RESEARCHS / INVESTIGACIÓN

Chemical diversity and antibacterial activity of volatile compounds from two Centrolobium paraense Tul. varieties.

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Abstract: This work presents a comparative study of the chemical composition of the essential oils obtained from two varieties of Centrolobium paraense (orinocense EO o and paraense EO p) from Venezuela. GC analyzed the oils, and the constituents were identified by GC-MS and retention indices. Eighteen compounds were identified in EO o, which made up 88.9 % of the oil, but only sixteen compounds were identified in EO p, which made up 95.5 % of the total oil. β-caryophyllene (35.1 %) and -humulene (35.3 %) are the main constituents of EO p. The main constituents of EO o are α-humulene (24.8 %), β-caryophyllene (14.3 %), caryophyllene oxide (18.33 %), and humulene epoxide II (16.86 %). The biological activity of both oils was assayed. They were found to be equally active against Staphylococcus aureus and Enterococcus faecalis, with MIC of 100 and 600 µL/mL respectively. This is the first report describing the chemical composition of the essential oil of these species and their antibacterial activity.

KeyWords: antibacterial activity, caryophyllene oxide, Centrolobium paraense, essential oil, humulene epoxide II, α-humulene, β-caryophyllene.

Introduction

The neotropical genus *Centrolobium* Mart. ex Benth (Leguminosae, Papilionoideae) comprises seven trees species: C. *robustum, C. microchaete, C. tomentosum, C. ochroxylum, C. sclerophyllum, C. paraense*, and *C. yavizanum* which grow in Brasil, Bolivia, Ecuador, Peru, Colombia, The Guianas, Panama, Trinidad and Venezuela between 50 and 350 meters above sea level. Some *Centrolobium* species are highly valued because of their enduring and beautiful wood, which has an orange-yellow color with dark red or black stripes[1]. In Venezuela *Centrolobium paraense* is the only *Centrolobium* species that have been described, which is popularly known as "cartán" or "Colorado".

Because its wood is highly appreciated *C. paraense* has been overexploited to the point that it is becoming scarce and it is considered to be in danger of extinction according to the ideas presented on the book "Libro Rojo de la Flora Venezolana"[2]. Since research on this valuable tree is very scarce it was considered convenient to start a research program in order to devise methods to increase its population.

Two varieties have been identified within this species: *C. paraense var. paraense* and *C. paraense var. orinocense.* Taxonomic differences between both varieties are difficult to establish, mainly regarding the shape of the leaves at their base and the amount of indument at their surface[1, 3].

Previous studies on the secondary metabolites present on the wood of *Centrolobium species*, (*C. paraense* included), reported diarylheptanoids and isoflavonoides such as centrolobol, centrolobine and methylcentrolobine[4, 5, 6, 7]. Our laboratory is making an overall study of *C. paraense*, which includes physico-chemical and anatomical aspects of its wood[8, 9]. Since no reports have been found on the chemical composition of the essential oil of the leaves, we have obtained, by hydrodistillation, the essential oils from the leaves of both varieties and compare their compositions. At the same time, their possible biological activity against human pathogens has been tested.

Materials and methods

Plant material

Fresh aerial parts of *C. paraense var. orinocense* and paraense (laminar portion) were collected in May 2014, from plants growing wild in similar environmental conditions near to Upata locality, state Bolívar, Venezuela at 345 m above sea level. Dr. Elio Sanoja made botanical identification. Voucher specimens were deposited at the MERF herbarium, Faculty of Pharmacy, University of Los Andes Merida, Venezuela (Luis Beltrán Rojas 063 and 064 *paraense* and *orinocense* respectively). The extraction conditions were identical for both species.

Isolation of essential oils

Aerial parts of both varieties were collected separately from random points on the trees under investigation. Leaves laminar portion (300 g) from each variety were separated and subjected to hydrodistillation in a Clevenger-type apparatus for 4h. The quantity of essential oil was measured directly in the extraction burette of the apparatus and content (%) was calculated as volume (mL) of essential oil per 100 g of plant material (v/w %). The oil sample was dried over sodium sulfate (Na_2SO_4), and stored in a dark vial at 4ºC until they were analyzed chromatographically.

Gas Chromatography GC

Analyses were performed using a Perkin-Elmer Autosystem gas chromatography equipped with an FID detector and

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data-handling system. A 5 % phenylmethyl polysiloxane fused-silica capillary column was used (30 m x 0.25 mm i.d., film thickness 0.25 m; HP-5, Hewlett-Packard, CA, USA). The oven temperature was programmed from 60 °C to 260 °C, rising at 4 °C/min. The injector and detector temperatures were 200 °C and 280 °C, respectively. The carrier gas was helium at 0.8 mL/min. The sample (1.0 µL) was injected using a split ratio of 10:1. Retention indices were calculated concerning C_8-C_{24} *n*-alkanes. The percentage composition of the oil was calculated by the normalization method from the GC peak areas. The percentage composition of the oil was calculated by the normalization method from the GC peak areas.

Gas chromatography-mass spectrometry

GC-MS analyses were carried out on a Model 5973 Hewlett-Packard GC-MS system fitted with an HP- 5MS fused silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm, Hewlett-Packard). The oven temperature program was the same as that used for the HP-5 column for GC analysis; the transfer line temperature was programmed from 60 °C to 260 °C; rising at 4 °C/min, source temperature, 230 °C; quadrupole temperature 150 °C; carrier gas, helium adjusted to a linear velocity of 34 cm/s; ionization energy, 70 eV; scan range, 40 to 500 amu; 3.9 scans/s. Sample (1.0 µL) was injected using a Hewlett-Packard ALS injector with a split ratio of 50:1. The identity of the oil components was established from their GC retention indices, by comparison of their MS with those of standard compounds available in the laboratory, and by a library search (Nist 05 and Wiley MS Data Library, 6th edn)[10, 11].

Antimicrobial Assay

The antibacterial activity and minimum inhibitory concentration (MIC) were evaluated by the agar disk diffusion method described by Velasco *et al.* (2007)[12]. The bacterial strains used in experiments were as follows: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25992), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (ATCC 23357). Every bacterial inoculum was spread over plates containing Mueller-Hinton agar and a paper filter disc (6 mm) saturated with 10 µL of essential oil and then incubated at 37 ºC for 24 h. Positive control was also assayed to check the sensitivity of the tested organisms using the following antibiotics: Oxacillin®, Vancomycin®, Tobramycin®, Cefepime® and Aztreonam®. 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) by pipetting 10 µL of each dilution onto a filter paper disc. Dilutions of the oil within a concentration range of 10-350 µg/mL were also carried out.

Statistical analysis

Data obtained from antibacterial activity were expressed as mean values. The statistical analyses were carried out employing one way ANOVA, results with P <0.05 were considered to be statistically significant. A statistical package (SPSS version 19.0) was used for the data analysis.

Results and Discussion

The results reported here on the essential oil chemical compositions of these varieties grow in Venezuela are novel, and show an interesting chemical diversification. Gas chromatographic analyses of both essential oils tested showed different composition. The yields of essential oil from *orinocense* (EO_o) and *paraense* (EO_p) were in the range of 0.25 - 0.22 %, respectively. EO_p showed a yellowish oil, and EO_o was a colorless oil. The identified components, percentages composition, and their Retention indices (RI) values listed in order of elution time on the HP 5 capillary column are reported in Table 1. A total of eighteen compounds were identified in the oil of the *orinocense* variety, which represents 88.9% of the oil. In the case of the *paraense* variety only sixteen constituents were identified, which in this case, represented 95.5 % of the total oil.

All the identified compounds from the essential oil from both varieties were sesquiterpenes. The main constituents of the *paraense* variety were β-caryophyllene (35.1 %) and α-humulene (35.1 %), which, with the contribution of other six minor constituents add up to the total sesquiterpene hydrocarbon fraction (77.4 %). Eight minor constituents, which account for 18.0% of the *paraense* variety oil, are oxygenated sesquiterpenes, being nerolidol (4.6 %) the most abundant one. In the oil of the orinocense variety, α-humulene (24.8 %), β-**ββ** (14.3 %) were the most abundant sesquiterpene hydrocarbons, the contribution of other seven minor compounds added up to a total sesquiterpene hydrocarbon fraction of 44.3 %. Oxigenated sesquiterpenoids made, in this case 44.6 % of the oil. Caryophyllene oxide (17.6 %) and humulene epoxide II (16.3 %) were the most abundant oxygenated sesquiterpenes in the oil of the *orinocense* variety, which along with seven other minor oxygenated constituents made a total oxygenated sesquiterpene fraction of 44.7 %.

In spite of this fact, both oils are different because the *paraense* variety contains 77.4 % of hydrocarbon sesquiterpenes and only 18 % of oxygenated sesquiterpenes. On the other hand, the *orinocense* variety oil contains the same proportion of hydrocarbon sesquiterpenes (44.3%) and oxygenated sesquiterpenes (44.6 %). β-caryophyllene and α-humulene, the compounds that dominate the hydrocarbon fraction of both oils, are twice as abundant in the *paraense* variety oil. On the other hand caryophyllene oxide and humulene epoxide II are six times more abundant in the oxygenated fraction of the *orinocence* variety oil than in the paraense variety oil. Finally ɑ-copaene, *cis* calamenene, and Epi-ɑ-Cadinol, which are minor constituents of the *orinocense* variety, are not present in the *paraense* variety oil. On the other hand δ cadinene, a minor constituent of the *paraense* variety oil is absent from the *orinocense* oil.

Table 2 shows the results obtained from the antibacterial evaluation of both essential oils (EO_o and EO_p). The results revealed that the oil possessed antibacterial activity with varying magnitudes towards different strains. The essential oils were active only against *S. aureus* and *E. faecalis* and completely ineffective against *E. coli*, *K. pneumoniae* and *P. aeruginosa*. This behavior is possible because Gram-negative bacteria are less susceptible to essential oils than the Gram-positive strains because the former possess an outer membrane surrounding the cell membrane which restricts the diffusion of hydrophobic compounds through its lipopolysaccharide covering[13].

Biological activity of both oils was statistically superior (P<0.05) on the bacterial growth of *S. aureus* compared with *E. faecalis* with inhibition halos of 13 mm and 8 mm, respectively (Table 2). The MIC value of the oils was 100 µL/mL on *S. aureus* and 600 µL/mL against *E. faecalis*. These results suggest that the oils were more active against *S. aureus*. Although both oils (EO_o and EO_p) possess different chemical composition, the antimicrobial activity of the essential oils did not show statistically significant differences (p=0.841) against *S. aureus* (Ta-

N°	Component	$IR_{Lit.}$	IR_{Exp}	*Centrolobium* varieties	
				EO_o	EO_p
1	Cyclosativene	1368	1372	0.7	0.7
2	α-copaene	1376	1382	0.5	0.6
3	β-caryophyllene	1418	1428	14.3	35.1
4	β-copaene	1430	1437	0.3	-
5	α-humulene	1454	1466	24.8	35.3
6	γ- muurolene	1478	1486	1.2	1.2
7	α-selinene	1494	1496	0.4	0.5
8	α-muurolene	1500	1509	1.7	2.6
9	δ cadinene	1524	1522	-	1.4
10	Cis calamenene	1528	1530	0.5	-
11	Nerolidol	1564	1566	4.4	4.6
12	Caryophyllenyl alcohol	1570	1573	0.34	1.28
13	Caryophylene oxide	1581	1586	17.6	2.9
14	1,5,8,8-tetramethyl-3,7-cycloundecadien-1-ol	1607	1596	0.9	2.3
15	Humulene epoxide II	1608	1610	16.3	2.6
16	Epi-α-Cadinol	1640	1642	0.8	-
17	α-muurolol	1646	1649	1.6	1.3
18	β-eudesmol	1650	1654	1.4	1.2
19	α-eudesmol	1654	1657	1.2	1.8
	Class composition				
	Sesquiterpene hydrocarbons (%)			44.3	77.4
	Sesquiterpene /oxigenate (%)			44.6	18.0
	Total Identified (%)			88.9	95.4

Notes: Retention indices in literature (IR_{Lit}), Retention indices experimental (IR_{Exp}), Essential oil *Centrolobium paraense* var. *orinocense* (EO_o),: Essential oil of *Centrolobium paraense* var. *paraense* (EO_p

Table 1. Chemical composition of the essential oil (%) of two varieties of *Centrolobium paraense* from Venezuela.

Microorganisms	Inhibition zone (mm)*							EO_o MIC µL/mL	EO_p MIC µL/mL
	EO_o	EO_p	Reference compounds						
			OX	VA	TOB	AZT	CEP		
Staphylococcus aureus (ATCC 29923)	13*	13*	23*					100	**100**
Enterococcus faecalis (ATCC 29212)	8*	8*		20*				600	**600**
Escherichia coli (ATCC 25922)	NA	NA			26*			NT	**NT**
Klebsiella pneumoniae (ATCC 23357)	NA	NA				30*		NT	**NT**
Pseudomonas aeruginosa (ATCC 27853)	NA	NA					32*	NT	**NT**

Notes: EO_o: Essential Oil of *Centrolobium paraense* var. *orinocense*, EO_p: Essential Oil of *Centrolobium paraense* var. *paraense*, Oxacillin® (30 µg), VA: Vancomycin® (30 µg), TOB: Tobramycin® (10 µg), AZT: Aztreonam® (30 µg), CEF: Cefepime® (30 µg), NA: Not Active, NT: Not Tested, *inhibition zone, diameter measured in mm, disc diameter 6 mm, average of two consecutive trials. MIC: Minimal Inhibitory Concentration of the essential oil, concentration range: 10-700 µL/mL.

Table 2. Antibacterial activity of essential oils of two varieties of *Centrolobium paraense* from Venezuela.

Bacterium	Analysis of variances (ANOVA)				Turkey's multiple comparisons			
	Sum of squares	Mean squares	*F*	*Sig.*	Compound		Mean difference	*Sig.*
S.aureus								
Inter-groups					EO$_a$	EO$_b$	0.250	0.841
Intra-groups	287.167	143.583	369.214	0.000		Ox	-10.250*	0.000
					EO$_b$	EO$_a$	-0.250	0.841
Total	3.500	0.389				Ox	-10.500*	0.000
					Ox	EO$_a$	10.250*	0.000
	290.667					EO$_b$	10.500*	0.000
E.faecalis								
Inter-groups					EO$_a$	EO$_b$	-0.2500	0.674
Intra-groups	360.500	180.250	1081.50	0.000		Va	-11.75*	0.000
					EO$_b$	EO$_a$	0.2500	0.674
Total	1.500	0.167				Va	-11.50*	0.000
					Va	EO$_a$	11.750*	0.000
	362.000					EO$_b$	11.500*	0.000

Notes: EO_o: Essential Oil of *Centrolobiumparaense* var. *orinocense*, EO_p: Essential Oil of *Centrolobiumparaense* var. *paraense*. Ox (Oxacillin®), Va (Vancomycin®). (*)The difference in means is significant at the level of *Sig.* p= 0.05.

Table 3. ANOVA and Turkey's multiple comparisons of biological activity of essential oils of *Centrolobium* (EO_o, EO_p), against *S. aerus* and *E. faecalis*.

ble 3). Similar behavior was observed on *E. faecalis* (*P*=0.674). The statistical analysis indicated that the inhibitory effect of the essential oils against the two bacteria tested was in most cases not as strong as that of the reference compounds. (Oxacillin® and Vancomycin® respectively).

The abundance, interaction mechanism, and presence of several reactive chemical components in the essential oils of both varieties, could have applications in the pharmaceutical and chemical industries. Previous investigations have shown that individual compounds detected in the essential oil of C. paraense show important biological activity. For example, β-caryophyllene has anti-inflamatory, anticarcinogenic, antibiotic, antioxidant, and local anesthetic activities[14]. The α-humulene showed anticancer activity[15], anti-inflammatory effects[16] and mixed with con β-caryophyllene increases its cytotoxicity against human tumour cell lines in vitro[14]. Nerolidol has an impact on the protein prenylation, and it can reduce adenomas in rats[17]. Caryophyllene oxide in combination with other sesquiterpenes showed higher anticancer activity against several cancer cell lines as human lung carcinoma, human colon adenocarcinoma, human leukemia cancer, human cervical adenocarcinoma, human gastric cancer, and human stomach cancer[18, 19, 20]. The specific antibacterial activity of both oils on the tested microorganisms could be attributed to its high sesquiterpene content and the synergistic and antagonistic effects of these compounds. However, further studies are needed to obtain a better understanding of their biological activity.

Conclusions

This article is the first report on the differences in the chemical composition of the essential oil of these two varieties of *Centrolobium paraense* and their antibacterial activity, information that could be used with chemotaxonomic purposes in this genus. Results suggest that essential oils from both varieties of *C. paraense* could contribute to the control of infections caused by *S. aureus* and *E. faecalis*.

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