is reduced. Methicillin-resistant *Staphylococcus aureus* (MRSA) and enterobacteria producing extended-spectrum β-lactamase (ESBL) as *Escherichia coli* and *Enterobacter cloacae* (Oteo et al., 2016) are the most dangerous microorganisms present in nosocomial infections, therefore the need for searching new alternatives in natural products.

The aim of this investigation was to determine the antimicrobial activity of two essential oils of different densities, obtained by hydrodistillation of fresh leaves of *Pimenta racemosa* var. *racemosa* collected from Táchira, Venezuela against different multirresistant bacterial strains of nosocomial origin. Authors consider that *P. racemosa* var *racemosa* study is very important since it confirms, scientifically, its popular use of local people to aid skin infections produced by Gram positive microorganisms such as *Staphylococcus*.

MATERIAL AND METHODS

Botanical material

Fresh leaves of *Pimenta racemosa* var. *racemosa* were collected in April 2012, near to Sector "Los Corredores de la Palmita", Junín Municipality, Rubio town, located in southwestern Táchira state, Venezuela, altitude 859 m.a.s.l. Botanical identification was carried out by Dr Leslie R. Landrum, Herbarium Curator, School of Life Sciences, Arizona State University, USA. Specimens collected in the field are housed in the MERF Herbarium of the Faculty of Pharmacy and Bioanalysis, University of Los Andes (BC-01 code), Venezuela, and at the Herbarium of Arizona State University (ASU0075448 code), USA.

Chemicals

Müeller-Hinton Agar, Müeller-Hinton Broth and Sabouraud Dextrose Agar from BBL™ (BD, Maryland, USA); sodium chloride from Riedel-de-Haën (Hannover, Germany); dimethyl sulfoxide from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA); glucose from Merck (Darmstadt, Germany); azurfosin-methylene-blue from Lab-Line Chemical Product (LAB-LINE CA, Barquisimeto, Venezuela).

Essential oils

The essential oils of different densities used in this study, light (LO) and heavy (HO), were isolated by hydrodistillation of fresh leaves of *Pimenta racemosa* var. *racemosa* collected from Táchira, Venezuela and their chemical composition (Table 1) were previously reported by Contreras-Moreno et al. (2014a). Both oils, analyzed by GC/MS, revealed the presence of 17 and 13 components for LO and HO, respectively, being eugenol for both oils the major compound with 60.4% (LO) and 82.9% (HO) (Contreras-Moreno et al., 2014a).

Table 1. Volatile compounds (% total peak area) of LO and HO essential oil obtained from *Pimenta racemosa* var. *racemosa* leaves collected in Táchira, Venezuela.

Compound	Essential oil (%)		RI
	LO*	HO*	
3-Hexen-1-ol, (*Z*)	0.6	-	849
α-Pinene	0.5	-	936
1-Octen-3-ol	2.2	0.3	977
Myrcene	**11.7**	1.5	989
α-phellandrene	0.8	-	1003
p-cymene	1.0	0.2	1025
Limonene	**5.4**	0.9	1030
1,8-cineole	2.9	0.3	1033
β-ocimene	0.2	-	1049
Linalool	**4.4**	0.7	1100
4-Terpineol	0.9	0.2	1178
α-Terpineol	1.3	0.6	1190
Chavicol	**6.0**	**9.3**	1259
Eugenol	**60.4**	**82.9**	1364
α-Copaene	0.3	0.2	1377
Trans-(*β*)- caryophyllene	0.7	0.5	1417
δ-cadinene	0.8	0.7	1524

RI, retention indices relative to C_6–C_{24} n-alkanes on the HP-5 MS column; MS, mass spectrum.

* Taken from Contreras-Moreno et al. (2014a).

Antibiotics and fungicides

Trimethoprin-Sulfamethoxazol® 10 µg, Vancomycin® 30 µg, Gentamycin® 10 µg, Aztreonam® 30 µg, Cefepime® 30 µg were purchased from BBL™ (BD, Maryland, USA), and Fluconazole® 100 µg from Liofilchem (Roseto degli Abruzzi, Italy). Vorcum® 200 mg (400 µg/mL Voriconazole) was adquired in Pfizer (Caracas, Venezuela).

Microbiological analysis

Bacterial strains

MRSA (525), *E. coli* ESBL (7532) and *E. cloacae* ESBL (10221) were isolated from patients with nosocomial infections, hospitalized at the Neonatology Service (P28), Autonomous Institute University Hospital of Los Andes (Mérida, Venezuela). All yeasts and bacteria tested in this investigation are described in Table 2.

Antimicrobial method

The antibacterial activity was evaluated following the disc diffusion method described by Velasco et al. (2007). Antifungal assay was carried out according to the NCCLS (2004) disc diffusion methodology with some modifications, each yeast inoculum (2.5 mL) was incubated in Sabouraud's dextrose agar with chloramphenicol at 37°C for 18 h and the turbidity was adjusted to McFarland Nº 1 (3 x 10^8 CFU/mL) (NCCLS, 2004; Lozina et al., 2005; Narvaez-Florez et al., 2008). Twenty mL Müeller-Hinton agar (BBL™) supplemented with methylene blue (0.5 µg/mL) and glucose (2%, w/v) were mixed with 1 mL of each yeast inoculum. The contents of Petri dishes were allowed to solidify at room temperature and kept at 4°C until analysis. A sterile control was also prepared (Pemán et al., 2006; CLSI, 2013; Buitrago et al., 2015).

The minimum inhibitory concentration (MIC) was determined with all tested microorganisms by dilution of essential oil in dimethylsulphoxide (DMSO) within a range between 10-500 µL/mL concentrations. MIC was defined as the lowest concentration that inhibited bacterial growth visible (CLSI, 2013); a negative control using a saturated disc with DMSO to check the possible activity of this solvent against the microorganism tested was also included. The inhibitory zone around the disc was measured and expressed in mm. Experiments were performed by duplicate.

Statistical analysis

Statistical analyses were performed by using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA) to determine the statistical significance of differences ($p < 0.05$) in antimicrobial activity using the one-way analysis of variance (ANOVA) with two post-hoc analyses. The post-hoc Dunnett's test was performed for the comparison of inhibition zones (Zi) between the control group (Trimethoprin-Sulfamethoxazol®, Vancomycin®, Gentamycin®, Aztreonam®, Cefepime®, Fluconazole® and Vorcum®) and the test groups (antimicrobial activities for both oils LO and HO) against test microorganisms

(*S. aureus, E. faecalis, E. coli, K. pneumonia, P. aeruginosa, C. albicans and C. krusei*); while the post-hoc Tukey test was performed for the comparisons of MIC between each microorganisms treated with oils (LO, HO, DMSO). All results (Table 2) were expressed as mean and standard deviation (SD) values of two parallel measurements.

RESULTS

Results observed in present investigation revealed that antimicrobial activity of essential oils (low-density oil, LO) and (high-density oil HO) of *P. racemosa* var. *racemosa* showed growth inhibition of all microorganisms tested with inhibition zones ranging between 14 to 32 mm and MIC values between 20-400 µL/mL. In general, there was a statistically significant difference between the Zi of both oils (for all reference bacterial strains) and their respective positive control ($p<0.05$) and between the MIC of LO and HO with the microorganisms *S. aureus, E. coli, K. pneumoniae, P. aeruginosa* and *C. krusei* ($p<0.05$). According to these results, essential oil (HO) could be considered as the best antimicrobial treatment compared to LO, since HO showed more activity than LO against *S. aureus, E. coli and K. pneumoniae* with MIC values of 20 µL/mL and against *C. krusei* with MIC value of 50 µL/mL. The complete results of these assays are detailed in Table 2.

Both oils tested revealed activity against three nosocomial bacterial strains of MRSA, *E. coli* ESBL and *E. cloacae* ESBL (Table 2).

DISCUSSION

The oils composition obtained from *P. racemosa* var. *racemosa* previously published (Contreras-Moreno et al., 2014a), showed eugenol 60.4%, chavicol 6.0 %, myrcene 11.7%, limonene 5.4% and linalool 4.4% as main components for the light density oil (LO), while the more heavy density oil (HO) was composed mainly by eugenol 82.9% and chavicol 9.3% (Table 1). Terpenoids are considered molecules capable of causing death of bacterial cells by three possibly mechanisms; increasing the membrane permeability, affecting structural stability of the membrane or disrupting the lipid bilayer packing (Maguna et al., 2006), thus, the activity of oils tested in this investigation might be due to any of these proposed mechanisms. According to Garcia et al. (2010), the antimicrobial activity of essential oils is due to the lipophilic nature of the hydrocarbon backbone and possibly the hydrophilicity of its functional groups, being eugenol, chavicol, and linalool among the most common terpenoids involved in such activity (Burt, 2004). Furthermore, eugenol is capable of generating morphological deformations in some enzymes present in the cell wall, such as chitinases and glucanases (Kalemba and Kunicka, 2003) due to the formation of a hydrogen bond between the hydroxyl group of the molecule and the enzyme site of action (Garcia et al., 2010).

On the other hand, previous investigations have revealed antimicrobial activity in essential oils of three different chemotypes of *P. racemosa* var. *racemosa* such as neral/geranial (lemon scent), chavicol/eugenol (clove smell) and methylchavicol/methyleugenol (anise smell). Those oils were active against five bacterial strains *S. aureus*, *Enterococcus faecium*, *E. coli*, *P. aeruginosa* and *Mycobacterlum smegmatis* (MIC of 500-8000 µg/mL) and five fungal strains *C. albicans*, *Aspergillus niger*, *Corymbifera absidia*, *Penicillium* and *Cladosporium cladosporioides verrucosum* (MIC 100-1000 µg/mL) (Aurore et al., 1998).

According to results reported by Aurore et al. (1998) for eugenol/chavicol chemotype, which is the same chemotype of the LO and HO oils tested in this study (Contreras-Moreno et al., 2014a), are superior since the MIC reported by Aurore et al. (1998) was 1000 µg/mL (*S. aureus*), 2000 µg/mL (*E. coli*), 8000 µg/mL (*P. aeruginosa*) and 500 µg/mL (*C. albicans*), whereas in this investigation ranged from 20 to 60 µL/mL (*S. aureus*), 20 and 40 µL/mL (*E. coli*), 200 to 400 µL/mL (*P. aeruginosa*) and 100 µL/mL (*C. albicans*).

Furthermore, the inhibitory power of both oils against multirresistant bacteria, which causes infections in hospitals and represent a public health problem, providing an alternative to the pharmaceutical industry for the exploration of novel metabolites with broad spectrum antimicrobial activity at low concentrations or be used as an alternative to commercial antimicrobials to treat infections caused by both pathogenic bacteria and multirresistant pathogenic bacteria.

Table 2. Antimicrobial activity of LO and HO essential oil obtained from *Pimenta racemose* var. *racemose* leaves collected in Táchira, Venezuela.

Microorganisms	Inhibition zone (mm)#				MIC (µL/mL)	
	Essential oil		Positive control		Essential oil	
	LO	HO			LO	HO
S. aureus (ATCC 25923)	20.3 ± 0.4[a]	21.8 ± 0.4[a]	SXT	42.0 ± 0.0[a]	60.0 ± 0.0[b,**]	20.0 ± 7.1[b,**]
E. faecalis (ATCC 29212)	16.5 ± 0.7[a]	18.0 ± 1.4[a]	VA	24.0 ± 0.0[a]	100.0 ± 0.0	100.0 ± 0.0
E. coli (ATCC 25922)	17.8 ± 0.4[a]	17.5 ± 0.7[a]	GM	30.0 ± 0.0[a]	40.0 ± 0.0[b,*]	20.0 ± 7.1[b,*]
K. pneumoniae (ATCC 23357)	13.8 ± 0.4[a]	15.3 ± 0.4[a]	AZT	42.0 ± 0.0[a]	60.0 ± 0.0[b,**]	20.0 ± 7.1[b,**]
P. aeruginosa (ATCC 27853)	14.0 ± 1.4[a]	14.0 ± 0.0[a]	CEF	35.0 ± 0.0[a]	200.0 ± 14.1[b,***]	400.0 ± 0.0[b,***]
MSRA (Nº 525)	17.5 ± 0.7	18.0 ± 1.4	nt	-	65.0 ± 7.1	45.0 ± 7.1
E. coli ESBL (Nº 7532)	17.8 ± 0.4	17.5 ± 0.7	nt	-	50.0 ± 14.1	50.0 ± 14.1
E. cloacae ESBL (Nº 10221)	17.5 ± 0.7	18.0 ± 0.7	nt	-	50.0 ± 14.1	50.0 ± 14.1
C. albicans (CDC-B385)	30.5 ± 0.7	31.5 ± 0.7	FLU	32.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
C. krusei (ATCC 6258)	29.5 ± 0.7	31.0 ± 0.0	VOR	30.0 ± 0.0	200.0 ± 14.1[b,**]	50.0 ± 14.1[b,**]

Data represented as mean ± SD of two independent readings. [a]Values are statistically significant (p≤0.05) between the Positive control group and the test groups (LO and HO) by means of Dunnett's test. [b]Values are statistically significant (p≤0.05) between LO and HO groups by means of Tukey test. *p≤0.05, **p≤0.005, ***p≤0.001 statistically significant between LO v.s. HO in specific pathogen species.

#Discs of 6 mm diameter and 2 mm thick; LO: Essential oil of light density; HO: Essential oil of heavy density; SXT: Trimethoprin-Sulfamethoxazol®; VA: Vancomycin®; GM: Gentamycin®; AZT: Aztreonam®; CEF: Cefepime®; FLU: Fluconazole®; VOR: Vorcum® (400 µg/mL Voriconazole); MRSA: Methicillin-resistant *S. aureus*; ESBL: extended-spectrum β-lactamase; MIC: Minimum Inhibitory Concentration, Range: 10 to 500 µL/mL; Initial concentration: 1000 µL/mL; nt: no-tested positive control. The positive control group was only evaluated with inhibition zone.

CONCLUSIONS

Present results are consider an important contribution to the natural products research and are a possible alternative to new antimicrobials against multirresistant bacterial strains, because both oils tested had activity against three nosocomial bacterial strains of MRSA (LO: 65 µL/mL and HO: 45 µL/mL), *E. coli* ESBL (LO and HO: 50 µL/mL) and *E. cloacae* ESBL (LO and HO: 50 µL/mL), however, it is necessary to conduct toxicity studies to discard any toxicity of these oils on normal cells. According to literature consulted, to date, there are no reports describing antimicrobial activity of different densities essential oils obtained in the same extraction of *P. racemosa* var. *racemosa*, similarly, there is no information about biological activities of *P. racemosa* var. *racemosa* from Táchira state (Venezuela).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Concepts or Ideas	X	X	X		
Design	X	X	X		
Definition of intellectual content	X	X	X		
Literature search	X	X		X	X
Experimental studies	X	X			
Data acquisition	X	X			
Data analysis	X	X			
Statistical analysis	X	X			
Manuscript preparation	X	X	X		
Manuscript editing	X	X	X		X
Manuscript review	X	X	X	X	X

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