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Article

Microclimatic Factors and Phenology Influences in the Chemical Composition of the Essential Oils from *Pittosporum undulatum* Vent. Leaves

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O óleo essencial das folhas de um espécime brasileiro de *Pittosporum undulatum* Vent. foi analisado através de cromatografia a gás-espectrometria de massas (CG-EM), além de análise por RMN após separação cromatográfica. As folhas de *P. undulatum* foram coletadas durante um ano (Janeiro, Março, Maio, Julho, Setembro e Novembro de 2004) e os óleos essenciais obtidos foram submetidos à análise por CG e CG/EM. O óleo mostrou-se composto por hidrocarbonetos monoterpênicos e sesquiterpênicos, sendo o (+)-limoneno o constituinte principal. Através dos resultados obtidos por essa análise, observou-se uma variação significativa na proporção relativa de (+)-limoneno em cada uma das coletas realizadas, a qual poderia estar correlacionada a parâmetros microclimáticos (temperatura e índice pluviométrico) além de fenologia da espécie estudada.

The essential oil from leaves of a Brazilian specimen of *Pittosporum undulatum* Vent. was analyzed by means of gas chromatography-mass spectrometry (GC-MS) and NMR analysis after chromatographic separation. The leaves of *P. undulatum* were collected during one year (January, March, May, July, September and November, 2004) and the obtained essential oils were analyzed. The oil is rich in hydrocarbon monoterpenes and sesquiterpenes, being (+)-limonene the main constituent. It was observed a significant variation on the relative amount of (+)-limonene in these collections, which could be associated to microclimatic parameters (temperature and pluviometric index) despite of phenology of the studied species.

Keywords: *Pittosporum undulatum,* essential oil, chemical variability

Introduction

Pittosporum undulatum (Pittosporaceae), named "pau-incenso" in Brazil because its leaves and fruits present a characteristic smell, is a tree of 12 m in its natural habitat but usually smaller in cultivation (7-10 m). Small, white, fragrant flowers occur in terminal clusters in spring and early summer and are followed by orange-tan berries 1 cm in diameter in autumn, which persist for several months. This species has been found as a wild plant in tropical forest from Africa, Asia and New Zealand and have been also planted as ornamental specie in other tropical regions of the world such as Brazilian cities, for example São Paulo.[1,2]

The chemical composition of fruits and leaves of *P. undulatum* have been extensively studied and several terpenoid derivatives were found, mainly triterpenoid sapogenins.[3-5]

The essential oils from several species of *Pittosporum* have previously been described. The oil from leaves of *P. senacia* collected in Madagascar was composed by 20.4% of monoterpenes, 31.7% of sesquiterpene hydrocarbons and 37.8% of sesquiterpene alcohols being the major compounds δ-cadinene (11.3%), α-muurolol (15.9%) and α-cadinol (19.0%).[6] From leaves of *P. balfourii*, collected in Island of Rodrigues, east of Madagascar, was obtained an essential oil composed by 86.2% of monoterpenes and 4.9% of sesquiterpenes, and the major component was identified as myrcene (47.5%).[7] In the essential oil from leaves of *P. viridiflorum*, the occurrence of monoterpenes, hydrocarbon sesquiterpenes and oxygenated sesquiterpenes were reported being δ-cadinene (10.6%) and α-cadinol (18.3%) the main constituents.[8] Recently, an extensive study with essential oils from several species of *Pittosporum* collected on New Zealand have been described.[9] The oils from *P. crassifolium*, *P. fairchildii*, *P. tenuifolium* and *P. umhellatum* was constituted mainly by sesquiterpenes and monoterpenes in low abundance. Other

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species such as *P. anomalum*, *P. eugenioides* and *P. dallii* were also studied and their essential oils are rich in sesquiterpene alcohols besides esters such as octyl and phytyl acetate.[9]

The essential oil from leaves of *P. undulatum* has been subject of one published research in which the plant material was collected in San Miguel Island (Azores). This report describes that 17 compounds were identified and the relative amount of each class of compounds have been described. Monoterpenes (1.6%), hydrocarbon sesquiterpenes (61.5%), sesquiterpene alcohols (16.5%), diterpene (10.7%) and alkanes (6.5%) were found, being the main constituents the sesquiterpenes calamenene (41.4%), farnesol (10.9%), spathulenol (5.6%) and β-selinene (5.2%) and the diterpene (8β,13β)-kaur-16-ene (10.7%).[10]

In continuation with our studies on essential oils from aromatic Brazilian species, the present investigation reports the chemical composition of the essentials from leaves of *P. undulatum* from Brazil, and compares the difference in the oil content and composition of other *Pittosporum* species. Despite of the chemical composition of essential oil from *P. undulatum* had been described previously, the effect of the harvesting period in the oil composition had not been reported in the literature.

Effects of environmental factors on the variation on the chemical composition of essential oils were observed in several species such as *Virola surinamensis,*[11] *Santolina rosmarinifolia*[12] and *Helichrysum italicum*.[13] Therefore, we describe here the analysis of the relative percentage of the leaves oils components of this species obtained in six different periods of one year (January, March, May, July, September and November) and a possible relationship with the microclimatic conditions and phenological state of the studied species.

Experimental

General experimental procedures

NMR (Bruker DPX-300): ^{1}H (300 MHz) and ^{13}C (75 MHz) in $CDCl_3$ (Sigma-Aldrich) and TMS as internal standard; LREIMS were obtained at 70 eV (INCOS 50 Finnigan-Mat-quadrupole); optical rotation was measured in EtOH in a digital polarimeter JASCO DIP-370 (Na Filter, λ = 588 nm); column chromatography: silica gel 60 (Merck, 60-200 μm).

Plant material

The leaves of *Pittosporum undulatum* Vent. (Pittosporaceae) were collected in the Universidade Presbiteriana Mackenzie Campus, São Paulo, SP, on January, March, May, July, September and November, 15th, 2004, in four times during a day (8 a.m., 12 a.m., 4 p.m., 8 p.m.). The leaves were harvested before (January, March, May and July), during (September) and after (November) the flowering stage. The specie was identified by Prof. Dr. Lúcia Rossi from Instituto de Botânica/SP and a voucher specimen was deposited at the Herbarium of the Prefeitura Municipal de São Paulo (PMSP) under code number 8767.

Essential oil distillation

The fresh leaves were hydro-distillated for 4 h in a Clevenger type apparatus. The essential oils were separated from water using CH_2Cl_2 as solvent, dried over anhydrous Na_2SO_4 and stored at 4 °C in the dark. For chromatographic separation of the oil components, the leaves were collected on July 15th, 2004 (500 g) and submitted to the same extraction procedure described above, to give 120 mg of crude essential oil (yield 0.02%).

Microclimatic factors

The registered values of temperature and relative humidity have been measured *in situ* with a digital Pocket Weather Meter Kestrel 3000 (Nielsen-Kellerman - USA). Precipitation values have been registered using a pluviometer made in our laboratory (values in millimeters) from 12th to 18th day in each collection.

Gas chromatography analysis (GC)

The crude essential oils were analyzed by GC with a Hewlett-Packard 5980 Series II system using an HP-5 column (30m × 0.32 mm internal diameter, with 0.25 μm film thickness) and helium as carrier gas (1 mL min^{-1}). The oven temperature was kept at 60 °C and programmed to 280 °C at a rate of 3°C min^{-1} and kept constant at 280 °C for 10 min. The injector temperature was at 220 °C and detector (FID) at 280 °C. The percentage compositions were obtained from electronic integration measurements using flame ionization detector (FID). A serie of linear n-alkanes was used as reference points in the calculation of relative Kovats indices (KI).

Gas Chromatography - Mass Spectrometry analysis (GC-MS)

GC-MS analysis was carried out in a Shimadzu GC-17A chromatograph interfaced with a MS-QP-5050A mass

spectrometer. Helium was used as the carried gas. The MS operating conditions were: ionization voltage 70 eV, ion source 230 °C. The GC analysis was done with a DB-5 column (30m × 0.25 mm internal diameter, with 0.25 μm film thickness) and the operating conditions were identical with those of the GC analysis.

Retention indices for all compounds were determined according to the Kovats method relative to the linear n-alkanes series (C_8 to C_{20}). The identification of the compounds was done by comparison of Kovats indices and by matching their fragmentation patterns in mass spectra with those of standard compounds and published mass spectra data[14] along with mass spectra of authentic compounds.

Chiral phase-Gas Chromatography analysis (GC)

The enantiomeric excess of limonene was determined by GC in a Shimadzu GC17A system using a Varian W_{cot} fused silica coated with CP Chirasil-dex CB (25 m × 0.25 mm internal diameter, with 0.25 μm film thickness) as stationary phase and helium as carried gas (1 mL min^{-1}). The oven temperature was kept at 60 °C and programmed to 280 °C at a rate of 5 °C min^{-1} and kept constant at 280 °C for 5 min. The injector temperature was at 220 °C and detector (FID) at 280 °C.

Essential oil separation

Part of the crude essential oil (80 mg) was submitted to separation in a silica gel coated with $AgNO_3$ column,[15] using pentane, a mixture of pentane:CH_2Cl_2 (1:1), CH_2Cl_2 and CH_2Cl_2:acetone 1:1 as eluents[16,17] giving 25 fractions which were individually analyzed by gas chromatography. GC chromatogram of fractions 3-7 (20 mg) indicated that these were composed by one major component (99%) which was identified as (+)-limonene by ^{1}H and ^{13}C NMR spectral analysis, specific optical rotation and comparison with literature data.[18,19] The enantiomeric excess was determined by chiral GC analysis which indicated the predominance of (+)-limonene (99% e.e.).

(+)-limonene. Oil; $[\alpha]_D^{25}$ = +44.0° (*c* 5.0, EtOH); ^{1}H NMR ($CDCl_3$, δ, 300 MHz): 5.39 (m, 1H, H-2), 4.70 (s, 2H, H-9), 2.2-1.4 (m, H-3, H-4, H-5 and H-6), 1.73 (s, 3H, H-7), 1.64 (s, 3H, H-10). ^{13}C NMR ($CDCl_3$, δ, 75 MHz): 149.6 (s, C-8), 133.1 (s, C-1), 120.8 (d, C-2), 108.4 (t, C-9), 41.2 (d, C-4), 30.8 (t, C-6), 30.6 (d, C-3), 28.0 (t, C-5), 23.7 (q, C-7), 20.5 (t, C-10). LREIMS (70 eV) *m/z* (int. rel.) 136 $[M]^+$ (14), 121 (16), 107 (17), 93 (60), 79 (33), 68 (100), 53 (29), 39 (40).

Results and Discussion

The crude essential oils from leaves of *Pittosporum undulatum* were analyzed by GC (DB-5 capillary column) and GC-MS associated to determination of the Kovats indexes. The oil contained ten identified components, corresponding to monoterpenes (β-pinene, β-myrcene, limonene, δ-elemene) and sesquiterpenes (α-copaene, β-elemene, β-caryophyllene, aromadendrene, bicyclogermacrene, δ-cadinene), being limonene the main constituent. Literature data indicated that (+)-limonene showed insecticidal activity against *Rhyzopertha dominica* (F.), lesser grain borer, and *Tribolium castaneum* (Herbst), red flour beetle, which are important pests of stored grain.[19]

Additionally, the crude oil was fractionated by SiO_2/$AgNO_3$ column followed by GC analysis, which indicated that fractions 3-7 were composed by a pure component (99.7%). These fractions were pooled together and submitted to chiral gas chromatography analysis, measurement of specific optical rotation, LREIMS and ^{1}H and ^{13}C NMR spectrometry to confirm the identity of the main component as (+)-limonene, in comparison with literature data.[18,19]

To verify the accumulation of this monoterpene as major component, the crude oils of *P. undulatum* were obtained from leaves collected on the 15th day in January, March, May, July, September and November, 2004 (four samples in each collection) and submitted to GC and GC/MS analysis. The medium values of components in the essential oils obtained from each collection, in the six months of analysis, corresponding to monoterpenes (68±10 – 83.9±0.6%) and sesquiterpenes (7±1 – 11±1%), as showed in Table 1. The yields of the essential oils appear in Table 2, along with registered precipitation, relative humidity, temperature values and phenology of the analyzed specimen.

We have detected quantitative but not qualitative variations on the yields and chemical constituents of the essential oil during the period of study. The yields of the essential oils were constant when the leaves were collected in the specimen sterile period (0.02%), and increase in the flowering and fruiting stages (0.05 - 0.06%, respectively).

During the sterile period (January, March, May and July) the relative level of (+)-limonene, was not constant (67±10 to 80.8±0.4%), which could be related to microclimatic factors such as temperature, air relative humidity and precipitation.[19] As showed in Table 1, in March and July the relative proportion of (+)-limonene was lower (67±10 and 69±3%, respectively) in comparison with other collection periods. In these months the precipitation values

Table 1. Compounds identified and percentage composition from the volatile oil of the leaves of *Pittosporum undulatum*

compounds[a]	KI[b]	January	March	May	July	September	November
β-pinene	980	1.9 ± 0.2	0.8 ± 0.2	1.7 ± 0.3	2.8 ± 0.4	1.3 ± 0.2	1.8 ± 0.3
β-myrcene	991	1.19 ± 0.06	0.67 ± 0.08	0.3 ± 0.3	0.86 ± 0.01	0.7 ± 0.1	0.88 ± 0.02
(+)-limonene	1031	80.8 ± 0.4	67 ± 10	76 ± 4	69 ± 3	78 ± 6	76 ± 6
δ-elemene	1339	0.08 ± 0.01	0.12 ± 0.02	-	0.09 ± 0.01	0.16 ± 0.02	0.16 ± 0.03
α-copaene	1376	0.41 ± 0.04	0.56 ± 0.05	0.35 ± 0.02	0.39 ± 0.04	0.7 ± 0.1	0.77 ± 0.06
β-elemene	1391	0.54 ± 0.03	1.2 ± 0.4	0.59 ± 0.06	0.55 ± 0.05	0.7 ± 0.1	0.50 ± 0.09
β-caryophyllene	1418	0.14 ± 0.01	0.32 ± 0.04	0.21 ± 0.06	0.19 ± 0.01	0.30 ± 0.07	0.23 ± 0.02
aromadendrene	1439	0.33 ± 0.04	1.3 ± 0.3	0.24 ± 0.03	0.36 ± 0.03	0.39 ± 0.03	0.36 ± 0.03
biclyclogermacrene	1494	5.3 ± 0.1	6.8 ± 0.8	6.2 ± 0.8	5.6 ± 0.9	4.1 ± 0.8	6.7 ± 0.8
γ-cadinene	1513	0.15 ± 0.02	0.53 ± 0.08	0.4 ± 0.2	0.33 ± 0.04	0.47 ± 0.09	0.40 ± 0.02
Monoterpenes		83.9 ± 0.6	68 ± 10	79 ± 4	73 ± 2	80 ± 6	79 ± 6
Sesquiterpenes		7.0 ± 0.1	11 ± 1	8 ± 1	8 ± 1	7 ± 1	9.1 ± 0.7
TOTAL		90.9 ± 0.6	79 ± 9	87 ± 4	80 ± 1	87 ± 6	88 ± 6

[a]Order of elution from the GC column. [b]Retention index on DB-5 capillary coated column.

Table 2. Essential oil yield, microclimatic (precipitation, air relative humidity and temperature) and phenological factors registered in the collection periods

	January	March	May	July	September	November
Essential oil yield /%	0.02	0.02	0.02	0.02	0.06	0.05
Precipitation / mm	0	33	8	65	16	39
Air relative humidity /%	64 ± 5	76 ± 5	81 ± 1	72 ± 9	64 ± 5	77 ± 4
Temperature / °C	23 ± 3	21 ± 2	16.3 ± 0.8	14 ± 2	25 ± 3	24 ± 2
Phenological factor	sterile	sterile	sterile	sterile	flowering	fructification

were high (33 and 65 mm, respectively) which suggest an influence of this microclimatic factor in the accumulation of (+)-limonene in the crude essential oil.[12] However, in September and November (78±6 and 76±6%, respectively), in the flowering and fructification periods, the relative amount of (+)-limonene was higher than March and July (67±10 and 69±3%, respectively) despite of the high precipitation index in November (39 mm). High values of limonene in the essential oil from leaves of *Helichrysum italicum* in flowering stage were observed previously.[13] These data suggest that in the flowering and fructification periods the production of more volatile derivatives was intensified, in agreement to literature data.[11,12]

Besides, a relationship between production of more volatile compounds and air relative humidity has been observed.[20] In the present study we detected a higher production of monoterpenes, mainly (+)-limonene, in a dry season (January) when the air relative humidity was 64 ± 5% (sterile period).

Therefore, although precipitation, temperature and relative humidity might be expected to affect the oil chemical composition, it also could be partially depend on the phenological state.[22]

It is important to mention that in the *P. undulatum* leaves oil previously analyzed by Mananjarasoa *et al.*[6] was observed a predominance of hydrocarbon sesquiterpenes, being calamenene the main constituent (41.4%), and a low amount of oxygenated derivatives. In the present work the main constituents were the hydrocarbon monoterpenes, being (+)-limonene the major component. The occurrence of one monoterpene (myrcene 47.5%) as the most abundant component has been described previously by Gurib-Fakim and Demarne,[7] in the essential oil from the leaves of *P. balfourii*. Therefore, the composition of the essential oil of *P. undulatum* collect in Brazil is quite different from the oils extracted from others *Pittosporum* species, which should be associated to several environmental factors.

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Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br, as PDF file.

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