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Article

Screening of the Odour-Activity and Bioactivity of the Essential Oils of Leaves and Flowers of *Hyptis Passerina* Mart. from the Brazilian Cerrado

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A composição química dos óleos essenciais das folhas e flores de *Hyptis passerina* Mart., uma espécie rara do cerrado brasileiro, está sendo descrita pela primeira vez. A análise por CG-EM revelou sesquiterpenos como constituintes majoritários. β-*epi*-acorenol (35.7% e 32.8%, respectivamente no óleo essencial das folhas e flores) foi isolado e identificado por RMN uni e bidimensionais. O óleo das flores apresentou maior concentração de monoterpenos (hidrocarbonetos e oxigenados), enquanto que o óleo das folhas foi mais rico em diterpenos. O aroma dos óleos foi investigado por análise olfatométrica direta e por CG-EM-O. Foi observado, para ambos os óleos, aroma herbáceo, com o óleo das folhas contendo nuances aromáticas de verde, cozido e madeiroso, lembrando chá, enquanto o óleo das flores apresentou aspectos de aroma condimentado, madeiroso e mentolado. β-epi-acorenol, espatulenol, β-cariofileno e óxido de cariofileno mostraram-se relevantes para a atividade odorífera dos óleos, bem como, constituintes minoritários como o linalol. A atividade antimicrobiana dos óleos foi investigada pelos métodos de difusão em disco de agar e bioautografia de contato contra bactérias Gram-positivas, negativas e fungos. Os óleos apresentaram-se ativos contra os microorganismos com nível de inibição significativo.

The chemical profile of the essential oils obtained from the leaves and flowers of *Hyptis passerina* Mart., a rare species of the Brazilian Cerrado, has been determined for the first time. Analyses by GC-MS showed sesquiterpenes as major compounds. β-*epi*-acorenol (35.7% and 32.8%, respectively from leaf and flower essential oils), was isolated and identified by 1D and 2D NMR. The flower-derived oil presented a higher concentration of hydrocarbon and oxygenated monoterpenes, while the leaf-oil was richer in diterpenes. The global odour impressions of both oils were given by direct analysis and GC-MS-O and were characterized as herbaceous with tea notes, and green, cooked and woody impressions for leaf-oil; herbaceous, with spicy, woody and minty notes for flower-oil. β-*epi*-acorenol, spathulenol, β-caryophyllene, and caryophyllene oxide were relevant for the odour-activity of both oils, as well as minor constituents, such as linalool. The antimicrobial activity was investigated by means of agar diffusion disc method and contact bioautography, against Gram-positive and negative bacteria and yeast. Both oils presented to be bioactive against the tested microorganisms with significant inhibition level.

Keywords: *Hyptis passerina* Mart., leaf and flower derived essential oil, terpenes, odour-active compounds, antimicrobial activity

Introduction

The rocky fields of the Brazilian Cerrado represent a mosaic constituted of peculiar savannah-type vegetation, associated to a sandy soil and flowery rocks (quartzite) that are inserted in the bioma of this region. Despite occupying restricted areas, this mosaic possesses a flora with a wide richness in species, including families as Lamiaceae (Labiatae), Asteraceae, Eriocaulaceae, Melastomataceae, Orchidaceae, Velloziaceae and Xyridaceae, among others.

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It is well-known that several Lamiaceae genera are widely used in the manufacture of infusions and tinctures applied in the treatment of several infections, as gastrointestinal infections, as well as skin disorders, cramps and pain,[1,2] while the odoriferous species of this family are abundantly used as spices, and also as raw material for food and cosmetic industries. From the 200 known genera, approximately 40% possess aromatic properties, and the essential oils of at least 30 species are being commercialized for over 70 years, such as *Rosmarinus officinalis, Thymus vulgaris* and *Salvia officinalis.*[3] Besides the olfactive characteristics, several species of this family present among their chemical constituents valuable bioactive molecules,[4] such as *Salvia officinalis*, species known for their antimicrobial properties as well as tannins' based adstringent activities, and therefore applied as active ingredient of dental-care medicinal preparations.[5]

The genus *Hyptis*, a member of the Lamiaceae family, is represented by well over 300 species and exhibits a major morphological diversity in the Brazilian Cerrado region.[6] Several species are reported as being extremely aromatic,[7] and presenting, as commonly reported for the genus belonging to the family Lamiaceae, medicinal properties; relevant pharmacological activities have already been studied.[1,8] Many essential oils of *Hyptis* species are reported in literature presenting under their constituents a wide range of monoterpenes, sesquiterpenes, and oxygenated derivatives of both classes, as well as some diterpenes. The most reported species is *H. suaveolens*, which composition and bioactivity have already been deeply studied. This species is traditionally used for the treatment of respiratory track infections, colds, pains, fever, cramps and skin diseases.[1,2,9] A research carried out on the comparison of nine *H. suaveolens* populations growing in the Brazilian Cerrado revealed that the oxygenated sesquiterpenes were the main group of constituents in most of the populations; however, a chemical polymorphism among these populations was observed. Constituents as 1,8-cineole, sabinene, spathulenol, β-caryophyllene, caryophyllene oxide and bicyclogermacrene were identified in different concentrations.[2] Also from Brazilian Cerrado, the essential oil of *H. ovalifolia* Benth. showed as major compound (*R*)-6-[(*Z*)-1-heptenyl]-5,6-dihydro-2*H*-pyran-2-one.[10] Other 6-substituted pyranones with acetoxy and hydroxy functions in the side-chain have been isolated from other species of *Hyptis*.[11] Regarding the chemical constitution of most frequently reported species of *Hyptis*, bicyclogermacrene was reported to be the major compound of *H. lantanifolia*, followed by β-caryophyllene and spathulenol.[12] 1,8-cineole is also commonly observed in *H. mutabilis* and *H. martiusii.*[9,13] Furthermore, Jirovetz *et al.*[14] investigated the chemical profile and established a comparison between the odour of leaf and flower-derived essential oils of *H. pectinata*, considering their possible use in medicinal and cosmetic applications. The composition of both oils differed significantly, as also the odour, being the leaf-derived one weak fresh, woody with fruity notes and major represented by oxygenated sesquiterpenes, while in the one obtained from the flowers the sesquiterpenes predominated presenting to be woody and weak fresh with cedarwood and vetiver-notes.

The species studied in the present work, *H. passerina* Mart. is a rare species that grows wildly in the rocky mountains of Minas Gerais State, Brazil, a region belonging to the Brazilian Cerrado. Able to grow in a sandy and stony ground resulting from the differential erosion of quartzite, *H. passerina* is until now a specimen not commonly deposited in herbarium collections.

In general, essential oils are widely used as flavourings for foods, confections and spices, as well as in perfume and cosmetic industries as fragrance materials. They are incorporated in the manufacture of several skin products due to their active compound's complexity, significant fragrant properties and marketing value. According to the trends of the 1990s, the well established antimicrobial activities of essential oils lead to several proposals for their usage as natural conservation agents in cosmetic preparations[15] and in prophylactic or therapeutic topical application products for the treatment of skin disorders.[16,17] Furthermore, the use of an essential oil in a phytocosmetic formulation would not only exert the function of an antiseptic agent, but also of the perfume raw material giving the product a pleasant odour.

To the author's knowledge, no previous research has been carried out on the chemical composition of leaf and flower-derived essential oils of *H. passerina* and their antimicrobial activity, neither on the odour-active contributors to the aroma of both oils. The aim of the present work is to investigate the chemical profile of these hydrodistilled oils from the leaves and flowers of this rare species of the rocky fields of the Brazilian Cerrado by means of gas chromatography-mass spectrometry (GC-MS) analyses, on both polar and non-polar capillary columns, followed by direct olfactive and GC-MS-Olfactometry (GC-MS-O) analyses. In addition, their antimicrobial activities were also investigated. Further, the development of effective topical antibacterial formulations with both the oils acting as antiseptic agents for the treatment of minor wounds, boils and pimples were also considered.

Experimental

Plant material

Hyptis passerina Mart. was collected in September 2003, 1.100 m above the sea level, in the Serra de São José,

Tiradentes, Minas Gerais, Brazil. A voucher specimen has been deposited in the Herbarium of the National Museum, Rio de Janeiro, RJ, Brazil [Reference number R. J. V. Alves 1028, 4829, 5477, 7051 (R)].

Refractive index

Three aliquots of both neat oils were measured in a Carl Zeiss model of an Abbey refractometer (Carl Zeiss, Jena, Germany), thermostated at 24 °C.

Sample preparation

The essential oils of fresh leaves (220.1 g) and flowers (235.4 g) of *H. passerina* were extracted by hydrodistillation using a Clevenger type apparatus for a period of 2.5 h. Each essential oil was dried with anhydrous sodium sulfate, stored in amber coloured vials and kept under refrigeration (4 ºC). Prior to the GC analyses, each oil was diluted 1:10 (w/v) in dichloromethane.

GC-MS analyses

GC-MS analyses were carried out on a gas chromatograph Agilent Technologies 6890N Network GC Systems hyphenated to a mass spectrometer Agilent 5973 Network Mass Selective Detector (Agilent, Palo Alto, USA), and equipped with the mass spectra database *NIST MS Search Program*, version 1.7, 2000 (Gaithersburg, USA) and Wiley 275 (Palo Alto, USA). The analyses were performed using two analytical columns with distinct stationary phases. The following operational conditions were used: capillary column HP5-MS (5% phenylpolymethylsiloxane) 30 m × 0.25 mm i.d. × 0.25 μm film thickness (Hewlett Packard Co., PA, USA); temperature program: from 32 °C (1 min) to 115 °C at 50.0 °C min^{-1}, to 145 °C at 1.5 °C min^{-1}, to 240 °C (10 min) at 12.0 °C min^{-1}. Further analyses were carried out with a column HP20M (polyethyleneglycol) 25 m × 0.20 mm i.d. × 0.20 μm film thickness (Hewlett Packard Co., PA, USA); temperature program: from 30 °C (1 min) to 110 °C at 50.0 °C min^{-1}, to 145 °C at 1.5 °C min^{-1}, to 220 °C (10 min) at 4.0 °C min^{-1}. The GC-MS was equipped with a split/splitless injector (240 °C): splitless mode for 1 min; injection volume: 1.0 μL; carrier gas: He; constant flow: 1 mL min^{-1}; interface temperature, 250 °C; MS ionization mode: electron ionization; detector voltage: 70 eV; acquisition mode: scan, mass range *m/z* 40 to 750.

GC-MS-O analyses

GC-MS-Olfactometric analyses were carried out on a GC-MS instrument identical to the aforementioned system, hyphenated to a sniffing port device (heated transfer line) developed in this laboratory. The presence of a splitter on the outlet of the analytical column enables the division of the chromatographic flow to the MS detector, and through the heated stainless steel transfer line (sniffing port) to the human nose. Furthermore, the system was equipped with both previously mentioned mass spectral databases. The applied temperature program and columns were identical to the GC-MS analyses. The system was equipped with a split/splitless injector (240 °C): splitless mode for 1 min; injection volume: 1.0 μL; carrier gas: He; constant flow: 1 mL min^{-1}; interface temperature: 250 °C; MS ionization mode: electron ionization; detector voltage: 70 eV; acquisition mode: scan. The sniffing port was held at 220 °C.

Four assessors were selected to evaluate the samples according to their sensitivity and ability to recognise odours. All sniffing analyses were divided in 15 min sessions with 15 min interval in order to avoid the discomfort of the panellists and the dry out of the nasal mucosa. All analyses were carried out in triplicate.

Olfactometry global analysis (frequency response)

This frequency detection method was carried out using an adaptation of the method presented by Pollien *et al*.[18] This adaptation concerns not only in the registration by each assessor of an odour perception, but also on the simultaneous description of the odour quality according to a glossary of descriptors adopted by Vittaflavor (São Paulo, Brazil), a Brazilian flavour industry which kindly performed the training in sensorial analyses of the members of the research group.

Adaptation of OSME (sensorial technique for the evaluation of time versus activity)

The adaptation of the method consisted on the evaluation of the intensity perceived by each panellist, rated on a five-point intensity interval scale (1 = extremely weak, 2 = weak, 3 = moderate, 4 = strong, 5 = extremely strong), and processed by means of a software *SigmaStat-Statistical Software* (Jandel Corporation, California, U.S.A.), while the classic OSME method, developed by McDaniel and cols., is based on the compilation of the results generating a consensual aromagram.[19]

Standard compounds and essential oils

Standards of limonene, α- and β-pinenes, methyl salycilate, β-damascenone, linalool, β-*trans*-caryophyllene,

δ-cadinene and caryophyllene oxide were used for co-injection in GC-MS analyses. To confirm the identity of other compounds identified as being odour-active, essential oils, presenting their chemical composition well reported in literature, were used for co-injection with the essential oils under investigation such as the essential oils of clary sage (*Salvia sclareae*, Lamiaceae) (Roth, Karlsruhe, Germany) and olibanum (*Boswellia carterii*, Burseraceae) (Firmenich, São Paulo, Brazil). δ-cadinene was kindly offered by Dr. Margaret Essenberg (Biochemistry and Molecular Biology Department, Oklahoma State University).

Linear retention index (LRI)

Linear aliphatic hydrocarbons (*n*-C_9 to *n*-C_{22}) (Aldrich, Bellefonte, U.S.A.), 1000 ppm in dichloromethane were injected under the abovementioned chromatographic conditions to calculate the LRIs by using the equation proposed by van den Dool and Kratz.[20]

Isolation of the major compound from the leaves of H. passerina

The essential oil obtained from the leaves (1 g) was fractionated on a silica gel column chromatography by elution with a gradient of pentane, dichloromethane and ethyl acetate. From pentane: CH_2Cl_2 fraction (25%) a white solid was obtained and recrystallized with methanol to give β-*epi*-acorenol, **I** (22 mg).

β-*epi-acorenol,* ***I***

IR (neat) ν_{max}/cm^{-1}: 3460; ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.32 (1H, br s), 2.20-1.50 (12H, m), 1.65 (3H, s), 1.29 (3H, s), 1.26 (3H, s), 0.92 (3H, d, *J* 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 133.9 (C-8), 121.2 (C-7), 73.7 (C-14), 61.3 (C-1), 44.9 (C-5), 42.7 (C-4), 40.9 (C-6), 31.3 (C-13), 30.7 (C-9), 29.5 (C-15), 29.2 (C-10), 26.3 (C-3), 24.9 (C-2), 23.3 (C-11), 15.9 (C-12); EIMS *m/z* (rel. int.): 222 [$M^{+\cdot}$] (3), 204 [M-H_2O] (25), 161 (27), 121 (40), 119 (100), 107 (20), 105 (30), 93 (35), 91 (25), 79 (20), 59 (40).

Sensorial analyses

The essential oils were submitted to direct analyses by a panel of seven judges with previous experience in sensorial evaluation. The analyses were carried out in triplicate, at room temperature, through the application of each essential oil, previously diluted in dichloromethane in proportion of 1:10, to smelling strips. The aforementioned glossary of descriptors was also adopted for these analyses.

Statistical evaluation for olfactometric analyses

The data obtained from the GC-MS-O and sensory analyses were subjected to statistical treatment using one way ANOVA analysis. A significance level of 5% was used throughout the study. The evaluation was executed using the software *SigmaStat-tatistical Software* (Jandel Corporation, California, U.S.A.).

Antimicrobial assays

The Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *S. epidermidis* ATCC 14990, Gram-negative bacterium *Pseudomonas aeruginosa* ATCC 15422, and the yeast *Candida albicans* ATCC 10231 were used as test microorganisms, all kindly supplied by Prof. Dr. Alane Beatriz Vermelho from the Microbiology Institute of the Federal University of Rio de Janeiro, Brazil.

The antimicrobial activity was studied by agar diffusion disc method performed in three duplicates. Luria Bertani (LB) agar and peptone yeast dextrose (YPD) broth were used according to the growth requirements of the test organism. A base layer of 10 mL was prepared of the aforementioned media, and 5 mL of the appropriate molten agar were inoculated with microbial suspensions of known density for the seed layer. Prior to the performance of the agar diffusion disc method the essential oils were diluted in ethanol and filtered (filter pores of 0.22 μm) (Millipore, U.S.A.). The tests for both oils were conduced at four concentration levels (50.0, 25.0, 12.5, and 6.25 μm L^{-1}). Whatman No. 5 filter paper discs (diameter 6 mm) were soaked with 10 μm L^{-1} of each concentration level, and placed on the agar. Bacterial plates were incubated at 37 °C and 28 °C, respectively, for 24 h, after which the diameter of the growth inhibition zone was measured. Ethanol (95%) was used as a negative control in all plates, while standard antibiotic discs were used as positive controls; tetracycline (30 μm g^{-1}) for all bacteria, oxacyline (1 μg g^{-1}) for Gram-positive bacteria, carbeniciline (100 μm g^{-1}) for Gram-negative bacteria (all purchased from Oxoid, England), and ketoconazole (5 μm g^{-1}) for the yeast (Cecon, Brazil). Contact bioautography was performed with *S. aureus*, which showed a good sensitivity to the oils. The assay consisted on the diffusion to the agar of compounds separated previously on thin layer chromatography (TLC). The TLC plate was placed on the surface of the aforementioned agar plates inoculated with the bacteria strain. After 40 min, the chromatoplaques were removed and the plates incubated, under the same abovementioned conditions, until a thin film of the growing microorganisms is visible on the surface, and the zones of inhibition were evaluated.

In addition, the biological activity of the isolated β-*epi*-acorenol has also been investigated. The antibacterial evaluation was performed with Gram-positive (*Staphylococcus aureus* ATCC 25923) and Gram-negative (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853) bacteria as test-microorganisms. The cultures were maintained on Mueller Hinton agar at 8 °C. The inoculum suspension of each strain, in Mueller Hinton broth, was prepared to attain a final inoculum of 10^8 CFU mL^{-1} (OD = 0.08-0.1 at λ = 625 nm, ThermoSpectronic Genesys 10 UV) and diluted 1:10 for the broth micro-dilution procedure according to the M7-A6 document.[21] Subcultures of all strains were prepared to verify the cell viability.

The minimum inhibitory concentration (MIC) was determined using 96 well microtitre plates. All samples were dissolved in dimethylsulfoxide (DMSO)[22] and tested in eight concentrations (1000 to 7.8 μg mL^{-1}). Chloramphenicol (Aldrich) (c = 20 μg mL^{-1} in EtOH 95%), was used as positive control. Pure solvent and culture medium were used for sterility and growth controls, respectively. Subsequently, 5 μL of inoculum was applied into the wells and the microplates were incubated overnight at 37 °C. Afterwards, 40 μL of a 0.2 mg mL^{-1} *p*-iodonitrotetrazolium chloride (*p*-INT) (Sigma) aqueous solution was added and once more incubated for 1-2 hours at 37 °C.[23] The MIC was defined as the lowest concentration of the compounds that inhibited the antibacterial visible growth as indicated by the *p*-INT colorimetric reagent. The tests were performed in duplicate.

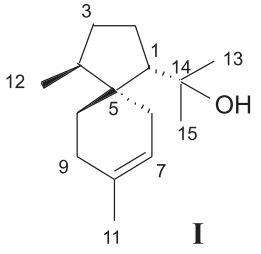
Statistical evaluation for antimicrobial assays

The measured inhibition zones diameters were subjected to statistical treatment using one way ANOVA analysis, followed by Student-Newman-Keuls test; significance level used was of 5%. Furthermore, graphic interpolations of dose-effect curves were created. The same abovementioned statistics software has been used.

Results and Discussion

The yields of the extraction of leaf and flower-derived essential oil were of 2.1% and 0.4%, respectively. Furthermore, the refraction indices of both oils were determined at 24 °C; 1.4989 ± 0.001 for the leaves and 1.4854 ± 0.001 for the flowers.

Both oils showed the same oxygenated sesquiterpene as major compound as pointed by mass fragmentation pattern and retention index in two different capillary columns. So, the leaf-oil of *H. passerina* was submitted to silicagel open column chromatography from which a white solid was obtained. Molecular formula of compound I was determined as $C_{15}H_{26}O$ from the NMR and EIMS (*m/z* 220). The absorption band in the IR spectrum at 3460 cm^{-1} was consistent with a hydroxyl group which, associated to *m/z* 59 (C_3H_7O), *m/z* 204 [$M^{+\cdot}$-18] and a small molecular ion of *m/z* 222 suggested the presence of a tertiary hydroxyl group on an isopropyl function. Analysis of the ^{1}H NMR spectrum indicated the presence of four methyl groups at δ 1.65 (H-11), 1.29 and 1.26 (H-15 and H-13) and 0.92 (H-12), together with an olefinic proton at δ 5.32 (H-7). ^{13}C NMR spectral data confirmed the presence of a trisubstituted double bond at δ 133.9 (C-8) and 121.2 (C-7), one quarternary hydroxylated carbon at δ 73.7 (C-14) and other at δ 44.9, characteristic of a spirocyclic ring system. The unequivocal assignment of **I** as β-*epi*-acorenol was established from COSY, HMBC and HMQC NMR experiments and comparison with previous works dealing with acorenol isomers.[24]



I

According to literature reports, four isomers of acorenol, *i.e.*, α-acorenol, β-acorenol, α-*epi*-acorenol and β-*epi*-acorenol have been isolated from plants. α- and β-Acorenol were first isolated from the wood of *Juniperus rigida*[25] and lately from *J. virginiana* and *J. mexicana*.[26] All four isomers are presented in Australian sandalwood oil, an important material used in perfumery obtained from *Santalum spicatum*.[27] The total synthesis of the four isomers was performed by Srikrishna and Babu.[24]

However, it presents to be a rather uncommon compound for the genus *Hyptis*, being identified here for the first time.

GC-MS associated to co-injection of standards and linear retention indices enabled the identification of the compounds. The detected compounds and their relative area are presented in Table 1. A total of 44 compounds were detected and 34 identified in the essential oil derived from the leaves of *H. passerina*, while in the oil extracted from the flowers 34 compounds were identified out of the 41 detected.

Taking in consideration the chemical profile obtained through the GC-MS analyses it must be emphasized that, although the major compounds of the essential oils

Table 1. Volatile compounds present in the essential oils of leaves and flowers of *H. passerina* Mart. by means of GC-MS (mean values of three analyses carried out on HP-5MS and HP20M columns)

No.	Compound	LRI[a]		FID relative % peak areas[b]	
		HP-5MS	HP-20M	Leaf oil	Flower oil
1	α-Thujene	927	1021	0.1	0.1
2	α-Pinene*	939	1032	4.6	12.1
3	Camphene	952	1075	tr[c]	0.6
4	β-Pinene*	981	1116	tr[c]	0.2
5	Myrcene*	992	1145	0.1	0.5
6	*p*-Cimene	1028	1261	tr[c]	-
7	Limonene*	1033	1178	0.5	1.7
8	γ-Terpinene	1062	1238	tr[c]	-
9	Fenchone*	1090	1410	0.1	-
10	Linalool*	1104	1500	0.1	0.7
11	n.i.	1128	1527	0.1	0.2
12	Borneol	1170	1677	-	tr[c]
13	Terpinen-4-ol	1181	1550	tr[c]	0.2
14	*p*-Cymenol	1188	1838	-	tr[c]
15	α -Terpineol	1193	1688	-	0.4
16	Methyl salycilate*	1200	1710	-	0.4
17	n.i.	1234	1757	-	0.2
18	Bornyl acetate*	1285	1559	-	0.5
19	*Trans*-pinocarvyl acetate*	1297	1600	-	0.2
20	α-Cedrene*	1383	1462	0.3	0.4
21	α-Damascenone*	1386	1813	tr[c]	tr[c]
22	β-Elemene	1390	1595	tr[c]	-
23	δ-Elemene*	1415	1539	3.1	2.6
24	β-Caryophyllene*	1421	1564	4.9	8.5
25	β-Gurjunene	1433	1567	0.4	0.4
26	Aromadendrene	1440	1600	0.6	0.35
27	Geranylacetone	1453	1802	1.5	0.3
28	α-Humulene*	1455	1663	-	0.8
29	β-Farnesene	1457	1628	0.3	-
30	n.i.	1461	1676	1.1	-
31	β-Acoradiene	1475	1672	0.8	1.0
32	α-Curcumene*	1485	1727	0.2	0.3
33	n.i.	1488	1764	0.8	1.0
34	Bicyclogermacrene	1499	1738	5.2	1.3
35	β-Bisabolene	1510	1686	1.9	1.5
36	γ-Acoradiene	1513	1689	1.0	1.3
37	δ-Cadinene*	1524	1710	0.4	0.4
38	n.i.	1538	1716	0.4	-
39	Nerolidol	1567	2009	-	0.5
40	Spathulenol*	1578	2129	11.6	12.8
41	Caryophyllene oxide*	1582	1890	6.2	4.4
42	Ledene oxide	1589	2062	0.4	-
43	n.i.	1598	1925	1.4	1.1
44	n.i.	1637	1987	0.7	-
45	Isospathulenol*	1644	2153	1.2	-
46	β-*epi*-Acorenol*	1655	2105	35.7	32.8
47	Acorenol isomer	1659	2191	1.5	1.0
48	n.i.	1680	2206	1.7	1.8
49	n.i.	1687	2225	0.5	0.5
50	n.i.	1690	2245	0.3	-
51	Farnesylacetone	1924	2300	0.3	0.2
52	Abietatriene	2073	2385	8.7	5.5
53	Beierene	2074	2314	0.7	-
	Monoterpenes[d]			5.4	15.2
	Oxygenated Monoterpenes[d]			0.2	2.1
	Sesquiterpenes[d]			19.3	19.0
	Oxygenated Sesquiterpenes[d]			56.9	50.9
	Diterpenes[d]			9.4	5.5
	Other or unidentified compounds[d]			8.8	7.3

[a]Linear retention indices were determined using *n*-hydrocarbons C_9–C_{22} as external references; [b]Relative percentage of GC peak area on HP-5MS column; [c]Trace amount less than 0.01% of total peak area; [d]Relative percentage based on GC peak area on HP-5MS column; *co-injected with standards or with certified essential oils of clary sage (*Salvia sclareae*, Lamiaceae) (Roth, Karlsruhe, Germany) or olibanum (*Boswellia carterii*, Burseraceae) (Firmenich, São Paulo, Brazil); n.i., not identified.

of leaves and flowers of *H. passerina* are very similar, the concentrations of these in each oil are distinct. Both oils are characterized by the presence of β-*epi*-acorenol (35.7% in the leaf- and 32.8% in the flower-derived oil), as their major compound, spathulenol, β-pinene, the latter especially in the oil extracted from the flowers, and abietatriene are of great abundance. Further compounds with expressive concentration values are caryophyllene oxide, β-caryophyllene, δ-elemene and bicyclogermacrene. Furthermore the essential oil obtained from the leaves of *H. passerina* presents a slightly higher concentration of sesquiterpenes, and an increased concentration of oxygenated sesquiterpenes and diterpenes, while the flower-derived oil is composed mainly of monoterpenes, oxygenated and hydrocarbons. The sesquiterpene concentration in both oils presented to be rather similar.

GC-MS-O and direct analyses

The essential oils were primarily evaluated through direct analyses by a panel of seven judges for the evaluation of their global aroma. The aroma elicited by the essential oil derived from the leaves was described as predominantly herbaceous with tea notes, accompanied by green, cooked and woody impressions, while the flower-derived oil was characterized as predominantly herbaceous, with spicy, woody and mint notes.

It is well known that several of the substances responsible for the aroma elicited by an essential oil are presented in low concentrations, or even trace amounts, fact that can be justified by the low perception limit of these volatiles, known as threshold.[28] It is worthwhile to emphasize that not all volatiles present in a matrix contribute to its odour,[29] and the intensity of a chromatographic peak does not correspond to the intensity of an odour-active substance; the latter can only be evaluated by means of analyses. In general, effective chromatographic techniques and methods are required for optimal separation and identification of the individual components. The hyphenation of GC-MS to olfactometry (GC-MS-O) applied in the present research enabled the acquisition of the mass spectra of the odour-active substances, leading to a more accurate investigation and identification of the components responsible for the essential oil's odour. The odour-active compounds of relevance present in the essential oils extracted from the leaves and the flowers of *H. passerina* are described in Table 2. It has to be mentioned that the reported results were obtained by using an HP-5MS column, since the analyses carried out on that stationary phase presented improved separation and increased reproducibility when compared to the HP-20M capillary column.

The results obtained by means of analyses enabled the characterization of the sensorial profile of odour-active substances present in the essential oils under investigation. It is important to point out that in both oils, the terpenes with oxygenated functional groups were characterized as responsible for the most intense impressions. It is known that the most expressive terpenes used in perfumery present functional groups containing oxygen,[30] and these when present in a matrix, lead the latter to assume an enriched organoleptic profile.[31] As observed, the woody, sweet, and floral note of β-*epi*-acorenol exerts an important role on the odour elicited by both essential oils, followed by the herbaceous, mouldy contribution of spathulenol and herbaceous, spice of caryophyllene oxide. Linalool and β-caryophyllene represent to be of enhanced importance for the oil of the flowers than for that of the leaves. Furthermore, δ-elemene, geranylacetone, bicyclogermacrene and isospathulenol are of major relevance for the leaf-derived oil, while α-pinene and methyl salycilate are for the flower-derived one. An unknown compound ($C_{15}H_{22}O$, LRI 1488, 218($M^{+\bullet}$, 55), 119(100), 135(84), 134(75), 91(57), 107(55), 43(47), 147(28)) also presented odour-activity in both oils. The majority of the substances that revealed a significant influence on the odour of both the oils (intensity scores 4 and 5) are well described in literature and worldwide used fragrance materials. The presence of a higher concentration of sesquiterpenes in both oils can induce their usage as fixative essential oils for the formulation of fragrances as proposed by Sant'Anna and co-workers. for copaiba oil, also known for its rich sesquiterpene composition and well employed by Amazonian people in perfumery.[29] Fixative compounds are generally less volatile substances, with higher molecular weight, able to interact physico-chemically with molecules of increased volatility, and equalize the different evaporation rates of fragrance constituents, enlarging the permanence of an odour.[32] Sesquiterpenes can represent a proper choice for the fulfilment of this task.

Establishing a relation between the impressions reported in the direct analyses and the data obtained through GC-MS-O, it was possible to attribute the herbaceous note of both oils to compounds such as spathulenol and caryophyllene oxide, that besides being described as herbaceous, were also characterized as very intense. Furthermore, the odour of methyl salycilate can be considered as responsible for the refreshing and mint notes elicited by the oil extracted from the flowers. β-*epi*-acorenol is also an important odorant responsible for the woody notes elicited by both oils.

The aforementioned procedure of essential oils co-injection has been carried out to confirm the olfactometric

Table 2. Aroma active compounds of the essential oils of leaves and flowers of *H. passerina* Mart. characterized by means of GC-MS-O

No.[a]	Compound	LRI[b] HP-5MS	Olfactive description[c]	Relative intensity[d]		ID[e]
				Leaf oil	Flower oil	
2	β- Pinene	939	Pine, turpentine	3	4	A
7	Limonene	1033	Citric, fresh, lemon	3	3	A
10	Linalool	1104	Floral, perfumed, fresh, citric	4	5	A
16	Methyl salycilate	1200	Fresh, pungent, peppermint	-	4	A
18	Bornyl acetate	1285	Fresh, camphoraceous, pine	-	3	C
19	*Trans*-pinocarvyl acetate	1297	Floral	-	3	C
20	α-Cedrene	1383	Wood	-	3	A
21	β-Damascenone	1386	Floral (rose), sweet, honey-like	2	2	A
23	δ-Elemene	1415	Wood	4	3	C
24	β-Caryophyllene	1421	Wood, spice	4	5	A
25	α-Gurjunene	1433	Wood, balsamic	3	3	D
26	Aromadendrene	1440	Wood	3	2	C
27	Geranylacetone	1453	Floral, green	4	2	D
28	α-Humulene	1455	Wood	-	3	C
30	n.i.[f] ($C_{15}H_{24}$)	1461	Green, leafy	3	-	-
31	β-Acoradiene	1475	Green	3	3	D
32	α-Curcumene	1485	Herbaceous	2	2	B
33	n.i.[f]	1488	Floral, sweet	4	4	-
34	Bicyclogermacrene	1499	Green, wood	4	2	B
36	γ-Acoradiene	1513	Green, wood	3	3	D
37	δ-Cadinene	1524	Herbaceous, wood, medicinal	3	3	A
38	n.i.[f]	1538	Wood, tea-note	3	-	-
39	Nerolidol	1567	Wood, floral, waxy	-	3	D
40	Spathulenol	1578	Herbaceous, mouldy	4	4	B
41	Caryophyllene oxide	1582	Herbaceous, spice	4	4	A
42	n.i.[f]	1598	Green, wood	3	-	-
43	n.i.[f]	1637	Wood, spice	3	3	-
44	Isospathulenol	1644	Herbaceous	4	-	B
46	β-*epi*-Acorenol	1655	woody, sweet, floral	5	5	A
47	Acorenol isomer	1659	Spice, tobacco-like	3	3	D
48	n.i.[f]	1680	Wood, sweet	3	3	-
49	n.i.[f]	1687	Green, perfumed, dry	-	3	-
51	Farnesylacetone	1924	Floral, oily	3	3	D
52	Abietatriene	2073	Green, wood	3	2	D
Mass spectra (*m/z*) of unidentified compounds[f]	LRI 1461: 204($M^{+\bullet}$,31), 91(100), 119(89), 79(80), 105(76), 41(67), 159(64), 93(54), 131(51), 189(44), 67(39), 133(39); LRI 1488: 218($M^{+\bullet}$,55), 119(100), 135(84), 134(75), 91(57), 107(55), 43(47), 147(28); LRI 1538: 205($M^{+\bullet}$,13), 162(100), 105(96), 81(77), 43(66), 147(65), 119(43), 41(39); LRI 1598: 202($M^{+\bullet}$,44), 119(100), 121(94), 43(86), 134(67), 123(66), 202(44), 159(43), 174(43), 91(40); LRI 1637: 204($M^{+\bullet}$,18), 119(100), 59(70), 91(59), 105(56), 93(50), 43(42), 41(40); LRI 1680: 204($M^{+\bullet}$,31), 59(100), 93(69), 107(67), 161(63), 81(50); LRI 1687: 185($M^{+\bullet}$,5), 145(100), 118(99), 160(83), 59(35), 128(23).					

[a]Numbers correspond to those in Table 1. [b]Linear retention indices were determined using *n*-hydrocarbons C_9–C_{22} as external references. [c]Description according to the Olfactive Descriptor Glossary adopted by the panellists. [d]Averages of normalized intensities evaluated by three panellists in triplicate. A five-point interval scale was adopted (1, extremely weak; 2, weak; 3, moderate; 4, strong; 5, extremely strong). [e]Identification: MS data, LRI and odour were consistent with those of: A, an authentic standard; B, compound present in clary sage essential oil;[33-35] C, compound present in olibanum essential oil;[36-38] D, LRI was consistent with reported in literature[50-52] and MS data identical to that present in the MS databases. [f]Compound not identified.

results obtained through GC-MS-O. The oils of sage[33-35] and olibanum,[36-38] well reported in scientific literature, were used to confirm the identity of the substances described by the panellists as being of high intensity. The oil of sage confirmed the presence of α-curcumene, spathulenol and isospathulenol, while the olibanum oil enabled the positive identification of bornyl acetate, *trans*-pinocarvyl acetate, δ-elemene and α-humulene, which was also confirmed by co-injection. The other important aroma substances were confirmed by the respective co-injection with standards.

Antimicrobial activity

As well expressed in literature, no standardized criteria exist for the results expression of antimicrobial screening,[39] and the agar diffusion disc method illustrates this statement. No guideline exists on the inhibition zone diameter measurement; a series of parameters (inocula volume, culture medium composition, pH, incubation temperature etc.) are involved making the establishment of an universal minimum diameter impossible. In the experiments reported in this paper, the inhibition zone diameter values higher than 10 mm were arbitrarily considered as positive and the activities are expressed by the diameter of the developed inhibition zones. Furthermore, the selection of microorganisms was not only based on their capability of causing skin infections, but also for being of representativeness for the entire Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria classes, as for yeasts (*C. albicans*). Table SI (Supplementary Information) presents the antimicrobial activity of both investigated essential oils, in distinct concentrations, against the selected bacteria and yeast, accompanied by the positive controls. The obtained results demonstrated that the concentration levels of 500.0 μg and 250.0 μg of the leaf-derived oil inhibited the growth of *S. aureus* and *P. aeruginosa* in identical proportion, while the flower-derived presented identical activity against these strains only at 500.0 μg, at 250.0 μg the oil was more active against *P. aeruginosa*. Moreover, for the oil extracted from the leaves, the decrease in concentration level (125.0 μg and 62.5 μg) presented a reduced bioactivity, especially against *P. aeruginosa*; similar was observed for the flower-derived oil. Concerning the inhibition growth of *S. epidermidis*, the essential oil obtained from the flowers presented superior activity when compared to the leaf-derived one, at the concentration of 500.0 μg, while the latter oil stronger in activity at the other three concentration levels. The yeast, on the other hand, presented to be more susceptible to the flower-derived oil, giving a rather constant response; the leaf-derived oil presented to be not very active against *C. albicans*. Establishing a comparison between the antimicrobial activities against bacteria and yeast it is possible to point out that all bacteria strains are more sensible to both the oils than the yeast, however the oil extracted from the flowers presented a significant activity at 250.0, 125.0 and 62.5 μg. For each bacteria strain two positive controls were used and the results compared with standard activity values given for each antibiotic disc by their manufacturers. Both Gram-positive bacteria presented to be sensible to tetracycline and oxacyline, while the Gram-negative bacterium was resistant to tetracycline, and with intermediate activity against carbeniciline. Moreover, *C. albicans* was very sensible to ketoconazole. Based on the results obtained for the agar diffusion disc method dose-effect curves were plotted for both essential oils against each microorganism enabling to establish a relation between material concentration and inhibition growth zone. Furthermore, the isolated compound **I**, β-*epi*-acorenol, was assayed for antibacterial activity by broth micro-dilution technique. MICs results were classified according to Santos *et al.*[40] who proposed a classification as follows: MIC ≤ 100 μg mL^{-1}, good; 100 ≤ MIC ≥ 500 μg mL^{-1}, moderate; 500 ≤ MIC ≥ 1000 μg mL^{-1}, weak; MIC ≥ 1000 μg mL^{-1}, inactive. This study showed that the sample had a good activity (MIC 62.5 μg mL^{-1}) against all Gram-positive and Gram-negative bacteria tested.

The presence of terpenes and terpenoids in essential oils and a certain degree of lipophilicity are reported to be of relevance for the antibacterial activity enabling the interaction of the oil with the bacteria's membrane constituents and their arrangement.[41] Further important characteristics for a positive bioactivity are the presence of molecules with functional groups[42,43] and their solubility in water.[43] It is known that terpenic alcohols exert antimicrobial activity, possibly as protein denaturizing agents, dehydrating agents or solvents.[44] Considering this, it is worthwhile to observe that the essential oils under investigation possess among their major compounds, β-*epi*-acorenol and spathulenol. Furthermore, the presence of abietatriene in both the oils can also represent to be of importance for their bioactivity. That abietane diterpene has already been isolated from the bark of *Prumnopytis andina* (Podocarpaceae), and was tested for antimicrobial activity against some standard bacterial strains, as *S. aureus* and *Pseudomonas* sp, presenting valuable results.[45] Furthermore, this diterpene is also known to be present in low quantities in the genus *Salvia*, in which it presented to be bioactive.[46] In addition, α-pinene, which is present in relevant amounts in the flower-derived oil, also had its bioactivity reported.[42]

The other method performed, contact bioautography, is used for the screening of possible new antimicrobial agents, enabling the localization on the TLC of the compound responsible for the bioactivity.[47] Both essential oils of *H. passerina* presented similar profile, seven spots, on the chromatoplaques. Clear zones of bacterial growth inhibition were observed for both the oils, however it revealed to be not possible to attribute the activity to a precise zone or chemical nature. It has to be pointed out that the obtained results induce to the occurrence of synergic effect between the substances, so that each single compound or compound class presents bioactivity, which is magnified when the compounds are present in the same sample. Synergic effects between terpenes and terpenoids have already been reported by Escoubas *et al.*[48] and Iscan *et al.*,[49] among other authors.

Conclusions

An integrated approach by means of GC-MS and GC-MS-O provided useful information about the volatile components present in the leaf- and flower-derived essential oils of *H. passerina*. In particular, the sesquiterpene concentration present to be similar for both oils, which differed mainly in the more expressive presence of non-oxygenated and oxygenated monoterpenes for the flower-derived oil, and slightly higher oxygenated sesquiterpenes and diterpene concentration in the oil extracted from the leaves. Since oxygenated terpenes are used in perfumery due to their expressive odour, the essential oil derived from the flowers of *H. passerina* presents to be a valuable raw material for perfumery industries. On the other hand, the leaf-derived oil, with its high concentration of sesquiterpenes and diterpenes could be applied as fixative in perfume formulations.

Furthermore, both the investigated oils possessed activity against the selected strains of bacteria and yeast. Their bioactivities, supported by the results obtained in the olfactometric analyses, might justify a further use of these matrices in topical antiseptic application products for the prevention, relief or treatment of various types of skin disorders, or as a natural conservation agent.

Both the essential oils, native from the Brazilian Cerrado, may be considered as natural raw materials of interest for perfumery and cosmetic products, due to their richness in composition, odour profile and the exerted antimicrobial activities.

Supplementary Information

Supplementary information including the antimicrobial activity of the flower- and leaf-derived essential oils of *H. passerina* in different concentrations are available free of charge at http://jbcs.sbq.org.br, as PDF file.

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Screening of the Odour-Activity and Bioactivity of the Essential Oils of Leaves and Flowers of *Hyptis Passerina* Mart. from the Brazilian Cerrado

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Table S1. Zones of growth inhibition (mm) representing antimicrobial activity of the essential oils of leaves and flowers of *H. passerina* Mart

Sample	Inhibition zone / (mm)[a]			
	Gram-positive		Gram-negative	Yeast
	Staphylococcus aureus	*Staphylococcus epidermidis*	*Pseudomonas aeruginosa*	*Candida albicans*
Leaf-derived oil[b]	ATCC 25923	ATCC 14990	ATCC 15422	ATCC 10231
500.0 µg	20.5	17.5	20.5	14.0
250.0 µg	18.0	16.5	18.0	14.0
125.0 µg	15.5	13.5	13.0	12.5
62.5 µg	14.0	13.0	12.0	11.0
Flower-derived oil[b]				
500.0 µg	19.5	18.5	19.5	17.5
250.0 µg	15.5	13.0	16.5	17.5
125.0 µg	14.5	12.5	13.5	16.5
62.5 µg	14.0	12.0	12.5	15.5
Tetracycline (30 µg *per* disc)	32.0	33.0	12.0	n.p.[c]
Oxacyline (1µg *per* disc)	25.0	12.0	n.p.[c]	n.p.[c]
Carbeniciline (100 µg *per* disc)	n.p.[c]	n.p.[c]	20.0	n.p.[c]
Ketoconazole (50 µg *per* disc)	n.p.[c]	n.p.[c]	n.p.[c]	38.0

[a]Values are mean of two triplicates. [b]Real concentrations after application of 10 µL of each dilution (50.00, 25.00, 12.50, 6.25 µg μL^{-1}) on a disc. [c]Experiment not performed.

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