COMPOSITION AND ANTIBACTERIAL ACTIVITY OF THE ESSENTIAL OIL OF *Myrcia fallax* (Rich.) DC. FROM VENEZUELA

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ABSTRACT

Essential oils obtained from leaves and flowers of *Myrcia fallax* by hydrodistillation were analyzed by GC-MS and GC-FID. GC analysis revealed the presence of 30 compounds in the oil obtained from the leaves, 19 of these compounds were identified which represent 83.4 %. The oil from the flowers contained 15 compounds 12 of which were identified. The main constituents of the leaves´ oil were guaiol (31,0%) and carotol (9.9%), while in the flowers´ oil the most abundant compounds were guaiol (27.5%) and aristolone (24,5%). Carotol was separated by flash chromatography and its structure confirmed by 1D and 2D NMR experiments. The antibacterial properties of essential oils were investigated against five reference strains. The essential oil from flowers was effective only against Gram positive bacteria, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212, with MIC values of 50 and 400µg/mL, respectively.

Key words*: Myrcia fallax*; Myrtaceae; guaiol; aristolone; carotol; antibacterial activity**.**

COMPOSICIÓN Y ACTIVIDAD ANTIBACTERIANA DEL ACEITE ESENCIAL DE *Myrcia fallax* (Rich.) DC. DE VENEZUELA

RESUMEN

Los aceites esenciales obtenidos a partir de las hojas y flores de *Myrcia fallax* por hidrodestilación fueron analizados por cromatografía de gases acoplada a un espectrómetro de masas (CG-EM) y cromatografía de gases acoplada a un detector de ionización de llamas (CG-FID). El análisis por CG reveló la presencia de 30 componentes en el aceite esencial obtenido a partir de las hojas; 19 de estos compuestos fueron identificados, los cuales representan el 83,4 %. El aceite de las flores contiene 15 compuestos, 12 de los cuales fueron identificados. El mayor de los constituyentes de las hojas fue guaiol (31,0%) y carotol (9.9%), mientras que en el aceite esencial de las flores los componentes más abundantes fueron guaiol (27,5%) y aristolone (24,5%). El carotol fue separado por cromatografía de flash y su estructura confirmada por experimentos RMN uni y bidimensional. Las propiedades antibacterianas del aceite esencial obtenido a partir de las flores fueron evaluadas contra cinco cepas de referencia. El aceite esencial de las flores fue efectivo sólo contra las bacterias Gram positivas, *Staphylococcus aureus* ATCC 25923 y *Enterococcus faecalis* ATCC 29212, con valores de CMI de 50 y 400µg/mL, respectivamente.

Palabras clave: *Myrcia fallax*; myrtaceae, guaiol, aristolona; carotol; actividad antibacteriana.

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INTRODUCTION

There are more than 150 species of *Myrcia* distributed in tropical South America and Western India. *Myrcia fallax* (Rich.) DC., is a tree let widely distributed in the neotropics, ranging from Eastern Mexico to the Southeastern Brazilian coastal forests. In Táchira, Venezuela it grows near the rivers.[1]

Terpenes and sesquiterpenes have been found in the genus,[2] a patent covering the antitumor constituents of *M. fallax* has been issued [3] and leaves of some species of *Myrcia* have been used as antidiabetic, anti-inflammatory, desinfectant and antidiarrhoeal .[4]

A literature survey on *M. fallax* yielded only one publication on the comparative chemical composition of the essential oil from the leaves five Brazilian species.[5] As part of a series of studies on aromatic plants from Táchira State (Venezuela),[6,7] the chemical composition of the essential oil from leaves and flowers of *M. fallax* has been obtained.

EXPERIMENTAL

Plant Material and extraction

Fresh leaves and flowers were collected at Boca de Monte, Táchira State (Venezuela). Voucher specimens (PBM6) are deposited at the Herbarium of the Universidad Nacional Experimental del Táchira. The samples were hydrodistilled for 5 h using a Clevenger type apparatus.[7] Oil yields were determined using oil dried over Na_2SO_4 and related to fresh plant material.

GC analysis

Analytical gas chromatography was carried out on a Perkin Elmer Auto System gas chromatograph. A 5% phenyl, 95% methylpolysiloxane capillary column (AT-5, Alltech Associates, Inc., Deerfield-Illinois, USA, 60m x 0,25mm x 0,25m film thickness) programmed from an initial temperature of 60°C (5 min) to 200°C at 4 °C/min. followed by a second ramp of 10°C/min up to a final temperature to 280°C, and a polyethylenglycol (carbowax 20 M) capillary column of the same dimensions (AT-WAX, Alltech Associates, Inc., Deerfield-Illinois, USA) programmed from 60°C to 220°C at 4°C/min were used. Injector and FID temperatures were 250°C and a flow rate of 0,8mL/min of he was used. A 1,0 mL sample of a 25 % solute of the essential oil in diethylether was injected with split ratio, the retention indices[8] were determined relative to the retention times of a series of n-alkanes (C_7–C_{22}) with linear interpolation on both capillary columns. Quantitative data were obtained from FID area percentages without the use of correction factors.

GC-MS analysis

The analysis was carried out on a Hewlett-Packard MSD 5973 mass spectrometry system using a 5% phenyl methylpolysiloxane column (HP-5MS, 30m x 0,25mm x 0,25m film thickness). The carrier gas was Helium at 0,9mL/min. The initial column temperature was 60°C and then it was raised at 4°C/min to 200°C. Finally the temperature was raised to 280°C at 10 °C/min. The ionization energy was 70 eV. A 1.0L sample of a solution of 20L of oil in 1,0mL of diethylether was injected with split ratio of 100:1; the temperature of the injection block was 250°C.

Isolation of carotol

Carotol was isolated by flash column chromatography from the oil obtained from the leaves of *M. fallax*. Silica gel 60 (Scharlau, 0.04-0.06 mm) and the column was eluted with hexane and hexane containing 2%-30% diethylether. Fractions eluted with hexane-2% diethylether yielded 95% pure carotol. Other fractions yielded mixtures whose NMR spectra were not suitable for interpretation.

Nuclear Magnetic Resonance Spectroscopy

Carotol spectra were measured at 303 K in $CDCl_3$ solution on a 400 MHz Bruker Avance spectrometer. ^{1}H, ^{13}C, DEP, H,H-COSY, HMQC, and HMBC spectra were taken to assign ^{1}H and ^{13}C signals.

Microbiological analysis

Bacterial strains

The microorganisms used were *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* ATCC 23357 and *Pseudomonas aeruginosa* (ATCC 27853).

Antimicrobial method

The antimicrobial activity was carried out according to the disc diffusion assay,[9] the strains were maintained in agar at room temperature. 2,5 mL of every bacteria inoculum were incubated in Mueller-Hinton agar at 37ºC for 18 hours. The bacterial inoculum was adjusted with SSF to the Mac Farland Nº 0,5 turbidness patron (10^{6-8} ufc/mL). Every inoculum was spread over plates containing Mueller-Hinton agar and a paper filter disc (6 mm) saturated with 10 µL of essential oil. The plates were left for 30 min at room temperature and then incubated at 37 ºC for 24 h.

The inhibitory zone around the disc was measured and expressed in mm. A positive control was also assayed to check the sensitivity of the tested organisms using the following antibiotics: Sulbactam-Ampicillin® (10µg/10 µg), Vancomycin® (30 µg), Netilmicin® (10 µg), Cefoperazone® (75 µg), and Aztreonam® (30 µg) (table 3).

The minimal inhibitory concentration (MIC) was determined only with microorganisms that displayed inhibitory zones. MIC was determined by dilution of the essential oil in dimethyl sulphoxide (DMSO) pipetting 10 µl of each dilution onto a filter paper disc. Dilutions of the oil within a concentration range of 10-420 µg/mL were also carried out. MIC was defined as the lowest concentration that inhibited the visible bacterial growth.[10]

A negative control was also included in the test using a filter paper disc saturated with DMS to check possible activity of this solvent against the bacteria assayed. The experiments were repeated at least twice.

RESULTS AND DISCUSSION

M. fallax essential oils are greenish and have a pleasant persistent spicy odor. Fresh leaves and flowers yielded 0,25 and 0,30% (v/w) respectively. Table 1 gives the relative percentages of the components of *M. fallax* oils. Nineteen constituents were identified which represented 83,4% of oil from the leaves and 12 constituents (83.2%) from the flowers´ oil.

Table 1. Percentage of the essential oil composition from leaves and flowers *Myrcia fallax*.

Compound	R I[a]	RI[b]	% in leaves	% in flowers	Identification
α- Pinene	931	1039	7,7	6,0	GC-MS, RI
β- Pinene	973	1122	6,9	4,9	GC-MS, RI
Myrcene	991	1156	0,2	-	GC-MS, RI
Limonene	1031	1206	2,5	1,0	GC-MS, RI
Ocimene<(E)-β->	1037	1250	1,2	-	GC-MS, RI
α- Terpinolene	1087	1287	0,5	-	GC-MS, RI
α- Terpineol	1189	1661	-	4,7	GC-MS, RI
Methyl salicylate	1192	1754	-	1,0	GC-MS, RI
Chrysanthenyl acetate	1238		1,4	-	GC-MS, RI

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α- Terpinyl acetate	1347	1687	0,9	-	GC-MS, RI
Caryophyllene<(E)-β>	1419	1612	6,0	2,6	GC-MS, RI
α- Humulene	1455	1672	0,6	-	GC-MS, RI
Germacrene - D	1485	1712	1,5	1,5	GC-MS, RI
α- Selinene	1498	1719	0,4	-	GC-MS, RI
Liguloxide	1536		2,8	-	GC-MS
γ- Cadinene	1539	1792	2,0	2,6	GC-MS, RI
Caryophyllene oxide	1585	1896	3,6	-	GC-MS, RI
Carotol	1595		9,9	-	GC-MS, RI
Guaiol	1601		31,0	27,5	GC-MS, RI
10-epi-γ-eudesmol	1624	2121	-	4,1	GC-MS, RI
α-Cadinol	1654	2224	3,8	2,8	GC-MS, RI
Valerianol	1655	2231	0,5	-	GC-MS, RI
Aristolone	1763		-	24,5	GC-MS, RI

[a] RI= Retention index on AT-5 column [b] RI= Retention index on Carbowax 20M column.[7].

The oil from the leaves contained 19,0% of monoterpene hydrocarbons and 11% of sesquiterpene hydrocarbons. The main oxygen containing component was guaiol (31%), followed by carotol (9,9%).

The oil from the flowers contained only 11,9% of monoterpene hydrocarbons and 6,7% of sesquiterpene hydrocarbons. This oil is dominated by guaiol (27,5%) and aristolone (24,5%). It is noteworthy to notice that aristolone was absent from the leaves´ essential oil.

The mass spectrum of carotol showed the molecular ion at m/z 222, $[M-H_2O]^+$ 204 (21), 179 (49), 161 (100), 138 (19), 123 (35), 105 (28), 81 (24), 65 (14), 43 (19). The identity of carotol was confirmed by NMR analysis performed on 95% pure carotol isolated from the oil by flash chromatography. Table 2 present ^{1}H and ^{13}C NMR chemical shifts of carotol (figure 1). These values agree with those reported in the literature.[11]

Table 2. ^{1}H and ^{13}C NMR chemical shifts of carotol isolated from leave's *Myrcia fallax* essential oil.

	^{13}C (ppm) $CDCl_3$	^{1}H (δ) $CDCl_3$
C-1	39,4	1,54 and 1,29
C-2	24,4	1,30 and 1,32
C-3	52,4	1,71
C-3a	84,6	--
C-4	34,4	1,62 and 1,95
C-5	29,4	1,27 and 2,06
C-6	138,6	--
C-7	122,1	5,31 vinil proton
C-8	38,6	2,24
C-8a	49,0	--
C-9	27,6	1,79
C-10	21,4	0,93
C-11	21,4	0,94
C-12	25,2	1,71
C-13	24,0	1,00

Carotol, guaiol and aristolone have not been reported for the oil of other *Myrcia* species[2,5] but they have been reported in Umbelliferaceae, Asteraceae, Rosaceae and Valeranaceae.[11,12]
In comparison to results previously reported for the oil of *Myrcia fallax* of brazilian origin,[5] the composition of the essential oil from leaves of *M. fallax* grown in Venezuela differs considerably. The main differences are related to sesquiterpene hydrocarbons (11,0% Venezuela and 7,2% Brazil) and oxygenated sesquiterpenes (48,3% Venezuela and 86,5% Brazil). The oil of other *Myrcia* species have been reported for instance, the oil of *M. bracteata* has by a high content of (E)-nerolidol, while the oil of *M. cuprea* was dominated by myrcene and the major constituent in the oil of *M. sylvatica* was reported to be selin-11-en-4-α-ol.
Antibacterial activity of the essential oil was evaluated against Gram positive and Gram negative bacteria. These microorganisms are both morphologically and physiologically different, thus the results obtained are representative of the antibacterial activity of the oil. The results showed that this essential oil is active only against Gram positive bacteria, displaying MIC values for *Staphylococcus aureus* (50µg/mL) and *Enterococcus faecalis* (400µg/mL). The results of this experiment are shown in table 3.

Table 3: Antimicrobial activity of essential oil from flower *Myrcia fallax*.

MICROORGANISM	Inhibition zone*						MIC µg/mL
		Positive control					
		SAM (10/10 µg)	VA (30 µg)	NET (30 µg)	AZT (30 µg)	CEF (75 µg)	
Staphylococcus aureus ATCC 25923	10*	45*	-	-	-	-	50
Enterococcus faecalis ATCC 29212	9*	-	27*	-	-	-	400
Klebsiella pneumoniae ATCC 23357	NA	-	-	30*	-	-	NA
Escherichia coli ATCC 25922	NA	-	-	-	46*	-	NA
Pseudomona aeruginosa ATCC 27853	NA	-	-	-	-	34*	NA

SAM: Ampicillin- sulbactam, VA: Vancomycin, NET: Netilmicin, AZT: Aztreonam, CEF: Cefoperazone. *Inhibition zone, diameter measured in mm, disc diameter 6 mm; average of two consecutive assays. MIC: Minimal inhibitory concentration, concentration range 10-420 µg/mL. NA: Not active.

The microorganisms, *S. aureus* and *E. faecalis*, are consider being responsible for causing several human infections and are known to be resistant to some kind of antibacterial treatment using commercial patented antibiotics.[13,14]
The activity of the oils would be expected to relate to the respective composition of the plant volatile oils. A non-correlation of the antimicrobial activity of the compounds tested and their relative percentage composition in the plant volatile oil used in this study suggests a number of observations. The absence of components with phenolic structures in *M. fallax* oils, such as eugenol and thymol, that are highly active against the most microorganisms and non activity of the monoterpene cyclic hydrocarbon, may suggest that the antimicrobial activity observed

maybe related to sesquiterpene oxygen contribuing cmpounds.[15] This is the first time the antibacterial activity of the essential oil from leaves and flowers of *M. fallax* has been reported.

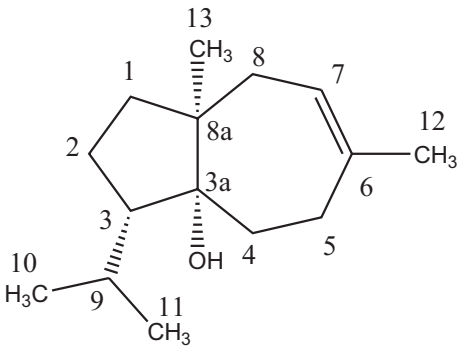

Figure 1. carotol

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REFERENCES

1. Pittier H. Manual de las Plantas de Venezuela y Suplemento. Barcelona: Fundación Eugenio Mendoza, Talleres Gráficos S.A., 1971. p.378.
2. Zoghbi MG, Andrade EH, Silva MH, Carreira LM, Maia JG. *Flavour Fragr. J.* 2003;18:421.
3. Hecht SM. US Pat. 4451459. 1984.
4. De Mello Cruz A, Coelho M. Floresta e Ambiente, 2004; 11:47.
5. Henriques AT, Sobral M, Bridi R. *J. Essent. Oil Res.* 1997; 9:13.
6. González de CN, Ojeda de RG, Prieto A, Crescente O, Cabrera L. *Ciencia*, 1998; 6:123.
7. González de CN, Sánchez F, Quintero A, Usubillaga A. Acta Horticulturae, 2002; 576:49.
8. Adams RP. Identification of Essential Oils by Gas Chromatography/Quadrupole Mass Spectroscopy. Allured: Carol Stream, IL, 2001.
9. Rondon, M., Velasco, J., Morales, A., Rojas, J., Carmona, J., Gualtieri, M., and Hernandez, V. (2005). Composition and antibacterial activity of the essential oil of *Salvia leucantha* Cav. cultivated in Venezuela Andes. *Rev. Lat. Quim* 33, 55-59.
10. CLSI. Clinical and Laboratory Standars Institute. Performance standars for antimicrobial susceptibility testing; Sixteenth informational supplement. CLSI document M100-S17 [ISBN 1-56238-625-5]. Clinical and Laboratory Standars Institute, 940 West Valley Road, Siute 1400, Wayne, Pennsylvania 19087-1898 USA, 2007.
11. Ghisalberti EL. *Phytochemistry*, 1994; 37:597.
12. Kong C, Wang P, Xu X. *Agriculture Ecosystems & Environment,* 2007; 119:416.

13. Hurtado M, De La Parte M, Brito A, Tapia I, Carmona C, GVRB. Resistencia de *Staphylococcus aureus* a los antimicrobianos en Venezuela 1988-1998. AVFT 2004, 23 (2): 159-165. AVFT: Archivos Venezolanos de Farmacología y Terapéutica
14. Guzmán, M., and Lozada, R. (2007). Detección de *Staphylococcus aureus* meticilino-resistentes de pacientes con infecciones nosocomiales y adquiridas en la comunidad. *Rev. Soc. Ven. Microbiol*., 27: 45-49.
15. Sotanaphun U, Lipipun V, Suttisri R, Bavovada R. *Planta Med,* 1999; 65:257.