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Article

Seasonal and Circadian Study of a Thymol/γ-Terpinene/*p*-Cymene Type Oil of *Ocimum gratissimum* L. and Its Antioxidant and Antifungal Effects

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Ocimum gratissimum oil is used for various medicinal, food and cosmetic applications due to its high chemical variability. Local businesses have considered the economic exploitation of this plant. A specimen sampled at São Luís, MA, Brazil, with thymol and derivatives oil, was subjected to seasonal and circadian study, and to antifungal and antioxidant assays. It was possible to observe that the weather conditions showed a direct influence on the oil yield and variation of its constituents, mainly oxygenated monoterpenes and monoterpene hydrocarbons, with the predominance of thymol, γ-terpinene, and *p*-cymene. Principal component analysis (PCA) was able to justify the oil chemical variability of the seasonal (70%) and circadian (86%) study. Oil displayed inhibition for the fungus *Corynespora cassiicola* at a concentration above 0.3 μL mL^{-1}, and it showed a more significant activity by comparison to thymol. Using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay, the oil antioxidant activity was about 75% of thymol.

Keywords: alfavaca, essential oil, seasonal and circadian variation, antifungal and antioxidant activity

Introduction

Ocimum species (Lamiaceae) are annual and perennial herbs or small shrubs native to the tropical and subtropical regions of Asia, Africa and Central South America. *Ocimum* comprises only 65 species, while other assignments associated with it should be considered synonyms.[1] It is known for having several medicinal, food and cosmetic applications due to its high chemical and morphological variability.[2] The ease of cross-pollination leads to a large number of subspecies, varieties, and forms, which differ in the essential oils composition and morphological characters of various *Ocimum* species.[3]

Ocimum gratissimum L. (syn. *O. guineense* Schumach. & Thonn., *O. suave* Willd., *O. urticifolium* Roth, *O. viride* Willd.)[4] has the African continent as the center of origin and forms a variable polymorphic complex,[3] although the present specimen has been adapted and sampled in the city of São Luís, state of Maranhão, at Eastern Amazon, Brazil, where it is known as "alfavaca". The leaf extract of *O. gratissimum* has been used in the treatment of respiratory infections, gastrointestinal disorder, skin and eye diseases,[5] as well as an antimicrobial and antifungal agent.[6-9] The leaf oil of *O. gratissimum* has been used for its antimicrobial, antifungal, antioxidant, larvicidal, antiprotozoal and therapeutic (anti-inflammatory, analgesic, and cardiovascular) properties.[10-12]

The recurring polymorphism observed in the *O. gratissimum* determines the presence of some chemical types for the essential oil of this species. The most common are the thymol and eugenol chemotypes,[13,14] followed by the geraniol,[15] linalool/methyl chavicol,[16]

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and methyl and ethyl cinnamate chemotypes.[17,18] Other thymol and eugenol rich oils, including different chemical variations, have been described, for instance, the methyl eugenol/eugenol,[19] thymol/γ-terpinene/*p*-cymene,[9,20] thymol/γ-terpinene/1,8-cineole/*p*-cymene,[21] and eugenol/(*Z*)-β-ocimene chemotypes.[2]

The principal constituents found in *O. gratissimum* essential oils are produced by two different biochemical pathways, via shikimic acid to phenylpropanoids, and via mevalonic acid to terpenes.[16] In addition to chemotypic and genetic factors, the season period, harvest time, phenological stage and part of the plant also affect the chemical composition of the oils. The objective of this work was to evaluate the seasonal and circadian variation of the thymol/γ-terpinene/*p*-cymene type oil of *O. gratissimum*, existing in São Luís, based on environmental factors and the high degree of polymorphism which occurs in *Ocimum* species. In addition, antioxidant and antifungal assays of the oil were conducted to compare their results with other previously reported plant chemotypes, with the aim of contributing to the valorization of the chemotype under study.

Experimental

Plant material and collection data

Leaf samples were collected in the medicinal garden of the Ático Seabra Herbarium, located at Universidade Federal do Maranhão (UFMA), São Luís, MA, Brazil, in the period from January to December 2014. The plant was identified by the botanist Prof Dr Eduardo de Almeida Junior and then deposited in the Ático Seabra Herbarium under the number 5150. A total of 22 plant samples were collected, 12 from monthly collections (seasonal study), all on the 24th of each month, at 9 a.m., plus 5 plants sampled in a single day of April (rainy season) and 5 plants collected in a single day of October (dry season), at 6, 9 and 12 a.m. and 3 and 6 p.m. (circadian study).

Essential oil distillation

The leaves (25 g) were ground and submitted to hydrodistillation using a Clevenger-type apparatus (3 h), after drying at room temperature for three days and loss of about 75% of the water content. The oils were dried over anhydrous sodium sulfate, and their yields were calculated by the plant dry weight. The moisture content of the samples was calculated using an infrared moisture balance for water loss measurement. The procedure was performed in duplicate.

Oil composition analysis

Analysis of the oils were carried on a Thermo Electron Corporation Focus DSQ II gas chromatograph (GC)-mass spectrometer (MS), under the following conditions: DB-5ms (30 m × 0.25 mm; 0.25 mm film thickness) fused-silica capillary column; programmed temperature, 60-240 °C (3 °C min^{-1}); injector temperature, 250 °C; carrier gas, helium, adjusted to a linear velocity of 32 cm s^{-1} (measured at 100 °C); injection type, split (1.0 μL), from 1:1000 hexane solution; split flow was adjusted to yield a 20:1 ratio; septum sweep was a constant 10 mL min^{-1}; electron impact MS (EIMS), electron energy 70 eV; temperature of the ion source and connection parts, 200 °C. The quantitative data regarding the volatile constituents were obtained by peak area normalization using a Thermo Scientific FOCUS GC/flame ionization detector (FID) operated under similar conditions as for the GC-MS, except the carrier gas, which was nitrogen. The retention index was calculated for all the volatiles constituents using a homologous series of *n*-alkanes (C_8-C_{32}; Sigma-Aldrich), according to van Den Dool and Kratz.[22] The oil components were identified by comparing their retention indices and mass spectra (molecular mass and fragmentation pattern) with those existing in the GC-MS system libraries and literature spectra.[23,24]

Antifungal assay

Corynespora cassiicola (Berk. & M. A. Curtis) C. T. Wei is an anamorphic fungus that causes severe leaf damage in more than 70 plant species worldwide. The isolate of *C. cassiicola* (MGSS108) was obtained from the mycology collection of Phytopathology Laboratory, Universidade Estadual do Maranhão, São Luís, MA, Brazil. The oils were dissolved in Tween 20 and incorporated to the potato dextrose agar (PDA) culture medium at concentrations 0.1, 0.3, 0.5, and 0.7 μL mL^{-1} (0.1% Tween). The medium (20 mL) containing the samples was poured into separate Petri dishes and inoculated in the center with an 8 mm diameter disk containing the fungal mycelia. The plates were incubated in a biochemical oxygen demand (BOD) incubator, under a 12 h photoperiod and 25 °C. The negative control was composed of plates containing the fungus but without the oil. The effect of the oil on the mycelial growth (mm) was determined by measuring the radial growth of the fungus in intervals from the 1st to 11th day, after the inoculation. The *in vitro* fungitoxic activity was expressed by the inhibition percentage (I%) of mycelial growth, calculated by the equation I% = [(NC – TP) / TP] × 100,

where NC and TP are the mycelial growth of the negative control and the treated plate, respectively. The statistical design was completely randomized, using three oil samples (March, May, and August) with distinct compositions, beyond pure thymol, the main constituent of these oils. Three replicates were performed and the data were also evaluated by mycelial growth rate index (MGRI), expressed in mm day^{-1} and calculated by the equation MGRI = (D – Db) / n, where D and Db are the diameters of the current day and the previous day, respectively, and n is the number of days of incubation.[25]

Antioxidant assay

A stock solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH; 0.5 mM) was prepared in ethanol. The solution was diluted to approximately 60 μM, measuring an initial absorbance of 0.62 ± 0.02, at 517 nm and room temperature. The radical scavenging activity was expressed as milligrams of Trolox equivalent (TE) *per* g of oil (mg TE g^{-1}), and it was calculated for oil samples with different chemical composition. The reaction mixture was composed of 900 μL of Tris-HCl (100 mM, pH 7.4), 40 μL of ethanol, 50 μL of Tween 20 solution (0.5% m/v), and 10 μL of Trolox in ethanol at concentrations of 0.25, 0.375, 0.50, 0.75, 1.00 and 1.25 mg mL^{-1}, followed by 1 mL of DPPH. The absorbance was measured at the start of the reaction, every 5 min during the first 20 min and, then, at 10 min intervals until constant absorbance value. The DPPH inhibition percentage (IDPPH%) was calculated by the equation IDPPH% = [1 – (AbsA / AbsB)] × 100, where AbsA and AbsB are the absorbance values of the sample and the control (blank) at the end of the reaction, respectively. The TE was obtained by replacing the Trolox solution with 10 μL of oil sample and it was calculated by the equation TE = (A – B) / (A – C) × 25 / 1000 × 250.29 / 1000 × 1000 / 10 × D, where A, B and C are the blank, sample and Trolox absorbance values in the reaction end, respectively, and D is the dilution factor.[26]

Statistical analysis

All data were submitted to analysis of variance (ANOVA) and compared by Tukey's test at 5% probability. Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were used to group the essential oils according to their chemical constituents above than 1%. Complete linkage and absolute correlation coefficient distance were selected as a measure of similarity. For the grouping of the oil samples, the agglomerative and hierarchical method was applied. PCA was performed on a correlation matrix for the visual comparison of the chemical compositions of the different *O. gratissimum* samples. All data were statistically analyzed using the Minitab 14.0 software.[27]

Results and Discussion

Oil composition analysis

A rational plantation of *O. gratissimum* could determine the best use for this species in the Eastern Amazon, based on the economic exploitation of the essential oil of some of its known chemical types. The cultivation of *O. gratissimum* in underutilized areas of secondary forests and savannas could lead to its densification and consequent commercial exploitation. In this work, the essential oil for circadian and seasonal studies was obtained from the leaves of a specimen growing in the campus area of UFMA. The climate of Amazon region is characterized by two well-defined seasons: the rainy season (January to June) and the dry season (August to November). July and December were considered transitional months between these two seasons. The annual survey was conducted during the twelve months of 2014, and the circadian evaluation was done in April (rainy season) and October (dry period) of the same year.

In general, the production of essential oils tends to increase at higher temperatures, which can lead to growth and development for the plant, since it is directly related to photosynthesis and other physiological, biochemical and morphological processes, although on warmer days there may be excessive loss of its volatile constituents. In the annual study, the yield of the essential oils of *O. gratissimum* leaves varied from 0.48 (January) to 0.70% (March, May and October), with an average of 0.63% in both rainy and dry season (Table 1). In the circadian study, the oils yield ranged from 0.46 (6 p.m.) to 0.58% (9 a.m.) in April (rainy season), with a mean of 0.52%. The variation in October (dry season) was 0.48 (6 p.m.) to 0.70% (9 a.m.), with an average of 0.57% (Table 2). It was observed that there was a visible difference in the average oil yield between the different collection time, including for the plant samples collected at 9 a.m. (dry season), where it was significantly larger. Therefore, the results showed that the climatic conditions, the seasonal period and the collection time affected the oil production. It is our understanding that the increase of the essential oil yield is due to the increase of the light radiation and is directly related to the increase of the production of the foliar biomass of *O. gratissimum*, as previously observed.[28]

GC and GC-MS analyzed leaf oils produced from the seasonal and circadian study, and about 99% of their

Table 1. Seasonal study and composition of the oils of leaves of *O. gratissimum*

	RI_{Calc}	RI_{Lit}	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Leaf oil yield / %			0.48	0.64	0.70	0.58	0.70	0.65	0.64	0.52	0.60	0.70	0.65	0.58
Leaf oil constituent / %														
α-Thujene	920	924	3.2	1.6	1.4	1.6	3.5	4.0	2.8	2.2	2.2	2.8	3.9	4.0
α-Pinene	928	932	–	0.4	0.4	0.5	0.8	0.9	0.7	0.5	0.5	0.6	0.4	0.3
Camphene	944	946	0.1	0.1	–	–	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sabinene	966	969	0.3	0.3	0.3	0.4	0.6	0.6	0.4	0.4	0.4	0.5	0.5	0.4
Myrcene	981	988	1.8	1.6	1.6	1.6	2.7	2.8	1.9	2.2	1.8	1.9	3.8	4.4
α-Phellandrene	1002	1002	0.2	0.2	0.2	0.3	0.4	0.5	0.4	0.1	0.4	0.4	0.3	0.2
α-Terpinene	1013	1014	–	1.8	1.8	2.1	2.8	2.7	2.2	2.5	2.4	2.8	1.7	1.9
p-Cymene	1019	1020	11.4	4.2	4.2	3.4	22.5	12.1	17.0	10.1	6.9	4.3	12.7	15.8
(*Z*)-β-Ocimene	1034	1032	0.1	0.1	0.1	0.1	–	–	–	–	–	–	0.1	0.1
(*E*)-β-Ocimene	1042	1044	–	–	–	–	0.1	0.2	0.1	0.1	0.1	0.1	–	–
γ-Terpinene	1052	1054	26.5	36.7	35.0	37.0	21.0	28.9	30.3	36.1	35.1	36.1	26.9	26.2
cis-Sabinene hydrate	1065	1065	0.1	0.5	0.5	0.5	1.1	0.8	0.9	0.9	0.8	0.7	0.3	0.6
Terpinolene	1083	1086	0.2	0.2	0.3	0.3	0.5	0.5	0.5	0.4	0.4	0.1	0.5	0.4
trans-Sabinene hydrate	1095	1098	0.2	0.2	0.2	0.2	0.4	0.3	0.3	0.3	0.3	0.5	0.4	0.2
p-1,3,8-Menthatriene	1107	1108	0.1	0.1	–	–	0.1	–	–	–	0.1	0.1	0.1	–
Borneol	1165	1165	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1
Terpinen-4-ol	1174	1174	0.4	0.3	0.3	0.4	0.5	0.6	0.5	0.4	0.5	0.5	0.3	0.4
p-Cymen-8-ol	1180	1179	0.1	0.1	0.1	–	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
α-Terpineol	1189	1186	0.1	–	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Thymol methyl ether	1222	1232	0.3	0.2	0.2	0.3	0.6	0.6	0.5	0.4	0.4	0.4	0.5	0.6
Thymol	1285	1289	49.7	47.8	48.8	47.1	33.2	36.6	33.4	36.2	39.1	39.3	40.2	37.7
Carvacrol	1291	1298	1.1	0.8	1.0	1.0	0.8	0.8	1.2	0.8	1.0	0.8	0.8	0.9
β-Elemene	1380	1389	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.1
(*E*)-Caryophyllene	1409	1417	1.3	0.7	0.9	0.9	1.6	1.2	1.7	1.5	2.3	2.2	1.6	1.2
α-Humulene	1445	1452	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1
Germacrene D	1470	1480	0.3	0.2	0.3	1.2	0.3	0.1	0.2	0.2	0.4	0.6	0.2	0.3
β-Selinene	1478	1489	1.3	0.9	1.2	0.4	2.0	1.8	1.8	1.5	2.1	2.2	2.1	1.8
Viridiflorene	1485	1496	0.4	0.2	0.4	0.1	0.6	0.5	0.6	0.5	0.7	0.8	0.5	0.4
β-Curcumene	1507	1514	0.1	0.1	0.1	–	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1
Spathulenol	1568	1577	0.2	0.1	0.1	–	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.1
Caryophyllene oxide	1573	1582	–	–	–	–	0.3	0.3	–	–	–	0.1	0.1	–
Monoterpene hydrocarbon / %			43.9	47.3	45.3	47.3	55.1	53.3	56.4	54.7	50.4	49.8	51.0	53.8
Oxygenated monterpene / %			52.1	50.0	51.3	49.7	37.0	40.1	37.1	39.3	42.4	42.5	42.8	40.7
Sesquiterpene hydrocarbon / %			3.6	2.3	3.1	2.8	5.1	4.1	4.9	4.3	6.1	6.4	4.8	4.0
Oxygenated sesquiterpene / %			0.2	0.1	0.1	–	0.6	0.6	0.3	0.2	0.2	0.3	0.3	0.1
Total / %			99.8	99.7	99.8	99.8	97.8	98.1	98.7	98.5	99.1	99.0	98.9	98.6

RI_{Calc}: calculated retention index; RI_{Lit}: literature retention index;[23,24] Jan: January; Feb: February; Mar: March; Apr: April; Jun: June; Jul: July; Aug: August; Sep: September; Oct: October; Nov: November; Dec: December.

constituents were identified (Tables 1 and 2). All the oil components belong to the terpenoids class, with the predominance of oxygenated monoterpenes (seasonal: 37.0 to 52.1%; circadian: 43.9 to 55.4%) and monoterpene hydrocarbons (seasonal: 43.9 to 56.4%; circadian: 31.7 to 52.9%), followed by sesquiterpene hydrocarbons (seasonal: 2.3 to 6.4%; circadian: 2.5 to 6.6%) (Tables 1 and 2). The main constituents of the oils of seasonal and circadian

Table 2. Circadian study and composition of the oils of leaves of *O. gratissimum*

	RI_{Calc}	RI_{Lit}	April collection					October collection				
			6 a.m.	9 a.m.	12 a.m.	3 p.m.	6 p.m.	6 a.m.	9 a.m.	12 a.m.	3 p.m.	6 p.m.
Leaf oil yield / %			0.51	0.58	0.57	0.47	0.46	0.64	0.70	0.55	0.48	0.48
Leaf oil constituent / %												
α-Thujene	918	924	1.2	1.6	1.3	1.1	0.9	2.6	2.8	2.5	2.7	1.7
α-Pinene	926	932	0.3	0.5	0.3	0.3	0.3	0.6	0.6	0.6	0.6	0.4
Camphene	947	946	–	–	–	–	–	0.1	0.1	0.1	0.1	–
Sabinene	964	969	0.3	0.4	0.3	1.5	0.2	0.6	0.5	0.6	0.6	0.3
Myrcene	982	988	1.1	1.6	1.3	0.2	1.0	1.9	1.9	1.7	1.9	1.5
α-Phellandrene	1001	1002	0.2	0.3	0.2	–	0.2	0.4	0.4	0.4	0.4	0.3
α-Terpinene	1010	1014	1.6	2.1	1.5	1.6	1.4	2.6	2.8	2.4	2.8	2.2
p-Cymene	1018	1020	2.1	3.4	2.5	2.1	2.3	3.8	4.3	4.8	4.3	3.4
Limonene	1026	1024	–	–	–	–	–	0.8	–	–	–	0.7
(*Z*)-β-Ocimene	1036	1032	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
(*E*)-β-Ocimene	1041	1044	–	–	–	–	–	0.1	–	–	–	0.1
γ-Terpinene	1051	1054	32.8	37.0	45.1	29.4	25.3	34.9	36.1	37.6	36.2	35.3
cis-Sabinene hydrate	1063	1065	0.5	0.5	–	0.5	–	0.9	0.7	0.8	0.7	0.9
Terpinolene	1082	1086	0.2	0.3	0.2	0.1	–	0.5	0.1	0.5	0.5	0.4
trans-Sabinene hydrate	1093	1098	0.1	0.2	0.1	–	0.1	0.3	0.5	0.3	0.2	0.3
p-1,3,8-Menthatriene	1108	1108	0.1	–	0.1	–	–	0.1	0.1	0.1	0.1	0.1
Borneol	1168	1165	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2
Terpinen-4-ol	1172	1174	0.2	0.4	0.3	0.3	0.2	0.4	0.5	0.4	0.5	0.4
p-Cymen-8-ol	1183	1179	–	–	–	–	–	0.1	0.1	0.1	0.1	0.1
α-Terpineol	1189	1186	–	0.1	0.1	–	–	0.1	0.1	0.1	0.1	0.1
Thymol methyl ether	1221	1232	0.1	0.3	0.2	0.1	0.1	0.4	0.4	0.4	0.4	0.4
Thymol	1284	1289	54.9	47.1	42.2	58.4	63.4	40.7	39.3	38.5	39.4	42.2
Carvacrol	1290	1298	0.9	1.0	0.9	0.8	1.0	0.9	0.8	0.9	0.8	0.8
β-Elemene	1379	1389	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.2
(*E*)-Caryophyllene	1407	1417	0.9	0.9	0.9	1.0	0.9	2.2	2.2	2.0	2.2	2.2
α-Humulene	1445	1452	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.3
Germacrene D	1472	1484	1.0	1.2	1.1	1.1	1.1	0.4	0.6	0.5	0.6	0.5
β-Selinene	1480	1489	0.3	0.4	0.4	0.4	0.3	2.0	2.2	2.0	2.2	2.4
Viridiflorene	1494	1496	0.1	0.1	0.1	0.1	0.1	0.7	0.8	0.6	0.8	0.8
β-Curcumene	1511	1514	–	–	–	–	–	0.2	0.2	0.2	0.2	0.2
Spathulenol	1568	1577	–	–	–	–	–	0.3	0.2	0.3	0.2	0.3
Caryophyllene oxide	1573	1582	–	–	–	–	–	0.2	0.1	0.2	0.1	0.2
Monoterpene hydrocarbon / %			40.0	47.3	52.9	36.4	31.7	49.1	49.8	51.4	50.3	46.5
Oxygenated monoterpene / %			56.8	49.7	43.9	60.2	64.9	43.9	42.5	41.7	42.3	45.4
Sesquiterpene hydrocarbon / %			2.5	2.8	2.7	2.8	2.6	5.9	6.4	5.7	6.4	6.6
Oxygenated sesquiterpene / %			–	–	–	–	–	0.5	0.3	0.5	0.3	0.5
Total / %			99.3	99.8	99.5	99.4	99.2	99.4	99.0	99.3	99.3	99.0

RI_{Calc}: calculated retention index; RI_{Lit}: literature retention index.[23,24]

studies were thymol (seasonal: 33.2 to 49.7%; circadian: 39.3 to 63.4%), γ-terpinene (seasonal: 21.0 to 37.0%; circadian: 25.3 to 45.1%), and *p*-cymene (seasonal: 4.2 to 22.5%; circadian: 2.1 to 4.8%).

The chemical composition of oils from *O. gratissimum* has been extensively investigated revealing that chemotypes containing thymol are among the most widely known. This subject was well documented in the Introduction section

of this paper. The composition of the chemotype that is presented in this paper, with thymol, γ-terpinene and *p*-cymene as the principal constituents, is very close to those previously registered to Togo and Benin (Africa),[9,20] and to Pará State (Brazil).[21] The occurrence of the higher percentage of the same constituents in these chemotypes may not be coincidental. Thus, it seems to us that the relatively high temperatures observed in the collection area of the plant (in Brazil) and hot countries like Benin and Togo (in Africa) may have contributed to the adaptation of the plant to the thymol and derivatives chemotype. Besides, these constituents with high content are derived from the same biosynthetic process occurring in the plant, where γ-terpinene is self-oxidized into *p*-cymene and, then, hydroxylated to the thymol.[29]

The variation of the main constituents of the *O. gratissimum* oil over a year (seasonal study) can be seen in Figure 1. Thymol showed the highest values in January (49.8%) and February (47.9%), and the lowest values in May (33.2%) and July (33.4%). γ-Terpinene showed a maximum percentage in February (36.8%) and August (36.1%), and a minimum percentage in May (21.0%). *p*-Cymene presented the highest content in May (22.5%), and the lowest content in February (4.3%) and March (4.2%).

Significant variation in the percentages of thymol, γ-terpinene, and *p*-cymene was observed in the circadian study, during the rainy (April) and dry (October) season, as can be seen in Figure 2. Regarding thymol, the highest values occur in the rainy season, during the afternoon (3 p.m., 58.4%; 6 p.m., 63.4%) and early in the morning (6 a.m., 54.9%), therefore, with lower solar radiation. The γ-terpinene profile was entirely different, showing significant and increasing values during the morning period and higher solar radiation, both in the rainy season (6 a.m., 32.8%; 9 a.m., 37.0%; 12 a.m., 45.1%) and dry season (6 a.m., 34.9%; 9 a.m., 36.1%; 12 a.m., 37.6%). The values for *p*-cymene, although below 5%, were more expressive during the dry season. Based on these data, we hypothesize that in the biosynthetic process of *O. gratissimum* the production of the precursor γ-terpinene occurs during the morning with higher solar radiation, whereas thymol, as a final product, is metabolized in the afternoon, or at night, with a low incidence of light. This hypothesis also justifies the low percentage of *p*-cymene in the oils, since it is rapidly hydroxylated to thymol.[30]

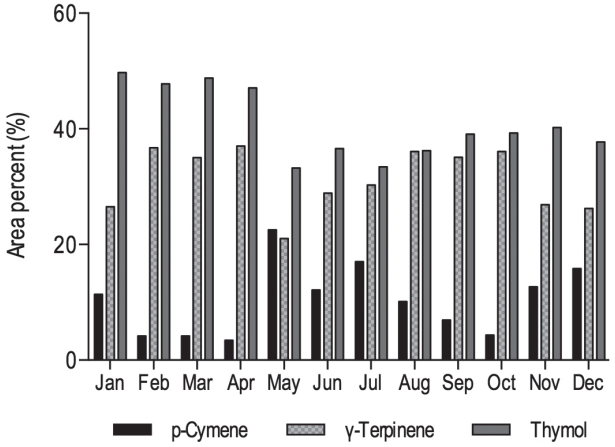

Figure 1. Percentage variation of thymol, γ-terpinene, and *p*-cymene in the annual study of the leaf oils of *O. gratissimum*.

Multivariate analysis of oils

PCA analysis of the seasonal study of the *O. gratissimum* oils showed that the components PC1 and PC2 have explained 70% of the chemical variation among all samples, which were classified into six groups (Figure 3). PC1 had positive correlations with α-thujene, myrcene, α-terpinene, *p*-cymene, *cis*-sabinene hydrate, (*E*)-caryophyllene and β-selinene, and negative correlation with γ-terpinene, thymol, carvacrol and germacrene D. The positive loadings were observed for samples collected from May to December and negative loadings for samples collected from January to April. On the other hand, PC2 displayed a positive correlation with α-thujene, myrcene, *p*-cymene, thymol and carvacrol and the more positive loadings were observed for samples collected from January to March.

Group I was characterized by the May sample, which displayed thymol (33.2%) and *p*-cymene (22.5%) as the main compounds. Group II had included the samples collected in November and December with similar amounts of thymol (40.2 and 37.7%), γ-terpinene (26.9 and 26.2%)

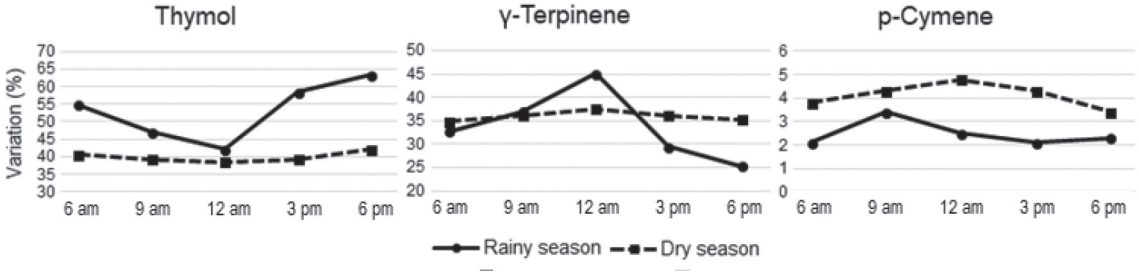

Figure 2. Percentage variation of thymol, γ-terpinene, and *p*-cymene during the dry and rainy season in the circadian study of the leaf oils of *O. gratissimum*.

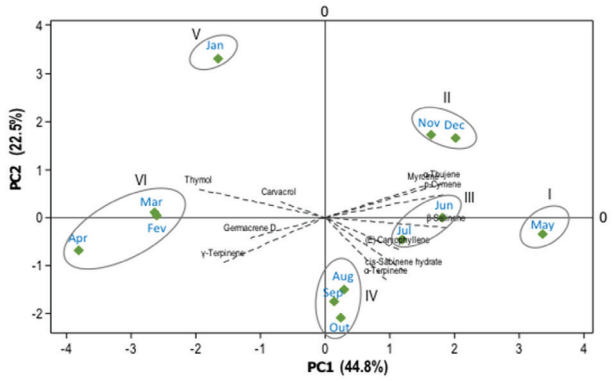

Figure 3. Bidimensional plot of the two components (PC1 and PC2) obtained by the PCA analysis of the leaf oils of *O. gratissimum*, based on the annual study.

and *p*-cymene (12.7 and 15.8%). Group III was composed of the samples collected in June and July, whose concentrations of thymol (36.6 and 33.4%), γ-terpinene (28.9 and 30.3%) and *p*-cymene (12.1 and 17.0%) were very close. Group IV showed equivalent proportions of thymol and γ-terpinene for the samples collected in August (36.2 and 36.1%), September (39.1 and 35.1%), and October (39.3 and 36.1%). Group V was composed of an individual sample collected in January, which displayed the highest amount of thymol (49.7%), followed by γ-terpinene (26.5%) and *p*-cymene (11.4%). Finally, Group VI included the samples collected in February, March, and April, which displayed relative amounts of thymol (47.8, 48.8, and 47.1%), γ-terpinene (36.7, 35.0, and 37.0%), and *p*-cymene (4.2, 4.2, and 3.4%). According to these results, the PCA analysis provided a separation among the samples collected in the rainy season (January, February, March, and April) and in the dry season (August, September, October, and November). Samples collected in July and December, considered transitional months, showed more similarity to samples from dry season, while samples from May and June can be associated with the rainy season.

PCA analysis of the circadian study of the *O. gratissimum* oils has explained 85.9% of the chemical variability (PC1, 72.2%; PC2, 13.7%) (Figure 4). In PC1, the positive loadings were observed for samples collected in October (from 6 a.m. to 6 p.m.) (dry season) which displayed significant amounts of thymol (38.5 to 42.2%). Similarity level among these samples was 65.1%, determined by HCA analysis. Samples collected in April (rainy season) displayed a higher variability with positive and negative loadings in PC2. The samples collected at 9 and 12 a.m. showed a similarity of 62.2% with the following amounts of thymol (47.1 and 42.2%) and γ-terpinene (37.0 and 45.1%). The oil samples collected at 6 a.m., 3 p.m. and 6 p.m. showed the higher amounts of thymol (54.9, 58.4 and 63.4%) and the lower amounts of γ-terpinene (32.8, 29.3 and 25.3%).

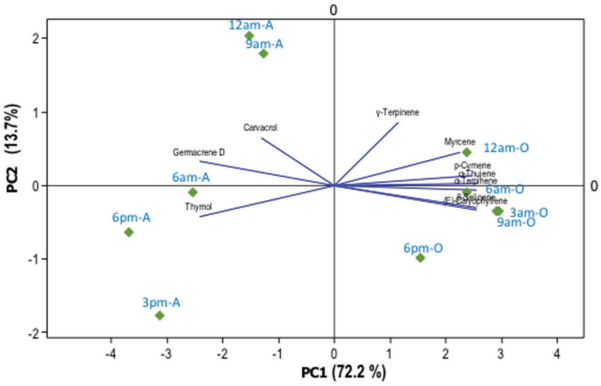

Figure 4. Bidimensional plot of the two components (PC1 and PC2) obtained by the PCA analysis of the leaf oils of *O. gratissimum*, during the rainy and dry season, based on the circadian study.

Antifungal activity

Corynespora cassiicola showed slow growth, with an average of 0.83 mm day^{-1} and the form of dark mycelium. The oil samples of seasonal study, collected in March, May, and August, were tested at different concentrations against the mycelial growth and the spore germination of *C. cassiicola*. All oil samples were active at a concentration above 0.3 μL mL^{-1} and displayed a linear inhibition for the mycelial growth (Table 3). Oil samples of March, May, and August and the thymol standard showed a correlation coefficient (R^2) of 0.94, 0.93, 0.92 and 0.79, respectively.

Table 3. Antifungal effects of the leaf oils of *O. gratissimum* from the seasonal study, against the fungus *C. cassiicola*

Concentration / (μL mL^{-1})	Mycelial growth inhibition[a] / %				Inhibition of sporulation / %			
	March	May	August	Thymol	March	May	August	Thymol
0.1	8.93 Aa	10.50 Aa	9.20 Aa	0.00 Ba	87.37	66.42	55.20	9.54
0.3	76.62 Ab	71.41 Ab	75.12 Ab	2.07 Ba	97.69	91.91	85.55	26.30
0.5	83.17 Ac	91.34 Bc	91.76 Bc	21.85 Cb	100.00	100.00	100.00	44.74
0.7	100.00 Ad	98.05 Ad	99.23 Ad	36.37 Bc	100.00	100.00	100.00	70.03

[a]Averages followed by the same lowercase letter in the column and averages followed by the same capital letter in the lines are not statistically different by Tukey's test at 5% probability.

The oil samples of March, May and August inhibited about 100% of mycelial growth and sporulation of *C. cassiicola*, at concentration of 0.7 μL mL^{-1}. All the oil samples presented more significant activity in comparison to the thymol, which means that a synergistic action with other constituents of the oils occurs. Antifungal activity of a thymol type oil of *O. gratissimum* was previously reported against dermatophytes, fungi and pathogenic yeasts, however, its derivatives γ-terpinene and *p*-cymene were not equally effective.[9] Therefore, in the *O. gratissimum* oil reported here, it is very likely that the significant fungicidal activity observed is due to thymol in association with minority constituents of the oil. Antifungal action of eugenol and methyl cinnamate types oils of *O. gratissimum*, against *Fusarium* and *Aspergillus* species, were also reported.[31,32] According to Pandey *et al.*,[12] the antifungal properties of oils extracted from *Ocimum* species are attributed to their terpene and phenol constituents. The antimicrobial actions of monoterpenes and phenolic compounds as thymol occurs in the cell membrane of the pathogens. Due to the lipophilic character, their accumulation promotes derangement in the structure, penetrating the cell and exerting inhibitory activity in the cell cytoplasm. This action causes the lysis and the release of intracellular adenosine triphosphate (ATP), with consequent cell death.[33]

Antioxidant activity

The oil samples of seasonal study, collected in January, March, May, August. and the thymol standard were assayed by the DPPH radical scavenging method. The reaction kinetics were considered slow, with the average of 60 min. All samples showed potential in reducing the DPPH radical, with inhibition percentages between 68.83 and 73.85%, at a concentration of 0.15 mg mL^{-1} (Figure 5). The total antioxidant activity was expressed as mg TE g^{-1} of oil and the oils collected in August (1631.5 ± 32.2 mg TE g^{-1}) and March (1674.1 ± 14.6 mg TE g^{-1}) showed the highest activities. The antioxidant capacity average for all samples was about 75% of the thymol activity.

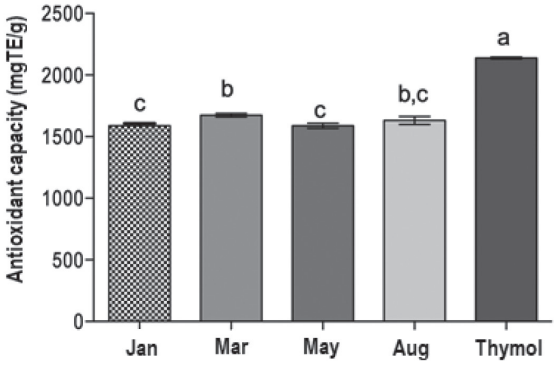

Figure 5. DPPH radical scavenging activity of the leaf oils of *O. gratissimum* from different months, in comparison to thymol. Error bars show the standard deviation (n = 3). Statistical differences by Tukey's test at 5% probability are represented by different letters.

The antioxidant activity of different chemotypes of the *O. gratissimum* oil has been reported in the literature. The methyl cinnamate (48.29%)/γ-terpinene (26.08%) chemotype showed antioxidant activity through the assays of DPPH radical scavenging and β-carotene/linoleic acid bleaching.[32] The protective effect of the oil of *O. gratissimum*, rich in thymol and γ-terpinene, was evaluated in flaxseed stored in an oven for 15 days, at 60 °C. The oil showed inhibition in the malonyl dialdehyde formation, at concentrations of 1 and 5 μL mL^{-1}, and the increase of the conjugated diene and triene formation, comparable to butylated hydroxytoluene (BHT), at 100 μg mL^{-1}.[34]

The oil samples of March and August showed significant amounts of thymol (48.8 and 35%) and γ-terpinene (36.2 and 36.1%), respectively. Thymol is a phenolic compound which has strong reducing properties because of its capacity to form stable intermediates, due to the conjugation of the aromatic ring. Also, the monoterpene γ-terpinene presented a DPPH radical scavenging about three times greater than Trolox.[35] The antioxidant potential of small molecules as thymol and γ-terpinene can be attributed to their greater steric accessibility to the DPPH radical site, when compared to other bigger molecules.

Conclusions

By the seasonal and circadian study of the leaf oils of *Ocimum gratissimum*, it was possible to check that the weather conditions have a direct influence on the oil yield and the content and variation of their volatile constituents. It has been found that the oils contain mainly oxygenated monoterpenes and monoterpene hydrocarbons, in which thymol, γ-terpinene, and *p*-cymene were the principal constituents. PCA analysis of the seasonal study of the *O. gratissimum* oils showed that its components PC1 and PC2 had explained 70% of the chemical variation among all the analyzed samples. Likewise, the PCA analysis of the circadian study of oils was able to justify 86% of its chemical variability. All oil samples were active at a concentration above 0.3 μL mL^{-1} and displayed a linear inhibition for the mycelial growth of the fungus *Corynespora cassiicola*. All the oil samples presented more significant activity in comparison to the thymol, which means that a probable synergistic action with the other constituents of the oils occurs. All oil samples showed potential in reducing the DPPH radical, with inhibition

percentages between 68.83 and 73.85%, at a concentration of 0.15 mg mL^{-1}. The antioxidant capacity average for all oil samples was about 75% of the thymol activity.

Acknowledgments

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References

1. Paton, A.; Harley, R. M.; Harley, M. M. In *Medicinal and Aromatic Plants and Industrial Profiles*; Holm, Y.; Hiltunen, R., eds.; Harwood Academic Press: Amsterdam, 1999, p. 1-38.
2. Verma, R. S.; Padalia, R. C.; Chauhan, A.; Thul, S. T.; *Ind. Crops Prod.* **2013**, *45*, 7.
3. Vina, A.; Murillo, E.; *J. Braz. Chem. Soc.* **2003**, *14*, 744.
4. http://www.tropicos.org/Name/17600215, accessed in March 2018.
5. Lorenzi, H.; Matos, F. J. A.; *Plantas Medicinais no Brasil: Nativas e Exóticas*; Plantarum: Nova Odessa, 2002.
6. Nakamura, C. V.; Ueda-Nakamura, T.; Bando, E.; Melo, A. F. N.; Cortez, D. A. G.; Dias Filho, B. P.; *Mem. Inst. Oswaldo Cruz* **1999**, *94*, 675.
7. Adebolu, T. T.; Oladimeji, S. A.; *Afr. J. Biotechnol.* **2005**, *4*, 682.
8. Lemos, J. A.; Passos, X. S.; Fernandes, O. F. L.; Paula, J. R.; Ferri, P. H.; Souza, H. L. K.; Lemos, A. A.; Silva, M. R. R.; *Mem. Inst. Oswaldo Cruz* **2005**, *100*, 55.
9. Koba, K.; Poutouli, P. W.; Raynaud, C.; Sanda, K.; *J. Sci. Res.* **2009**, *1*, 164.
10. Freire, C. M. M.; Marques, M. O. M.; Costa, M.; *J. Ethnopharmacol.* **2006**, *105*, 161.
11. Faria, T. J.; Ferreira, R. S.; Yassumoto, L.; Souza, J. R. P.; Ishikawa, N. K.; Barbosa, A. M.; *Braz. Arch. Biol. Technol.* **2006**, *49*, 867.
12. Pandey, A. K.; Singh, P.; Tripathi, N. N.; *Asian Pac. J. Trop. Biomed.* **2014**, *4*, 682.
13. Yusuf, M.; Begum, J.; Mondello, L.; d'Alcontres, I. S.; *Flavour Fragrance J.* **1998**, *13*, 163.
14. Vieira, R. F.; Grayer, R. J.; Paton, A.; Simon, J. E.; *Biochem. Syst. Ecol.* **2001**, *29*, 287.
15. Charles, D. J.; Simon, J. E.; *J. Essent. Oil Res.* **1992**, *4*, 231.
16. Lawrence, B. M. In *Essential Oils 1988-1991*; Lawrence, B. M., ed.; Allured Publishing: Carol Stream, 1992, p. 186.
17. Fun, C. E.; Svendsen, A. B.; Baerheim-Svendsen, A.; *Flavour Fragrance J.* **1990**, *5*, 173.
18. Dubey, N. K.; Tiwari, T. N.; Mandin, D.; Andriamboavonjy, H.; Chaumont, J.-P.; *Fitoterapia* **2000**, *71*, 567.
19. Vostrowsky, O.; Garbe, W.; Bestmann, H.-J.; Maia, J. G. S.; *Z. Naturforsch., C: J. Biosci.* **1990**, *45*, 1073.
20. Yayi, E.; Moudachirou, M.; Chalchat, J.-C.; *J. Essent. Oil Res.* **1999**, *11*, 529.
21. Zoghbi, M. G. B.; Oliveira, J.; Andrade, E. H. A.; Trigo, J. R.; Fonseca, R. C. M.; Rocha, A. E. S.; *J. Essent. Oil-Bear. Plants* **2007**, *10*, 229.
22. van Den Dool, H.; Kratz, P. Dec.; *J. Chromatogr. A* **1963**, *11*, 463.
23. Adams, R. P.; *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, 4th ed.; Allured Publishing: Carol Stream, 2007.
24. National Institute of Standards and Technology (NIST); *Mass Spectral Library (NIST/EPA/NIH, v.2.0d)*; The NIST Mass Spectrometry Data Center: Gaithersburg, USA, 2005.
25. Ogbebor, N.; Adekunle, A. T.; *Afr. J. Biotechnol.* **2005**, *4*, 996.
26. Silva, J. K. R.; Andrade, E. H. A.; Kato, M. J.; Carreira, L. M. M.; Guimarães, E. F.; Maia, J. G. S.; *Nat. Prod. Commun.* **2011**, *6*, 1361.
27. *Minitab Statistical Software 14.0*; Minitab Inc., Pennsylvania, USA, 2014.
28. Fernandes, V. F.; Almeida, L. B.; Feijó, E. V. R. S.; Silva, D. C.; Oliveira, R. A; Mielke, M. S.; Costa, L. C. B.; *Braz. J. Pharmacog.* **2013**, *23*, 419.
29. Franco, C. S.; Ribeiro, A. F.; Carvalho, N. C. C.; Monteiro, O. S.; Silva, J. K. R.; Andrade, E. H. A.; Maia, J. G. S.; *Afr. J. Biotechnol.* **2014**, *13*, 3107.
30. Poulose, A. J.; Croteau, R.; *Arch. Biochem. Biophys.* **1978**, *187*, 307.
31. Dambolena, J. S.; Zunino, M. P.; López, A. G.; Rubinstein, H. R.; Zygadlo, J. A.; Mwangi, J. W.; Thoithi, G. N.; Kibwage, I. O.; Mwalukumbi, J. M.; Kariuki, S. T.; *Innovative Food Sci. Emerging Technol.* **2010**, *11*, 410.
32. Prakash, B.; Shukla, R.; Singh, P.; Mishra, P. K.; Dubey, N. K.; Kharwar, R. N.; *Food Res. Int.* **2011**, *44*, 385.
33. Matasyoh, L. G.; Matasyoh, J. C.; Wachira, F. N.; Kinyua, M. G.; Muigai, A. W. T.; Mukiama, T. K.; *Afr. J. Biotechnol.* **2007**, *6*, 760.
34. Hzounda, J. B. F.; Jazet, P. M. D.; Lazar, G.; Dumitra, R.; Caraman, I.; Bassene, E.; Boyom, F. F.; Lazar, I. M.; *Ind. Crops Prod.* **2016**, *80*, 101.
35. Choi, H. S.; Song, H. S.; Ukeda, H.; Sawamura, M.; *J. Agric. Food Chem.* **2000**, *48*, 4156.

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