

SUPPLEMENTARY MATERIAL

Antimycoplasmic activity and seasonal variation of essential oil of *Eugenia hiemalis* Cambess. (Myrtaceae)

Gabriele Andressa Zatelli^a, Priscila Zimath^b, Adrielli Tenfen^b, Caio Maurício Mendes de Cordova^b, Dilamara Riva Scharf^c, Edésio Luiz Simionatto^c, Michele Debiasi Alberton^b, Miriam de Barcellos Falkenberg^{a*}

^a*Programa de Pós-graduação em Farmácia, Universidade Federal de Santa Catarina, Rua Delfino Conti s/n, CEP 88040-900, Florianópolis, SC, Brazil*

^b*Departamento de Ciências Farmacêuticas, Universidade Regional de Blumenau, Rua São Paulo, 2171, CEP 89030-000, Blumenau, SC, Brazil*

^c*Laboratório de Cromatografia, Universidade Regional de Blumenau, Rua São Paulo, 3250, CEP 89030-000, Blumenau, SC, Brazil*

*Corresponding author. Address: CIF, CCS, UFSC, Rua Delfino Conti s/n. Florianópolis-SC, Brazil. CEP 88040-900. E-mail: miriam.falkenberg@ufsc.br
Telephone: +55 48 3721-5076.

Abstract

The purpose of this work was to study the chemical composition and antimycoplasmic and anticholinesterase activity of the essential oil of *Eugenia hiemalis* leaves collected throughout the year. A total of 42 compounds were identified by CG, and are present in almost every seasons. Sesquiterpenes were dominant (86.01 - 91.48 %), and non-functionalized sesquiterpenes comprised the major fraction, which increased in the summer; monoterpenes were not identified. The major components were spathulenol (5.36 - 16.06 %), δ -cadinene (7.50 - 15.93 %), bicyclogermacrene (5.70 - 14.24 %), and β -caryophyllene (4.80 - 9.43 %). The highest oil yield was obtained in summer and autumn. Essential oils presented activity against three evaluated *Mycoplasma* strains, but no activity was observed in the anticholinesterase assay.

Keywords: *Eugenia hiemalis*; essential oil; seasonal variation; *Mycoplasma*; antibacterial

1. Experimental

1.1. Chemicals

All chemicals were of analytical grade. Acetylcholinesterase (AChE) type VI-S, from electric eel (lyophilized powder, 411 U mg⁻¹ protein), 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB, ≥ 99 %), acetylthiocholine iodide (AChI, ≥ 98 %), galanthamine hydrobromide (≥ 96 %), tris[hydroxymethyl]aminomethane (Tris buffer), bovine serum albumin (BSA, ≥ 96 %) and dimethylsulfoxide (DMSO) were acquired from Sigma-Aldrich® (Steinheim, Germany).

1.2. Plant material

Leaves of *E. hiemalis* were collected in July and December 2013, and March and May 2014 in Blumenau (Santa Catarina, Brazil) (26°54'19.65"S, 49°04'44.00"W) for extraction of essential oil. Sample was identified by Morilo J. Rigon Jr (Fundação Universidade Regional de Blumenau). Voucher specimen was deposited at Herbarium Roberto Miguel Klein (Blumenau) under the number 41952.

1.3. Extraction procedure

Essential oil of leaves of *E. hiemalis* was obtained by hydrodistillation for 4 h in Clevenger-type apparatus. After extraction, the oils were dried with sodium sulfate and stored at a low temperature.

1.4. Analysis of quantitative and qualitative composition of the essential oils

The qualitative analysis were performed by gas chromatography coupled with mass spectrometry (GC-MS) in a Shimadzu® GCMS-QP2010 Plus chromatograph (nonpolar capillary column RTX®-5MS: 30 m x 0.25 mm x 0.25 µm), and the quantitative analysis by gas chromatography coupled with flame ionization detector (GC-FID) in a Shimadzu® GC-FID2010 chromatograph (nonpolar capillary column OV®-5: 30 m x 0.25 mm x 0.25 µm), being this the quantitative analysis performed in triplicate. The oven temperature program was 60 °C for 5 min, with increase of 3 °C per min until 240 °C remaining in this temperature for 5 min. Helium was used as carrier gas (flow rate of 1 mL min⁻¹), with the temperature of the injector of 250 °C (1:20 split) and an ion source of 70 eV on the MS, with interface at 280 °C.

Identification of the essential oil components was based upon their retention indexes (in comparison with an homologous series of alkanes from C₈ to C₁₉), and by comparison of these and their mass spectral patterns with those reported in the literature (Adams 2007) and stored in the MS library NIST 2008 (National Institute for Standards and Technology) database.

The quantitative variation of the constituents of the essential oils in the seasons was analysed using descriptive statistics, where the coefficient of variation was calculated for each compound. The compounds whose concentration could not be measured were considered as traces and for purposes of calculation equal to 0 %. The coefficient of variation was calculated for the compounds present in at least two

samples. Climatic data for the geographic region were provided by CIRAM/EPAGRI (Florianópolis-SC) and used for qualitative correlations with phytochemicals.

1.5. Evaluation of antimycoplasmic activity

Tests were performed against *Mycoplasma capricolum* subsp. *capricolum* (ATCC 27343), *M. genitalium* (ATCC 33530), *M. hominis* (ATCC 23114) and *M. pneumoniae* FH (ATCC 13883). The Laboratory of Clinical Microbiology from Universidade Regional de Blumenau (FURB) provided the mollicutes strains.

For the growth of bacterial strain, SP4 broth was used to *Mycoplasma capricolum* subsp. *capricolum*, *M. genitalium* and *M. pneumoniae* FH (Razin & Tully 1995), and MLA broth to *M. hominis* (Velleca et al. 1979).

The antimycoplasmic activity of the essential oils was evaluated by determination of the minimum inhibitory concentration (MIC). The microdilution broth assay was performed in sterile 96-well microplates (CLSI 2011; Bébéar & Robertson 1996).

The essential oils were properly prepared and transferred to each microplate well with the appropriate culture medium, in order to obtain a twofold serial dilution of the original extract in a 10 % H₂O/dimethyl sulfoxide (DMSO) solution. The inoculum containing 10⁴ to 10⁵ microorganisms per mL were then added to each well. A number of wells were reserved in each plate to test for sterility control (no inoculum added), positive control (levofloxacin), inoculum viability (no extract added), and the DMSO inhibitory effect.

The microplates were incubated at 37 ± 1 °C for 48 or 72 hours (the latter one for *M. pneumoniae* FH). Thereafter, growth of mollicutes strains was detected by observing the colour change in the medium. The MIC was defined as the lowest concentration of the essential oil able to inhibit bacterial growth.

1.6. Determination of anticholinesterase activity

The enzymatic activity was measured using the method described by Mata et al. (2007). Briefly, 325 µL of 50 mM Tris-HCl buffer (pH 8), 100 µL of a buffer solution of sample (0.1 mg mL⁻¹ in methanol) and 25 µL of AChE solution containing 0.28 U mL⁻¹ (50 mM Tris-HCl, 0.1% BSA) were incubated for 15 min. Subsequently, 75 µL of a solution of AChI (0.023 mg mL⁻¹ in water) and 475 µL of DTNB (3 mM in Tris-HCl) were added and the final mixture incubated for another 30 min at room temperature. Absorbance of the mixture was measured at 405 nm. A control mixture was prepared, using 100 µL of methanol instead of sample. Inhibition (%) was calculated as follows: I (%) = 100 - (A_{sample}/A_{control}) x 100; where A_{sample} and A_{control} are the absorbance of the respective terms. Tests were carried out in triplicate. The sample concentration providing 50% inhibition (IC₅₀) was obtained by plotting the inhibition percentage against the sample solution concentrations. Galanthamine was used as the positive control.

Table S1. Identified compounds (%) from the essential oils of leaves from *E. hiemalis* collected in the four seasons.

Compounds	Rt ^a	Relative Concentration (%)				AI ^b		CV ^e
		Winter	Spring	Summer	Autumn	E ^c	L ^d	
1-octen-3-ol	9.05	5.19 ± 0.33	4.92 ± 0.05	5.56 ± 0.19	t	974	979	67
α-cubebene	26.21	0.40 ± 0.04	0.58 ± 0.01	0.75 ± 0.01	0.24 ± 0.03	1345	1348	45
α-ylangene	27.16	0.24 ± 0.02	0.33 ± 0.02	0.35 ± 0.01	0.24 ± 0.11	1373	1370	20
α-copaene	27.36	3.53 ± 0.08	5.28 ± 0.02	5.64 ± 0.04	2.22 ± 0.03	1374	1374	38
β-bourbonene	27.74	0.58 ± 0.03	0.78 ± 0.04	0.48 ± 0.01	0.26 ± 0.04	1387	1383	41
α-gurjunene	28.82	0.32 ± 0.02	0.63 ± 0.02	0.90 ± 0.01	0.30 ± 0.01	1409	1408	53
β-caryophyllene	29.24	6.19 ± 0.01	9.43 ± 0.03	9.18 ± 0.02	4.80 ± 0.09	1417	1418	31
β-copaene	29.63	1.57 ± 0.04	1.86 ± 0.05	2.21 ± 0.01	1.42 ± 0.01	1430	1427	20
Aromadendrene	30.04	3.43 ± 0.15	4.10 ± 0.05	4.69 ± 0.02	2.91 ± 0.16	1439	1437	21
α-guaiene	30.23	0.30 ± 0.01	0.35 ± 0.05	0.34 ± 0.01	0.28 ± 0.07	1437	1442	10
α-humulene	30.65	1.59 ± 0.03	2.10 ± 0.01	2.23 ± 0.02	1.70 ± 0.06	1452	1451	16
Allo-aromadendrene	30.96	0.41 ± 0.09	0.50 ± 0.03	1.15 ± 0.01	0.44 ± 0.02	1458	1459	56
Trans-cadina-1(6),4-diene	31.50	0.28 ± 0.02	0.39 ± 0.03	0.48 ± 0.01	0.44 ± 0.00	1475	1472	22
γ-murolene	31.67	4.39 ± 0.07	4.80 ± 0.04	4.78 ± 0.07	5.27 ± 0.04	1478	1475	7
Germacrene D	31.79	1.15 ± 0.02	4.61 ± 0.03	4.02 ± 0.07	1.87 ± 0.03	1484	1479	57
β-selinene	32.01	0.24 ± 0.01	0.25 ± 0.01	0.21 ± 0.01	0.25 ± 0.02	1489	1484	8
Trans-muurolo-4(14),5-diene	32.23	0.27 ± 0.00	0.31 ± 0.00	0.33 ± 0.01	0.32 ± 0.01	1493	1489	9
Bicyclogermacrene	32.42	5.70 ± 0.08	12.75 ± 0.09	14.24 ± 0.04	9.37 ± 0.02	1500	1494	36
α-murolene	32.60	1.30 ± 0.11	1.44 ± 0.06	1.64 ± 0.02	1.72 ± 0.05	1500	1499	13
Trans-β-guaiene	32.88	0.26 ± 0.01	0.37 ± 0.03	0.50 ± 0.01	0.37 ± 0.02	1502	1503	26
γ-cadinene	33.14	2.69 ± 0.04	3.52 ± 0.03	3.20 ± 0.02	3.86 ± 0.12	1513	1511	15
δ-cadinene	33.54	10.74 ± 0.07	7.50 ± 0.04	13.06 ± 0.07	15.93 ± 0.12	1522	1521	30
Trans-cadina-1,4-diene	33.87	0.38 ± 0.00	0.39 ± 0.03	0.62 ± 0.02	0.65 ± 0.04	1533	1530	28
α-cadinene	34.07	0.41 ± 0.01	0.42 ± 0.01	0.46 ± 0.00	0.56 ± 0.03	1537	1535	15
α-calacorene	34.29	0.82 ± 0.03	0.74 ± 0.03	0.53 ± 0.01	0.91 ± 0.06	1544	1541	22
Germacrene B	34.91	0.33 ± 0.05	0.50 ± 0.03	0.42 ± 0.01	0.75 ± 0.02	1559	1557	36
Maaliol	35.24	0.86 ± 0.04	0.76 ± 0.03	0.67 ± 0.01	0.96 ± 0.02	1566	1565	15
Spathulenol	35.67	16.06 ± 0.31	9.43 ± 0.02	5.36 ± 0.03	12.47 ± 0.14	1577	1577	42
Globulol	35.90	3.87 ± 0.60	3.62 ± 0.04	3.27 ± 0.05	5.26 ± 0.10	1590	1583	22
Viridiflorol	36.20	1.41 ± 0.04	1.20 ± 0.02	1.03 ± 0.02	1.51 ± 0.05	1592	1590	17
Cubeban-11-ol	36.28	0.27 ± 0.03	0.23 ± 0.01	0.23 ± 0.00	0.38 ± 0.04	1595	1593	26
Rosifolol	36.60	1.48 ± 0.02	1.10 ± 0.03	0.97 ± 0.01	1.58 ± 0.10	1600	1600	23
Humulene epoxide II	36.90	0.57 ± 0.02	0.26 ± 0.01	-	-	1608	1607	68
1,10-di-epi-cubenol	37.09	0.55 ± 0.24	0.24 ± 0.01	0.14 ± 0.0	0.38 ± 0.02	1618	1614	54
Junenol	37.20	t	0.05 ± 0.01	0.04 ± 0.00	0.02 ± 0.00	1618	1618	81
1-epi-cubenol	37.59	3.02 ± 0.07	2.13 ± 0.02	1.20 ± 0.01	2.37 ± 0.03	1627	1627	35
Epi-α-muurolo	38.11	3.61 ± 0.11	2.87 ± 0.02	2.33 ± 0.03	4.13 ± 0.08	1640	1641	25
α-muurolo	38.28	1.34 ± 0.02	0.88 ± 0.04	0.74 ± 0.02	1.35 ± 0.11	1644	1645	29
α-cadinol	38.58	3.98 ± 0.73	2.55 ± 0.08	2.17 ± 0.02	3.99 ± 0.05	1652	1654	30
Cadalene	39.33	0.44 ± 0.02	0.38 ± 0.02	t	-	1675	1673	87
Khusinol	39.74	1.03 ± 0.06	0.64 ± 0.03	0.35 ± 0.05	-	1679	1684	51
n-heptadecane	40.41	0.68 ± 0.10	-	-	-	1700	1696	-
Monoterpene hydrocarbons		0.00	0.00	0.00	0.00			0
Oxygenated monoterpenes		0.00	0.00	0.00	0.00			0
Total monoterpenes		0.00	0.00	0.00	0.00			0
Sesquiterpene hydrocarbon		47.96	64.31	72.41	57.08			17
Oxygenated sesquiterpenos		38.05	25.96	18.5	34.40			30
Total sesquiterpenes		86.01	90.27	90.91	91.48			3
Others		5.87	4.92	5.56	0.00			67
TOTAL		91.88	95.19	96.47	91.48			3

Identified compounds listed by order of elution in nonpolar column (RTx[®]-5MS).

^aRt = Retention time in minutes; ^bAI = Arithmetic index; ^cE = Experimental data; ^dL = Adams 2007; ^eCV = Coefficient of variation in %; t = Traces, not quantified.

Table S2. Climatic data in the region and seasons of collection.

	Winter	Spring	Summer	Autumn
Total rainfall (mm)	521	345	679	485
Number of rainy days	33	46	47	38
Absolute maximum temperature (°C)	36.0	40.9	40.9	32.8
Minimum absolute temperature (°C)	1.4	11.9	16.9	7.8
Average temperature (°C)	19	26	29	22
Average relative humidity (%)	77	72	71	80

Source: CIRAM/EPAGRI.

Table S3. Antibacterial activity of essential oil from *E. hiemalis* against mollicutes strains.

	MIC ($\mu\text{g mL}^{-1}$)			
	Winter	Spring	Summer	Autumn
<i>M. capricolum</i> subsp. <i>capricolum</i>	333.33	208.33	333.33	333.33
<i>M. genitalium</i>	1,000	1,000	1,000	1,000
<i>M. hominis</i>	333.33	333.33	333.33	333.33
<i>M. pneumoniae</i> FH	416.67	333.33	416.67	416.67

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