J. Braz. Chem. Soc., Vol. 18, No. 1, 184-188, 2007.
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0103 - 5053 $6.00+0.00

Article

Phytochemical and Antibacterial Evaluation of Essential Oils from *Ottonia Martiana* Miq. (Piperaceae)

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Três óleos essenciais extraídos das folhas, frutos e raízes de *Ottonia martiana* Miq. (Piperaceae), espécie comum da floresta Atlântica brasileira, e conhecida popularmente por "anestésia", foram analisados por CG-EM e submetidos a um ensaio antibacteriano bioautográfico frente a *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Pseudomonas aerogenes* (ATCC 27853) e *Escherichia coli* (ATCC 25922). Setenta e sete compostos foram identificados e submetidos a uma análise comparativa, a qual revelou uma variabilidade no teor dos componentes majoritários desses óleos (espatulenol, óxido de cariofileno, (*E*)-nerolidol, viridiflorol, β-cariofileno, δ cadineno e aloaromadendreno). A presença de zonas de inibição de crescimento bacteriano nos bioautogramas analisados (R*fs* 0,29 e 0,34) revelou o potencial antibacteriano dos óleos analisados frente às bactérias Gram-positivas testadas e permitiu identificar alguns dos componentes bioativos.

Three essential oils extracted from leaves, fruits and roots of *Ottonia martiana* Miq. (Piperaceae), common species in Brazilian Rain Forest, known as "anestésia", were analyzed by GC-MS and tested in an antibacterial assay against *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Pseudomonas aerogenes* (ATCC 27853) and *Escherichia coli* (ATCC 25922). Seventy-seven compounds were identified and submitted to a comparative analysis, which revealed variability on the amount of principal components of these oils (spathulenol, caryophyllene oxide, (*E*)-nerolidol, viridiflorol, β-caryophyllene, δ cadinene and alloaromadendrene). Inhibition zones of bacterial growth in the bioautograms (R*fs* 0.29 and 0.34) showed antimicrobial activity of essential oils against tested Gram-positive bacteria and permitted to identify some bioactive components.

Keywords: antimicrobial activity, essential oil composition, Gram-positive bacteria, *Ottonia martiana*

Introduction

Actually, research of healthy habits for world population has promoted a progressive increase on production and consumption of natural products, as the case of essential oils from plants, source of bioactive substances.[1]

Chemical investigation of Piperaceae essential oils has revealed the presence of monoterpenes, sesquiterpenes and arylpropanoids that have shown interesting biological properties including psychotropic, antimicrobial, antioxidant and cytotoxic effects stimulating studies on plants within this family.[2]

Among Piperaceae species of medicinal interest, there is *Ottonia martiana* Miq, a characteristic shrub of the Brazilian Rain Forest[3,4] and known as "anestésia" for the natives of the Paraná coast due to its anesthetically action on the oral mucous.[5,6]

Phytochemical investigations with roots and aerial parts of "anestésia" revealed some isobutylamides (piperlonguminine, isopiperlonguminine and piperovatine), compounds of notable biological properties.[7-9]

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No chemical data on essential oil of *O. martiana* has been found, but two papers discussing on essential oils constitution of other species from genus *Ottonia* have been previously published.[10,11]

This work relates a phytochemical investigation on essential oils extracted from leaves, fruits and roots of *O. martiana* and their antibacterial activity.

Experimental

General

All reagents were of analytical grade. Column chromatography and TLC were performed on silica gel 60 F_{254} (Merck). Mass spectra was determined with a Varian 3800 Series GC-EIMS (Varian SATURN 2000), 70 eV, capillary column CP-SIL PONA CB (100 m × 0.25 mm I.D. × 0.25 μm film thickness) with helium as the carrier gas at a flow rate of 1.6 mL min^{-1}; the temperature program was 140 °C (10 min) and increased at a rate of 5 °C min^{-1} to 230 °C, standing 25 min at this temperature; injection in the split mode (1:200) at an injector, temperature of 200 °C; detector temperature at 200 °C; injection volume, 0.5 μL. Individual components were identified by comparison of both mass spectrum and their GC retention times (RT) and retention indices (RI) with those of authentic compounds previously analyzed and stored in the data system or existing in the literature.[10-12] The NIST (National Institute for Standard Technology – 62.235 compounds) was used for comparison of mass spectra. The retention indices were calculated for all volatile constituents using a homologous series (C_9 to C_{20}) recorded under the same operating conditions. Retention indices (RI) have been obtained according to the method of Van den Dool.[13] The quantitative data were obtained by electronic integration of the GC-FID peak areas.

The bacterial strains were provided by the Laboratory of Basic Pathology-Universidade Federal do Paraná. Tests were carried out in duplicate with strains of *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Pseudomonas aerogenes* (ATCC 27853) and *Escherichia coli* (ATCC 25922). New cultures of each strain were standardized in sterile saline solution following 0.5 MacFarland scale. Miller Hinton Agar (Difco Laboratories) was used for culture medium.

Plant material

Roots and aerial parts (leaves and fruits) of *O. martiana* were collected in February 2003, in Paraná State, Southern region of Brazil. The botanist Dr. Gerdt Hatschbach of the Museu Botânico Municipal - Prefeitura de Curitiba, Paraná (MBM) identified the plant. Voucher specimens are deposited at the Herbarium of the MBM under number 259057.

Extraction of the oils

Essential oils of leaves (188.4 g) and fruits (30.0 g) from *O. martiana* were obtained by hydrodistillation for four hours in a modified Clevenger-type apparatus, after cooling to 0 °C.

The essential oil of roots was obtained in previously phytochemical investigation. The air-dried and powdered roots of *O. martiana* (470.0 g) were extracted with 95% ethanol in a Soxhlet for 5 h at hot stage (470 g / 3 L). The obtained filtered material, after removal of the ethanol under reduced pressure was partitioned with 250 mL of hexane, dichloromethane, ethyl acetate and methanol, respectively. The fraction hexane (4.0 g) obtained after evaporation of the solvent under vacuum at 40 ºC, was submitted to column cromatography on a column of silica gel and developed with solvent mixtures of increasing polarity at 5% (hexane, hexane/ethyl acetate, ethyl acetate/ methanol and methanol), yielding 0.1 mL of the colorless essential oil (0.02%) correspondent to the grouped subfractions 5-13.

Antimicrobial activity assay

The bioautography method,[14] adapted from Hostettmann[15] was based on the application of 3 μL of the test oils on a TLC plate. The samples were first applied on a TLC plate GF_{254} (2.5 × 5.0 cm) and developed with toluene/EtOAc (97:3), transferred to a Petri dish, covered with Miller Hinton Agar containing triphenyltetrazolium chloride (TTC – 1%) and inoculated with bacterial strains previously standardized. Inhibition zones after 24 h of incubation (37 ºC) indicated the presence of active compounds. Chloramphenicol (Newprov - 3 μL) was used as positive control of growth inhibition.

MIC tests were carried out according to Eloff,[16] using a tissue culture testplate (96 wells). The stock solutions of the oils were diluted and transferred into the first well (100 μL), and serial dilutions were performed so that concentrations in the range of 1 - 0.001 mg mL^{-1} were obtained. Chloramphenicol (Merck) was used as the reference antibiotic control. The inoculum was added to all wells and the plates were incubated at 37 °C during 24 h. Antimicrobial activity was detected by adding 20 μL of 1% TTC aqueous solution. MIC was defined as the lowest concentration of oil that inhibited visible growth.

Results and Discussion

The oils were obtained from dried plant material yielding 0.21% (leaves), 0.33% (fruits) and 0.02% (roots).

A total of 77 compounds were identified, accounting for 68.22 to 78.87% of the constituents. The identification of the constituents was performed by computer library search, retention indices (RI) and visual interpretation of the mass spectra.[12] Results obtained for the qualitative and quantitative analysis of these essential oils are shown in Table 1.

The three oils showed some differences in their main constituents. The major components of root oil were spathulenol (17.83%), (*E*)-nerolidol (10.33%), viridiflorol (7.53%), caryophyllene oxide (6.41%), δ-cadinene (4.16%), myrcene (4.07%), α-copaene (3.86%), β-caryophyllene (3.21%), allaromadendrene (3.11%) and α-pinene (2.10%). The fruit oil was characterized by its high content of spathulenol (17.37%), (*E*)-nerolidol (9.14%), viridiflorol (7.38%), caryophyllene oxide (5.77%), δ-cadinene (3.90%), myrcene (3.84%), α-copaene (3.69%), β-caryophyllene (3.07%), alloaroma-

Table 1. Essential oils composition from *Ottonia martiana* Miq., Piperaceae

	Compounds	Formula	MW	Rt/min	RI	Leaves/(%)	Fruits/(%)	Roots/(%)
01	α-Thujene	$C_{10}H_{16}$	136	13.205	929	< 0.01	0.02	0.02
02	α-Pinene	$C_{10}H_{16}$	136	13.703	941	1.30	2.01	2.10
03	6-Methyl-5-hepten-2-one	$C_8H_{14}O$	126	14.431	958	0.02	0.54	0.56
04	Camphene	$C_{10}H_{16}$	136	14.448	958	0.02	0.04	0.05
05	Sabinene	$C_{10}H_{16}$	136	15.174	975	0.50	< 0.01	< 0.01
06	Myrcene	$C_{10}H_{16}$	136	15.407	981	1.01	3.84	4.07
07	β-Pinene	$C_{10}H_{16}$	136	15.656	987	0.37	0.72	0.76
08	α-Phellandrene	$C_{10}H_{16}$	136	16.734	1007	0.01	< 0.01	< 0.01
09	δ-3-Carene	$C_{10}H_{16}$	136	17.271	1015	< 0.01	0.03	0.03
10	α-Terpinene	$C_{10}H_{16}$	136	17.420	1017	< 0.01	< 0.01	< 0.01
11	*p*-Cymene	$C_{10}H_{14}$	134	17.537	1018	0.22	0.42	0.45
12	Limonene	$C_{10}H_{16}$	136	18.203	1028	0.37	0.59	0.63
13	β-phellandrene	$C_{10}H_{16}$	136	18.335	1030	0.04	0.08	0.09
14	*cis*-Ocimene	$C_{10}H_{16}$	136	18.655	1034	< 0.01	0.02	0.02
15	γ-Terpinene	$C_{10}H_{16}$	136	20.021	1053	0.01	0.02	0.02
16	(*Z*)-Linalool oxide	$C_{10}H_{18}O_2$	170	20.791	1064	< 0.01	0.21	0.21
17	*p*-Cymenene	$C_{10}H_{22}$	132	22.000	1081	< 0.01	0.03	0.03
18	Linalool	$C_{10}H_{18}O$	154	22.379	1086	0.06	0.19	0.19
19	α-Campholenal	$C_{10}H_{16}O$	152	24.471	1120	< 0.01	0.20	0.21
20	*trans*-Pinocarveol	$C_{10}H_{16}O$	152	25.778	1143	0.02	0.14	0.14
21	*trans*-Verbenol	$C_{10}H_{16}O$	152	25.832	1144	0.01	0.13	0.13
22	Pinocarvone	$C_{10}H_{14}O$	150	26.620	1158	< 0.01	0.03	0.03
23	*p*-Cymen-8-ol	$C_{10}H_{14}O$	150	27.342	1172	< 0.01	0.01	0.01
24	Terpinen-4-ol	$C_{10}H_{18}O$	154	27.643	1177	< 0.01	0.08	0.02
25	α-Terpineol	$C_{10}H_{18}O$	154	28.085	1185	< 0.01	0.03	0.05
26	Myrtenal	$C_{10}H_{14}O$	150	28.176	1187	0.03	0.02	0.03
27	Myrtenol	$C_{10}H_{16}O$	152	28.423	1191	0.02	0.03	0.04
28	Verbenone	$C_{10}H_{14}O$	150	28.718	1196	< 0.01	0.05	0.06
29	*trans*-Carveol	$C_{10}H_{16}O$	152	29.150	1206	< 0.01	0.03	0.05
30	Nerol (*cis*-geraniol)	$C_{10}H_{18}O$	154	29.508	1215	0.01	0.04	0.06
31	*p*-Anisaldehyde	$C_8H_8O_2$	136	29.961	1227	< 0.01	0.06	0.02
32	Carvone	$C_{10}H_{14}O$	150	30.066	1229	0.01	0.05	0.01
33	*trans*-Geraniol	$C_{10}H_{18}O$	154	30.424	1238	0.05	0.06	0.07
34	Piperitone	$C_{10}H_{16}O$	152	30.615	1243	0.06	0.02	0.05
35	Geranial (*a*-citral)	$C_{10}H_{16}O$	152	30.829	1249	< 0.01	0.01	0.01
36	*N*-decanol	$C_{16}H_{32}O$	256	31.093	1256	< 0.01	0.07	0.01
37	α-Terpinen-7-al	$C_{10}H_{14}O$	150	31.307	1261	0.06	0.50	0.01
38	*cis*-Verbenyl acetate	$C_{12}H_{18}O_2$	194	31.425	1264	< 0.01	0.01	0.01
39	Indole	C_8H_7N	117	31.471	1265	< 0.01	0.01	0.01
40	*p*-Cymen-7-ol	$C_{10}H_{14}O$	150	31.716	1271	0.06	0.01	0.02
41	Tymol	$C_{10}H_{14}O$	150	32.012	1279	0.07	0.01	< 0.01
42	Perilla alcohol	$C_{10}H_{16}O$	152	32.329	1287	< 0.01	0.01	< 0.01
43	*N*-tridecane	$C_{13}H_{28}$	184	32.861	1301	0.07	0.02	< 0.01
44	Piperonal	$C_8H_6O_3$	150	33.000	1305	< 0.01	0.03	< 0.01
45	δ-Elemene	$C_{15}H_{24}$	204	34.439	1351	< 0.01	0.60	0.10
46	α-Cubebene	$C_{15}H_{24}$	204	34.886	1366	0.48	0.28	0.30
47	Isoledene	$C_{15}H_{24}$	204	35.593	1388	< 0.01	< 0.01	< 0.01

Table 1. cont.

	Compounds	Formula	MW	Rt/min	RI	Leaves/(%)	Fruits/(%)	Roots/(%)
48	α-Ylangene	$C_{15}H_{24}$	204	35.714	1392	< 0.01	< 0.01	< 0.01
49	Cyclosativene	$C_{15}H_{24}$	204	35.782	1394	< 0.01	0.03	0.04
50	α-Copaene	$C_{15}H_{24}$	204	35.858	1397	4.41	3.69	3.86
51	β-Elemene	$C_{15}H_{24}$	204	36.108	1405	0.95	1.28	1.35
52	β-Bourbonene	$C_{15}H_{24}$	204	36.185	1408	< 0.01	0.18	0.18
53	Longifolene	$C_{15}H_{24}$	204	36.854	1432	0.12	0.03	0.09
54	α-Gurjunene	$C_{15}H_{24}$	204	36.977	1436	0.59	0.41	0.43
55	β-Caryophyllene	$C_{15}H_{24}$	204	37.276	1447	12.61	3.07	3.21
56	*epi*-bicyclosesquiphellandrene	$C_{15}H_{24}$	204	37.468	1454	0.88	0.35	0.35
57	β-Gurjunene	$C_{15}H_{24}$	204	37.732	1463	1.49	0.79	0.83
58	Aromadendrene	$C_{15}H_{24}$	204	37.880	1468	0.14	0.11	0.12
59	α-Humulene	$C_{15}H_{24}$	204	38.250	1482	0.31	0.57	0.76
60	Alloaromadendrene	$C_{15}H_{24}$	204	38.475	1490	3.15	2.97	3.11
61	γ-Muurolene	$C_{15}H_{24}$	204	38.596	1494	2.51	0.32	0.34
62	Germacrene D	$C_{15}H_{24}$	204	38.815	1501	0.19	0.47	0.49
63	γ-Cadinene	$C_{15}H_{24}$	204	38.915	1505	0.21	0.59	0.62
64	Valencene	$C_{15}H_{24}$	204	39.197	1515	0.21	0.15	0.15
65	*epi*- Cubebol	$C_{15}H_{26}O$	222	39.230	1517	0.33	1.17	1.21
66	Viridiflorene (ledene)	$C_{15}H_{24}$	204	39.316	1520	0.38	0.22	0.27
67	α-Selinene	$C_{15}H_{24}$	204	39.388	1523	0.83	0.46	0.47
68	*cis*-Calamenene	$C_{15}H_{22}$	202	39.705	1534	0.10	0.12	0.13
69	δ-Cadinene	$C_{15}H_{24}$	204	39.777	1537	3.74	3.90	4.16
70	(*E*)-Nerolidol	$C_{15}H_{26}O$	222	40.278	1555	8.18	9.14	10.33
71	Elemol	$C_{15}H_{26}O$	222	40.396	1559	0.48	0.82	0.85
72	Ledol	$C_{15}H_{26}O$	222	41.425	1597	0.19	0.76	0.70
73	Spathulenol	$C_{15}H_{24}O$	220	41.609	1603	8.59	17.37	17.83
74	Globulol	$C_{15}H_{26}O$	222	41.813	1610	0.44	0.44	0.43
75	Caryophyllene oxide	$C_{15}H_{24}O$	220	41.949	1614	7.37	5.77	6.41
76	Viridiflorol	$C_{15}H_{26}O$	222	42.637	1636	2.75	7.38	7.53
77	Cubenol	$C_{15}H_{26}O$	222	43.104	1651	2.19	2.35	1.94
	Total identified					68.22	75.77	78.87

The identified contituents are listed in their order of elution from a nonpolar column (CP-SIL PONA CB); Rt - retention times; RI - retention indices (experimental data).

dendrene (2.97%), cubenol (2.35%) and α-pinene (2.01%). The most important constituents in leaf oil were β-caryophyllene (12.61%), spathulenol (8.59%), (*E*)-nerolidol (8.18%), caryophyllene oxide (7.37%), α-copaene (4.41%), δ-cadinene (3.74%), alloaromadendrene (3.15%), viridiflorol (2.75%) and γ-muurolene (2.51%). Several compounds of the oils remained unidentified.

The comparative analysis between oils showed variability in chemical constitution related to content of their principal components in different organs (Figure 1).

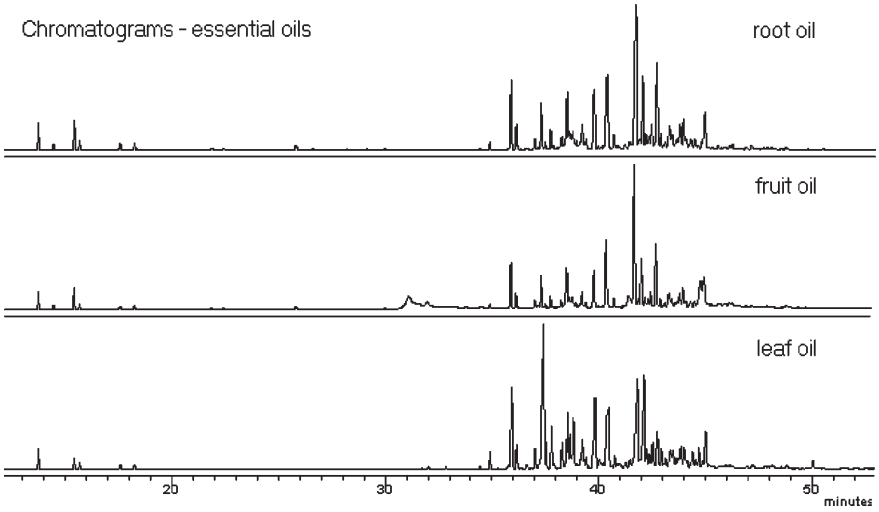

Figure 1. Chromatograms of GC-MS analysis of essential oils from the *Ottonia martiana.*

Table 2. Antibacterial activity of essential oils from *Ottonia martiana* on four bacterial species

Microrganisms(Bacteria)	Gram	Inhibition zones (IZ) (Bioautography)					
		leaves 3 μL		fruits 3 μL		roots 3 μL	
		IZ	R*fs*	IZ	R*fs*	IZ	R*fs*
Staphylococcus aureus (ATCC 25923))	Positive	++	0.29 and 0.34	++	0.29 and 0.34	++	0.29 and 0.34
Staphylococcus epidermidis (ATCC 12228)	Positive	++	0.29 and 0.34	++	0.29 and 0.34	++	0.29 and 0.34
Pseudomonas aerogenes (ATCC 27853)	Negative	-	-	-	-	-	-
Escherichia coli (ATCC 25922)	Negative	-	-	-	-	-	-

ATCC (American Type Culture Collection); + = inhibition zone; - = no inhibition zone.

Compounds present in oils indicated a preference of *Ottonia* to synthesize sesquiterpenes and the commonest have the *E,E*-farnesyl-PP as fundamental precursor.[10] Many described compounds as components of essential oils of *O. anisum* and *O. corcovadensis*[10,11] also were detected during analysis of this research. This is the first report over a detailed composition of the essential oil present in roots, leaves and fruits of *O. martiana*.

Bioautography assay permitted to detect and identify bioactive compounds of *O. martiana* against all Gram-positive bacteria tested (Table 2).

The bioautography method directioned isolation of the bioactive compounds towards their identification as α-pinene (R*f* 0.29), β-pinene (R*f* 0.29), myrcene (R*f* 0.29), α-copaene (R*f* 0.34), β-caryophyllene (R*f* 0.34) and δ-cadinene (R*f* 0.34). Some of these compounds are previously known for its antimicrobial activity.[17,18] The relative composition of each inhibition zone was characterised using GC-MS after preparative TLC. The inhibition zone with R*f* 0.34 also contained smaller quantities of α-cubebene, β-elemene, valencene, α-gurjunene, *epi*-bicyclosesquiphellandrene and germacrene D.

The MIC (Minimal Inhibitory Concentration) was determined for oils that presented positive results on bioautographic assays. The oils showed an activity against *S. aureus* with the MIC of 9 μg mL^{-1} (leaf oil), 5 μg mL^{-1} (fruit oil) and 5 μg mL^{-1} (root oil). This oils also showed an activity against *S. epidermidis* with the MIC of 9 μg mL^{-1} (leaf oil), 5 μg mL^{-1}(fruit oil) and 5 μg mL^{-1} (root oil).

These results indicated an effective *in vitro* activity of the essential oils from *O. martiana* and encourage further studies for its application in antibiotic therapy of infectious diseases.

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Received: May 5, 2005
Web Release Date: January 24, 2007