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Influence of environmental factors on the composition of essential oils from leaves of *Myrcia tomentosa* (Aubl.) DC.

[Influencia de los factores ambientales sobre la composición de los aceites esenciales de hojas de *Myrcia tomentosa* (Aubl.) DC.]

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Abstract
The leaves of *Myrcia tomentosa* were collected from five sites and four different months in the Brazilian Cerrado and their essential oils were obtained by hidrodistillation and analyzed by GC/MS. The aim of this work was to evaluate the influence of environmental factors on essential oils composition in the leaves of *M. tomentosa*. The results were submitted to stepwise Multiple Regression and Canonical Correlation Analysis that indicated a relationship between compounds in essential oils and some environmental factors (foliar nutrients, soil nutrients and climatic data). Cluster Analysis indicated a high chemovariability in the essential oils from different sites, also showed that the collection time had a minor effect on oil composition.

Keywords: Medicinal plants, Myrtaceae, essential oil composition, β-caryophyllene, environmental influence.

Resumen
Las hojas de *Myrcia tomentosa* se obtuvieron de cinco sitios diferentes y cuatro meses en el Cerrado brasileño y sus aceites esenciales fueron obtenidos por hidrodistillation y analizados por GC/MS. El objetivo de este trabajo fue evaluar la influencia de los factores ambientales sobre la composición de los aceites esenciales en las hojas de *M. tomentosa*. Los resultados fueron sometidos a múltiples etapas de regresión y análisis de correlación canónica indicó que la relación entre los compuestos en los aceites esenciales y algunos factores ambientales (nutrientes foliares, fertilizantes y datos climáticos). El análisis de agrupamiento indicó chemovariability un alto en los aceites esenciales de diferentes sitios, también mostró que el equipo de recolección tuvo un efecto menor sobre la composición del aceite.

Palabras Clave: Plantas medicinales, Myrtaceae, composición del aceite esencial, β-cariofileno, influencia del medio ambiente.

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INTRODUCTION

The Myrtaceae family is constituted by 140 genera and about 3000 species, which are widely distributed in America and Australia (Joly, 2002). The genus *Myrcia* comprises about 300 species found in Brazil. Ethnopharmacological data describes the use of genus *Myrcia* against diabetes, diarrhea, hemorrhages, ulcers of the mouth and several species present diuretic properties (Russo *et al.,* 1990*). Myrcia tomentosa* (Aubl.) DC., popularly known as "goiaba-brava", can be found from Panama, northem Venezuela and Guyana to southeast Brazil and is often cited in works on the flora, phytosociology and the characterization of the Cerrado (McVaugh, 1969; Judd *et al.*, 2009). Sá *et al.* (2012) analyzed the composition and chemical variability of essential oils from aerial parts from *Myrcia tomentosa*, however, more studies about environmental influence over essential oils are important, because these variations may influence the essential oils obtained.

Many works have demonstrated that the chemical composition of the essential oils, although genetically determined, can suffer influence of diverse environmental factors. These variations influence directly the quality of the plant for medicinal application (Azevedo *et al.*, 2001, Bergo *et al.*, 2005). This study analyses the possible influence of environmental factors on the chemical variability of the essential oils from leaves of *M. tomentosa* collected in five sites of Brazilian Cerrado every four months over a year.

MATERIALS AND METHODS

Plant material

Leaves of *M. tomentosa* were collected in April 2010, August 2010, December 2010 and April 2011 in five Brazilian cities: Hidrolândia, GO (16° 53' 59.4" S; 49° 13' 29.4" W; 786 m); Nova América, GO (15° 01'11.8" S; 49°52' 32.2" W; 756 m); Crixás, GO (15° 00' 30.2" S; 49° 58' 51.6" W; 755 m); Pires do Rio, GO (17° 12' 35.5" S; 49° 58' 51.6" W; 852 m) and São Gonçalo do Abaeté, MG (18° 20' 27.2" S; 45° 51' 36" W; 919 m). Specimens were identified by José Realino de Paula and vouchers were deposited in the Herbarium of Universidade Federal de Goiás (UFG), Goiânia, GO, Brazil under code numbers 45518, 43836 and 41318.

Essential oils isolation

Dried and powdered leaves were submitted to hydrodistillation in a Clevenger-type apparatus for 2 h. The essential oils were dried with anhydrous Na_2SO_4, transferred to glass flasks and stored at -18 °C for further analysis.

Analysis of the essential oils

Oil sample analyses were performed on a GC-MS Shimadzu QP5050A apparatus using a CBP-5 (Shimadzu) fused silica capillary column (30 m × 0.25 mm; 0.25 μm film thickness composed of 5% phenylmethylpolysiloxane) and the injector and interface temperatures were 220 ºC and 240 ºC, respectively, with a split ratio of 1:5. The injection volume was 0.5 mL (10% in hexane), and the oven temperature program consisted of ramping up from 60 ºC to 240 ºC at 3 ºC min^{-1}, followed by an increase to 280 ºC and 10 ºC min^{-1}, and ending with 5 min at 280 ºC. The carrier gas was He at a flow rate of 1.0 mL/min and the split mode had a ratio of 1:20. MS principal parameters were: temperature of injection port was set at 220 °C; Interface temperature 240 °C; energy for electron impact: 70 eV with scan mass range of 40-400 *m/z* at a sampling rate of 1.0 scan/s. Individual components were identified by a comparison of their linear retention indices and authentic mass spectra, relative to n-alkane series (C_8-C_{32}) in a temperature-programmed run and by the equation of Van Den Dool and Kratz (1963).

Soil and leaf analyses

Analysis of soil and leaves were performed in Solocria Agricultural Laboratory, following usual techniques (Silva, 2009). Soil samples were collected at a depth of 20 cm in four locations around each specimen of *M. tomentosa* in all sites and months. The pH was determined in a 1:1 soil/water volume ratio. Ca, Mg and Al were extracted with KCl 1M, and P, K, Zn, Cu, Fe and Mn were extracted with Mehlich's solution. Organic matter (OM), cation exchange capacity (CEC), potential acidity (H+Al), base saturation (V) and aluminum saturation (m) were determined by standard techniques (Silva, 2009) (Tables Nº 1 and 2).

The nitrogen (N) was extracted by digestion with H_2SO_4 and catalysts. The nutrients (P, K, Ca, Mg, S, Cu, Fe, Mn and Zn (Table Nº 3) were extracted by digestion with $HClO_4$ and HNO_3 (Silva, 2009).

Table Nº 1
Levels of mineral nutrients and fertility parameters of soil from each collection site

Sample	Cu mg/dm^3	Fe mg/dm^3	Mn mg/dm^3	Zn mg/dm^3	P mg/dm^3	K mg/dm^3	Ca mg/dm^3	Mg mg/dm^3	pH ($CaCl_2$)
H/April/2010	1.60	79.10	62.80	1.30	5.10	69.00	1.40	0.50	5.40
NA/April/2010	1.10	70.40	51.70	1.50	1.80	87.00	1.30	0.50	4.40
Cr /April/2010	0.40	46.50	25.00	1.00	2.10	151.00	3.90	0.70	5.50
PR/April/2010	0.30	100.60	24.40	2.20	2.70	89.00	1.90	0.40	4.20
SG/April/2010	0.40	175.70	12.30	1.30	1.50	27.00	0.90	0.20	4.00
H/August/2010	1.85	76.30	67.60	1.95	4.05	101.50	1.65	0.80	4.90
NA/August/2010	1.20	153.35	52.05	1.75	2.10	91.50	1.85	0.65	4.50
Cr/August/2010	0.60	54.55	27.25	1.10	2.55	175.50	4.10	1.11	5.55
PR/August/2010	2.35	82.20	25.50	1.85	1.95	118.50	1.85	0.80	4.50
SG/August/2010	0.75	128.95	8.00	1.10	1.50	31.00	0.60	0.20	3.85
H/December/2010	2.10	73.50	72.40	2.60	3.00	134.00	1.90	1.11	4.40
NA/December/2010	1.30	236.30	52.40	2.00	2.40	96.00	2.40	0.80	4.60
Cr/December/2010	0.80	62.60	29.50	1.20	3.00	20.00-	4.30	1.50	5.60
PR/December/2010	1.70	63.80	26.60	1.50	1.20	148.00	1.80	1.20	4.70
SG/December/2010	1.10	82.20	3.70	0.90	1.50	35.00	0.30	0.20	3.70
H/April/2011	1.60	84.70	100.80	2.10	2.10	8.00	1.20	0.30	4.60
NA/April/2011	1.50	445.00	31.90	1.40	1.20	52.00	0.70	0.30	4.50
Cr/April/2011	1.70	66.80	11.80	0.50	1.20	14.00-	1.30	0.80	4.10
PR/April/2011	1.50	56.80	6.20	0.80	0.80	32.00	0.30	0.20	4.80
SG/April/2011	1.30	84.00	11.00	0.60	-	-	-	-	3.90

H-Hidrolândia; NA- Nova América; Cr- Crixás; PR-Pires do Rio; SG- São Gonçalo do Abaeté.

Table Nº 2
Levels of mineral nutrients and fertility parameters of soil from each collection site

Sample	H+Al	Al	CEC	M.O.	m	V	Ca/CTC	Mg/CTC	K/CTC
H/April/2010	3.90	-	6.00	1.30	-	34.70	23.40	8.40	3.00
NA/April/2010	3.80	0.70	5.84	2.60	25.74	34.89	22.26	8.56	3.77
Cr /April/2010	2.60	-	7.62	3.70	25.00	65.83	51.18	9.19	5.12
PR/April/2010	4.60	0.70	7.15	2.60	21.67	35.69	26.57	5.59	3.22
SG/April/2010	3.40	0.60	4.58	2.50	33.90	25.83	19.65	4.37	1.53
H/August/2010	4.75	0.25	7.48	2.50	6.51	36.08	22.32	10.35	3.40
NA/August/2010	3.80	0.45	6.56	2.40	15.61	41.32	27.64	9.78	3.60
Cr/August/2010	2.35	-	7.79	3.00	12.50	70.00-	51.18	13.49	5.60
PR/August/2010	3.55	0.40	6.52	2.15	12.27	46.70	28.60	13.00	4.84
SG/August/2010	3.60	0.60	4.50	2.50	42.16	19.85	13.23	4.46	1.79
H/December/2010	5.60	0.50	8.95	3.70	13.02	37.46	21.23	12.29	3.80
NA/December/2010	3.80	0.20	7.27	2.20	5.48	47.75	33.01	11.00	3.44
Cr/December/2010	2.10	-	8.43	2.40	-	75.06	51.01	17.79	6.05
PR/December/2010	2.50	0.10	5.89	1.70	2.87	57.61	30.56	20.37	6.45
SG/December/2010	3.80	0.60	4.41	2.40	50.42	13.87	6.80	4.54	2.04
H/April/2011	4.40	0.20	8.40	6.50	4.78	47.59	28.57	14.29	4.52
NA/April/2011	3.00	0.40	4.71	1.90	19.05	36.28	25.48	6.37	4.25
Cr/April/2011	5.20	1.40	6.33	1.80	55.34	17.92	11.06	4.74	2.05
PR/April/2011	3.10	0.10	5.58	3.00	3.91	44.40	23.30	14.34	6.45
SG/April/2011	3.50	0.60	4.09	2.40	50.85	14.39	7.33	4.89	1.96

H+Al= Potential acidity. Al= Aluminum, CEC= Cation Exchange Capacity.
H-Hidrolândia; NA- Nova América; Cr- Crixás; PR-Pires do Rio; SG- São Gonçalo do Abaeté.

Table Nº 3

Levels of macronutrients (N_l, P_l, K_l, Ca_l, Mg_l, S_l in g/kg) and micronutrients (Cu_l, Fe_l, Mn_l, Zn_l in mg/kg) in the leaves of *Myrcia tomentosa* from each collection site over the period from April 2010 to April 2011.

Sample	N	P	K	Ca	Mg	S	Cu	Fe	Mn	Zn
H/April/2010	16.00	0.80	12.80	14.00	2.50	1.20	6.00	162.00	420.00	19.00
NA/April/2010	18.00	1.30	9.20	10.50	3.00	1.60	9.00	247.00	308.00	25.00
Cr /April/2010	17.00	1.10	10.40	23.00	4.30	1.40	8.00	162.00	137.00	16.00
PR/April/2010	17.50	1.20	7.60	13.50	4.20	1.50	10.00	183.00	320.00	18.00
SG/April/2010	19.50	1.10	8.00	14.20	1.80	1.50	7.00	141.00	215.00	16.00
H/August/2010	9.60	1.00	5.60	16.10	1.60	1.00	8.00	329.00	340.00	16.00
NA/August/2010	16.80	1.20	0.80	6.30	2.80	1.10	4.00	199.00	87.00	18.00
Cr/August/2010	12.00	1.00	5.00	23.40	3.20	1.00	5.00	257.00	123.00	14.00
PR/August/2010	16.00	1.20	8.00	6.10	4.50	1.40	8.00	217.00	109.00	20.00
SG/August/2010	15.00	0.90	5.20	9.60	2.10	1.10	7.00	165.00	218.00	15.00
H/December/2010	16.60	1.10	6.80	8.00	2.80	1.20	8.00	179.00	172.00	18.00
NA/December/2010	15.80	1.10	6.00	5.80	3.80	1.30	6.00	213.00	168.00	16.00
Cr/December/2010	16.00	1.20	5.20	12.20	4.60	1.20	9.00	99.00	68.00	13.00
PR/December/2010	19.00	1.10	8.80	7.10	2.10	1.10	9.00	131.00	54.00	16.00
SG/December/2010	12.00	1.00	6.00	7.40	2.10	0.80	5.00	116.00	407.00	15.00
H/April/2011	16.80	1.00	8.00	18.00	2.00	1.10	11.00	180.00	500.00	19.00
NA/April/2011	15.60	1.00	8.00	12.00	2.00	1.20	5.00	264.00	485.00	16.00
Cr/April/2011	17.00	1.10	6.60	31.00	2.60	1.50	8.00	144.00	117.00	16.00
PR/April/2011	18.00	1.10	8.40	9.40	1.50	1.00	7.00	174.00	80.00	17.00
SG/April/2011	15.60	1.00	8.00	8.00	1.70	1.10	8.00	147.00	401.00	18.00

H-Hidrolândia; NA- Nova América; Cr- Crixás; PR-Pires do Rio; SG- São Gonçalo do Abaeté.

Table Nº 4

Climate data for the collection sites over the period from April 2010 to April 2011 - Mean precipitation (mm) and mean temperature (ºC).

Sample	Precipitation (mm)	Temperature (ºC)
H/April/2010	4.58	24.00
NA/April/2010	1.71	26.51
Cr /April/2010	1.71	26.51
PR/April/2010	1.75	23.39
SG/April/2010	3.87	22.75
H/August/2010	-	21.50
NA/August/2010	-	23.67
Cr/August/2010	-	23.67
PR/August/2010	-	21.25
SG/August/2010	-	20.25
H/December/2010	9.87	25.30
NA/December/2010	6.85	27.75
Cr/December/2010	6.85	27.75
PR/December/2010	4.03	24.31
SG/December/2010	12.10	25.33
H/April/2011	1.08	24.10
NA/April/2011	1.28	27.25
Cr/April/2011	1.28	27.25
PR/April/2011	-	24.00
SG/April/2011	1.42	22.75

H-Hidrolândia; NA- Nova América; Cr- Crixás; PR-Pires do Rio; SG- São Gonçalo do Abaeté.

Climate data

The average of temperature and daily precipitation (Table Nº 4) were collected from the official site of National Institute for Space Research (Instituto Nacional de Pesquisas Espaciais-INPE).

Chemical variability

The relationship between components found in leaf essential oils (dependent variables) from *M. tomentosa* and environmental factors (independent variables) were investigated by stepwise Multiple Regression and Canonical Correlation Analysis implemented using SAS GLM and SAS CANCORR procedure, respectively (Draper and Smith, 1981). Cluster Analysis was also applied to the study of similarity of samples on the basis of constituent distribution and hierarchical clustering was performed according to Ward's variance minimizing method (Ward, 1963). For these procedures were used the softwares SAS (Statystical Analysis System) and STATISTICA 7.

RESULTS AND DISCUSSION

Essential oils yields varied yield of 0.1–0.8% (wt/wt), and these yields are similar to other members of Myrtaceae family (Gottlieb *et al.*, 1970, Pino *et al.*, 2002). In total, 40 compounds were identified, however just the components that appear in most amounts or with more frequency were chosen for statistical analysis. The following compounds were selected: β-caryophyllene (RI = 1414), γ-muurolene (RI = 1476), bicyclogermacrene (RI = 1491), δ-amorphene (RI = 1517), spathulene (RI = 1571), globulol (RI = 1586) and (2*E*, 6*E*)-methyl farnesoate (RI = 1776).

From the stepwise Multiple Regression, were obtained the following equations with the significative (*p*-value less than 0.05) variables (l = leaf and s = soil):

(Eq.1) β-Caryophyllene (%) = 27.445 – 0.7998Nl – 0.0543Fel (R^2= 0.6101)

(Eq.2) γ-Muurolene (%) = 86.656 – 4.4112Ps – 67.246Pl + 2.0748Rainfall (R^2=0.7042)

(Eq.3) Bicyclogermacrene (%) = 41.488 – 22.355Pl – 0.0581Fel (R^2=0.4480)

(Eq.4) δ-Amorphene (%) = 0.3354 + 0.0365Fes (R^2=0.4732)

(Eq.5) Spathulenol (%) = -1.226 + 6.8707Cus – 0.9145Rainfall (R^2=0.6047)

(Eq.6) Globulol (%) = 33.614 – 6.3543 (H+Al) – 2.895Kl + 2.4456Cal – 0.0329Mnl (R^2=0.8746)

(Eq.7) (2*E*, 6*E*)-Methyl farnesoate (%) = 26.975 – 23.2Als + 29.525Cus – 9.1929Mgl – 0.0812Mnl (R^2=0.8030)

The only compound found in all samples was the β-caryophyllene (Table Nº 5), while the others components suffered more variability.

Results of Canonical Correlation Analysis (Table Nº 6 and Figure Nº 1) indicate the linear combination of the two sets of variables: oil components and environmental factors. The coefficients for the first set of linear combinations showed high correlation (0.9593). In this analysis the set of variables: Cus, Mns, Zns, Cul, Mnl, Znl were evaluated and the first two eigenvalues obtained were significant.

From the Multiple Regression and Canonical Correlation Analysis it is clear that the main factors that can influence the levels of the compounds analyzed were: Mnl, Znl, Cul, Mgl, Mnl, Kl, Cal, Ps, Pl, Fel, Fes, Cus, Mns, Zns and Rainfall.

The observed positive correlation between Mnl and Mns with spathulenol (Table Nº 6 and Figure Nº 1) is in agreement with the requerement of sesquiterpene synthases for a divalent metal ion as cofactor (Picaud *et al.*, 2005). However, the formation of sesquiterpenes such as germacrenes D and B and bicyclogermacrene by germacrene D synthase in ginger (*Zingiber officinale* Roscoe) is stimulated by Mg^{2+} as cofactor and inactive with Cu^{2+} ions, that is in agreement with negative correlation between bicyclogermacrene and Cu present in soil (Picaud *et al.*, 2006).

As regards the relationship between P and essential oil compounds, it has been reported that reduced levels of P causes and increased production of different secondary metabolites (Kosiński, 1996). The observed correlation is in agreement with equations 2 and 3, wherein the two sesquiterpenes have the percentage increased in accordance with the reduction of P levels in leaves and soil.

Table Nº 5
Percentage of chemical constituents analyzed of *Myrcia tomentosa* leaf oils collected in five sampling sites of the Central Brazilian Cerrado.

Sample	β-Caryophyllene	γ-Muurolene	Bicyclogermacrene	δ-Amorphene	Spathulenol	Globulol	(2*E*, 6*E*)-Methyl farnesoate
H/April/2010	3.43	18.46	8.19	3.47	0.32	0.69	5.33
NA/April/2010	2.46	-	-	-	6.49	-	-
Cr /April/2010	7.48	-	10.39	3.60	-	33.93	-
PR/April/2010	1.74	-	-	-	20.09	-	44.23
SG/April/2010	3.69	-	-	-	-	9.09	1.51
H/August/2010	2.22	0.23	-	1.94	18.35	7.66	46.38
NA/August/2010	1.19	2.15	-	10.47	6.60	17.28	0.92
Cr/August/2010	1.36	1.86	-	1.71	3.69	67.46	-
PR/August/2010	1.74	-	-	-	20.00-	-	44.23
SG/August/2010	12.46	39.65	13.42	6.74	-	2.57	1.05
H/December/2010	5.01	25.78	-	2.97	0.73	2.30	35.50
NA/December/2010	1.92	14.20	14.38	18.83	-	2.92	-
Cr/December/2010	7.79	10.02	11.55	2.61	-	41.32	-
PR/December/2010	5.33	11.19	10.08	1.80	1.81	1.69	48.30
SG/December/2010	12.66	40.16	13.74	6.31	-	2.38	1.37
H/April/2011	7.10	-	14.72	4.85	10.68	-	-
NA/April/2011	0.80	5.14	5.92	13.07	5.55	3.85	-
Cr/April/2011	3.76	6.52	8.04	2.69	1.87	57.48	-
PR/April/2011	3.07	7.67	5.85	1.36	4.98	1.51	60.69
SG/April/2011	6.44	19.19	17.32	5.43	15.30	5.62	-

H-Hidrolândia; NA- Nova América; Cr- Crixás; PR-Pires do Rio; SG- São Gonçalo do Abaeté.

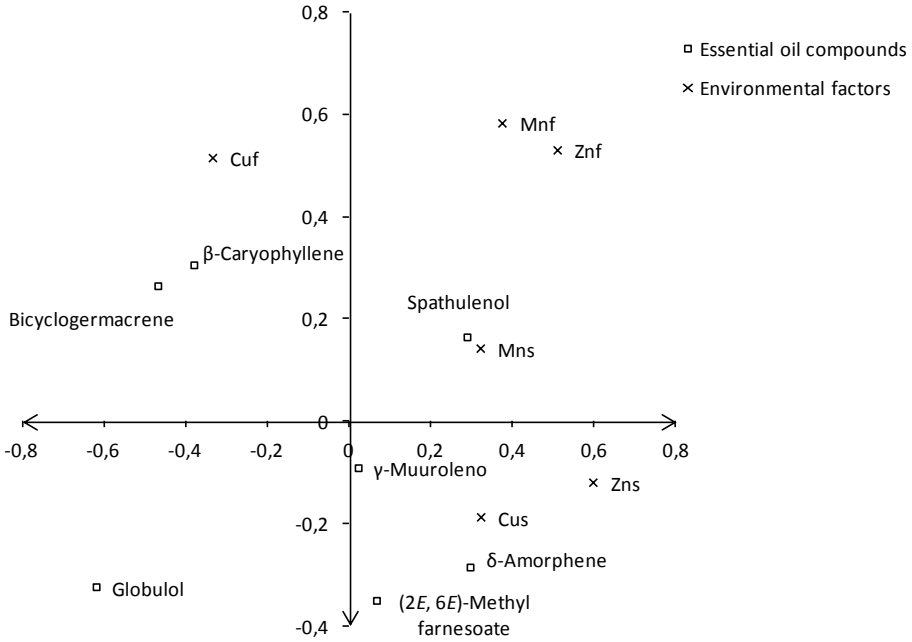

Figure Nº 1
Canonical Correlation Analysis scatterplot of *M. tomentosa* originating from five sampling sites. Axes refer to loadings from canonical coefficients.

Table Nº 6
Canonical Correlation Analysis summary of *Myrcia tomentosa* leaf essential oils.

Eigenvalues	Canonical correlation	F-ratio	P-value
(1) 11.5614	0.9593	2.63	0.0018
(2) 5.6083	0.9212	1.97	0.0288

Canonical variate (V1)	**Canonical variate (W1)**
β-Caryophyllene = -0.3805	Cus= 0.3216
γ-Muurolene = 0.0217	Mns= 0.3206
Bicyclogermacrene = - 0.4687	Zns= 0.5961
δ-Amorphene = 0.2956	Cul= - 0.3349
Spathulenol = 0.2881	Mnl= 0.3736
Globulol= - 0.6197	Znl= 0.5089
(2*E*, 6*E*)-Methyl farnesoate = 0.0664	

Canonical variate (V2)	**Canonical variate (W2)**
β-Caryophyllene = 0.3064	Cus= - 0.1867
γ-Muurolene = - 0.0906	Mns= 0.1434
Bicyclogermacrene = 0.2655	Zns= - 0.1189
δ-Amorphene = - 0.2849	Cul= 0.5162
Spathulenol = 0.1654	Mnl= 0.5841
Globulol= - 0.3234	Znl= 0.3213
(2*E*, 6*E*)-Methyl farnesoate = -0.3501	

Wilks' Lambda
0.0014

In fact, results obtained from Hierarchical Cluster Analysis employing Ward's method showed high chemical variability within the leaf essential oils of *M. tomentosa*. Dendrogram shown in Figure Nº 2, indicate the similarities on the basis of chemical composition, site and date of collection. Collection site is the main factor that affect chemical composition, while time effect it the minor proportion, due the similarities profile. The collection time seems to have a minor effect on the chemical variability of the samples due of the similarity found between the different months in each specimen. The Brazilian Cerrado is characterized by two seasons: dry (April to September) and wet (October to March), however was found a low influence of climatic data on chemical variability of leaf essential oils of *M. tomentosa*.

CONCLUSIONS

The chemical variability in leaf essential oils of *M. tomentosa* determined by multivariate techniques may reflect environmental influence on leaf oil composition, although it may also be influenced by genetic factors. The main factors which had relationship with essential oil components were: Nl, Fel, Pl, Kl, Fes, Cal, Mgl, Mnl, Als, Cus and Rainfall.

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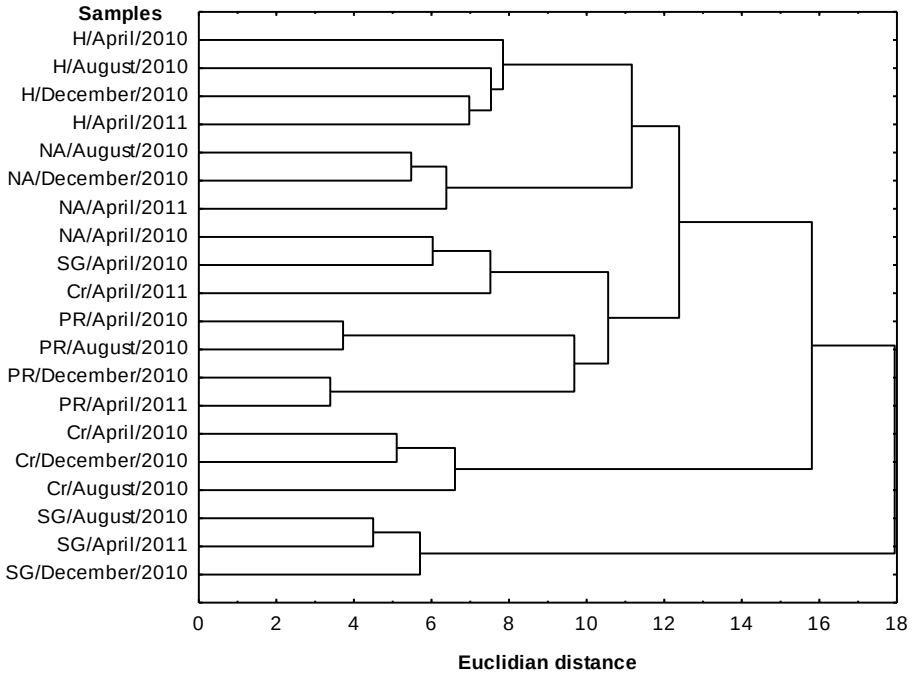

Figure Nº 2
Dendrogram representing the chemical composition similarity relationships of *Myrcia tomentosa* according to Ward's variance minimization method. H-Hidrolândia; NA- Nova América; Cr- Crixás; PR-Pires do Rio; SG- São Gonçalo do Abaeté.

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