Multivariate analysis of the essential oil components of the genus *Citrus* and their antifungal activity

Análise multivariada dos componentes dos óleos essenciais do gênero C*itrus* e sua atividade antifúngica

Marcos de Souza GOMES[1]; Maria das Graças CARDOSO[2], Paulo Estevão de SOUZA[3], Samísia Maria Fernades MACHADO[4], Lucilene Fernandes SILVA[5], Maria Luisa TEIXEIRA[6], Juliana de ANDRADE[7], Cíntia Alvarenga Santos Fraga de MIRANDA[8], Milene Aparecida ANDRADE[9]

[1] Doutorando; Universidade Federal de Lavras – UFLA; marcosopq@yahoo.com.br
[2] Doutora e Professora; Universidade Federal de Lavras – UFLA; mcardoso@dqi.ufla.br
[3] Doutor e Professor; Universidade Federal de Lavras – UFLA; pauleste@ufla.br
[4] Doutora e Professora; Universidade Federal de Sergipe – UFS; samisiamachado@yahoo.com.br
[5] Mestranda; Universidade Federal de Lavras – UFLA; lufernandes1000@hotmail.com
[6] Mestranda; Universidade Federal de Lavras – UFLA; teixeira_ml@hotmail.com
[7] Mestranda; Universidade Federal de Lavras – UFLA; juandrade_quimica@yahoo.com.br
[8] Doutoranda; Universidade Federal de Lavras – UFLA; cintiafmiranda@yahoo.com.br
[9] Doutoranda; Universidade Federal de Lavras – UFLA; mileneaandrade@hotmail.com

Abstract

The essential oils and their chemical components were analyzed using Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). The chemical identification and quantification of the essential oils based on gas chromatography coupled to mass spectrometry (GC/MS) displaying various substances correlated each other with limonene being the most predominant compound. We were able to clusterize the essential oils in three groups based on multivariate analysis. The first *Citrus sinensis* (orange) and *Citrus reticulata* (ponkan), the second *Citrus limonia* (lemon lime) and the third *Citrus aurantifolia* (lime) and *Citrus medica* (citron). Using the concentration 2000 µg mL^{-1}, the essential oil of the third group inhibited mycelial growth completely of the fungi *Fusarium oxysporum* and *Alternaria alternata*. On the other hand, using the same concentration, only the essential oil of the citron was able to inhibit the mycelial growth completely of fungi *Colletotrichum musae*. The study of essential oils is an important step to find and develop new biotechnology products using in the control of pests and other biotic (living) factors.

Additional keywords: Fungicide; limonene; natural products; phytopathogenic.

Resumo

Os óleos essenciais e seus componentes químicos foram analisados utilizando a análise de componentes principais (ACP) e a análise de agrupamentos hierárquicos (AAH). A identificação química e a quantificação dos óleos essenciais foram realizadas utilizando um cromatógrafo gasoso acoplado a um espectrômetro de massas (CG/EM) mostrando que várias substâncias se correlacionaram umas com as outras, sendo o limoneno o composto de maior predominância. Observou-se, por meio da análise multivariada de agrupamento, que os óleos essenciais foram divididos em três grupos. O primeiro *Citrus sinensis* (laranja) e *Citrus reticulata* (ponkan), o segundo *Citrus limonia* (limão-rosa) e o terceiro *Citrus aurantifolia* (limão-taiti) e *Citrus medica* (cidra). Utilizando a concentração de 2000 µg mL^{-1}, os óleos essenciais de terceiro grupo inibiram completamente o crescimento micelial dos fungos *Fusarium oxysporum* e *Alternaria alternata*. Por outro lado, utilizando a mesma concentração, somente o óleo essencial de cidra foi capaz de inibir completamente o crescimento micelial do fungo *Colletotrichum musae.* O estudo de óleos essenciais tem contribuído para obtenção de novos produtos biotecnológicos para uso de controle de pragas e outros bióticos.

Palavras-chave adicionais: Fitopatogênico; fungicida; limoneno; produtos naturais.

Introduction

The *Citrus* genus is native from the southeast region of the Asian continent, involving a group of plants belonging to Rutaceae family. Brazil stands out in the production of essential oils along with India, China and Indonesia, which are considered the four big world producers. The position of Brazil is due to the essential oils of *Citrus*, which are sub-products of the juice industry. In the world market, among the twelve main essential oils, orange, lemon and lime are highlighted (BIZZO & HOVELL, 2009).

The use of synthetic fungicides is one of the main control methods of plant diseases; therefore, its constant use can promote the selection of resistant phytopathogenic fungi, making it not have satisfactory efficiency anymore. Besides this problem, there is an increasing pressure of bodies and governmental institutions, mainly from developed countries in order to avoid the use of synthetic fungicides due to the high carcinogen effect of some of these products. Thus, a desirable alternative in relation to the traditional chemical treatment is the usage of natural products. The literature has registered in many works the efficiency of the essential oils of a large number of botanic species in promoting the development inhibition of several fungal plant pathogens (KIRBASLAR et al., 2009; SHARMA & TRIPATHI, 2008; VIUDA-MARTOS et al., 2008; CHUTIA et al., 2009). These, besides being viable, do not leave toxic residues to the humans and treated foods.

In many chemical experiments in order to obtain information about the results, the analysis of a large number of variables is necessary. The multivariate analysis aids to interpret these data in a more objective way, extracting from this quantity of variables and results, relevant information. The reduction of these variables, permitting the construction of bidimensional graphics containing further statistical information, can be obtained through the Principal Components Analysis (PCA). It is also possible, by using these variables, the construction of groupings among the samples according to their similarity, representing it in a bidimensional way through a dendogram.

Thus, the objective of this work was to realize an exploratory data analysis (essential oils of five species of *Citrus* genus and concentration of chemical constituents), using Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA), correlating them with their antifungal activity.

Experimental Part

Collect of the vegetal material and extraction of the essential oil

The vegetal materials, *C. aurantifolia* (lime), *C. limonia* (lemon lime) and *C. reticulata* (ponkan) , were collected in the orchard of the Federal University of Lavras (UFLA), and the *C. sinensis* (orange) peels and the *C. medica* (citron) were obtained from a restaurant of the city of Lavras-MG and a nearby property, respectively. The samplings were realized in the morning in the months of February and March of 2010. Healthy fruits and with a perfect and homogeneous appearance were collected.

The extraction process of the essential oils was realized in the Laboratory of Organic Chemistry of the Federal University of Lavras. The method used was the hydrodistillation, using a Clevenger device modified, coupled to a balloon with a round bottom with capacity of 5 L. The extraction was realized in triplicate during two hours, keeping the solution in boiling. After that, the hidrolact was collected, which was centrifuged in centrifugal horizontal crosshead at 965.36 X *g* for 10 minutes. Next, the essential oil was removed with a Pasteur micropipette, packed in amber glass bottle and stored under refrigeration. The extraction yield of the essential oils was calculated in p/p in moisture free basis (GUIMARÃES et al., 2008).

Chemical identification and quantification of the essential oils

The quantitative analysis and the identification of constituents of the essential oils were realized at the Federal University of Sergipe (UFS).

The quantitative analyses of the reaction mixture were realized in a gas chromatography equipped with detector of flames ionization (FID) Shimadzu GC-17A, under the following operational conditions: fused silica capillary column ZB-5MS (5% dimethylpolysiloxane) with 30 m x 0.25 mm i.d. x 0.25 µm of film, using He as gas carrier with flow of 1.2 mL min^{-1}. The temperature was kept at 50 °C for 2 minutes, and later increased 4°C min^{-1}, up to reaching 200°C. After that, increased 15 °C min^{-1}, up to reaching 300 °C, keeping this temperature steady for 15 minutes; the temperature of the injector was 250 °C and the temperature of the detector (or interface), 280 °C. It was injected a volume of 0.5 µL of the reaction mixture dissolved in ethyl acetate. The qualitative analysis of the reaction mixture was realized in gas chromatography coupled to a mass spectrometry GC-MS (Shimadzu, model QP 5050A), equipped with fused silica capillary column J&W Scientific (5%-phenyl-95%-dimethylpolysiloxane) 30 m x 0.25 mm i.d., 0.25 µm of film, using He as gas carrier, with flow of 1.2 mL min^{-1}. The chromatographic conditions of the analysis were the same used for GC-FID. The conditions of the MS were detector of ionic capture operating by electronic impact and impact energy of 70 eV; scan rate 1000; interval of decomposition of 0.50 fragments/s and detected fragments in the stripe from 40 to 500 Da. For the identification of the constituents, it was also used the comparison of their retention indexes with the ones of the literature (ADAMS, 2007). Two libraries NIST107 and NIST21 were also used, which permitted the comparison of the spectra database with the ones in the libraries.

Antifungal activity of the essential oils

The antifungal activity of the essential oils was realized at the Department of Epidemiology and Handling of the Phytopathology Department of the Federal University of Lavras. The phytopathogens were obtained at the mycology collection of the Phytopathology Department and registered with the following numbers: *F. oxysporum* CLM No. 1451; *A. alternata* CLM No. 1606; *C. musae* CLM No. 938. The biological essays were realized in triplicate using the fumigation method "in vitro", by which the effects of the essential oils of the five species of *Citrus* genus in different concentrations were evaluated, about the mycelial growth and / or inhibition of the fungal cultures. In a chapel aseptic of laminar flow, approximately 25 mL culture media potato-dextrose-ágar (PDA) was added in Petri plates with 9 cm of diameter. After the solidification of culture media, an inverted disk of 8 mm containing the mycelium of each fungus (removed from the colony with 12 days in BDA), was placed in the center of each plate. After that, the essential oil diluted in ethyl ether in a circular filter paper with 4 cm of diameter sticked to the up part of Petri plate was applied with a micropipette. The concentrations tested of the essential oils were 125, 250, 500, 1000 and 2000 µg mL^{-1}, according to PIMENTEL et al. (2010) with modifications. Parallely, two plates were prepared, one of them only with ethyl ether (relative witness) and the other one without treatment (absolute witness). The plates were sealed with plastic film, identified and incubated in germination chamber under photoperiod of 12 hours at 25 ^{o}C. The evaluations were realized after seven days of the assembling experiment, through measurements diametrically opposing (average of two measurements diametrically opposing) of the mycelial growth of the pathogen.

The effective concentration for inhibition of the mycelial growth in 50% (EC50) was estimate through the regression equation.

Statistical analysis

The experiments were submitted to the variance analysis; the significant quantitative variables by the F test were submitted to the regression analysis, and the qualitative ones to the medium test, compared by Tukey test. For the evaluation of extraction yield data, the completely randomized design was used, with three repetitions. For evaluation of the antifungal activity data, the completely randomized design was used with the treatments displaced in factorial scheme (5X6), being five essential oils and six concentrations with three repetitions. The data were analyzed by the statistical program Variance Analysis System of Balanced Data – SISVAR, according to FERREIRA (2003).

The Principal Components Analysis (PCA) and the Hierarchical Cluster Analysis (HCA) were used to comprehend the similarity among the essential oils in relation to the contents of their chemical constituents and their respective EC50 in relation to the pathogens. The analysis was realized by the program R (R DEVELOPMENT CORE TEAM, 2010).

Results and discussion

Yield of the essential oils

It was verified the variance analysis showed significant difference in relation to the yield (P>0.01), of the essential oils of the five species of *Citrus* genus. By the data of Table 1, it is observed there is no significant difference among the results of *C. sinensis* (orange), *C. reticulata* (ponkan) and *C. limonia* (lemon lime), which presented a higher yield than the ones of *C. aurantifolia* (lime) and *C. medica* (citron).

SHARMA & TRIPATHI (2006) found a yield of 1.8% of the essential oil of *C. sinensis*, similar to the one obtained in this work. Nevertheless, AHMAD et al. (2006), researching the essential oil of two varieties of *C. sinensis*, *C. limon* and *C. paradise*, found very different values from those obtained in this work, which was 1.21%, 0.98%, 1.12% and 0.73%, respectively. These variations can have occurred, according to BURT (2004), the difference of harvest period, type of soil, climate of the region and relative humidity of air on the day of the collect. According to MINH TU et al. (2003) the species yield of *Citrus* genus varied from 0.2 to 2%.

Table 1 - Essential oils yield of five species of the genus *Citrus: C. sinensis* (orange), *C. reticulata* (ponkan), *C. limonia* (lemon lime), *C. aurantifolia* (lime) and *C. medica* (citron).

Citrus species	Yield of essencial oils (%)
C. sinensis (Orange)	2.21 a
C. reticulata (Pokan)	2.08 a
C. limonia (Lemon lime)	1.81 a
C. aurantifolia (Lime)	0.73 b
C. medica (Citron)	0.71 b

Means followed by same letter in columns do not differ by Tukey test at 5% probability.

Identification and quantification of the constituents of the essential oils of the five species of *Citrus* genus

The essential oils of the five species of *Citrus* genus, analyzed by gas chromatography coupled to a mass spectrometry (GC/MS), showed a large variety of constituents. These results are in accordance with the considerations described by LADANIYA (2008), who reported the existence of more than 150 compounds found in essential oils of *Citrus* genus*.* By the data analyzed in Table 2, it was observed that the major component of the five essential oils was the limonene.

Studying the essential oil of the peels from 6 cultivars of *C. sinensis*, HOSNI et al. (2010) found as the major component the limonene, with a percentage from 96.0% to 97.3%, similar value obtained in this work. The results are also in accordance with the one found by CHOI et al. (2000), who reported in the research the presence of 92.0% of limonene and 1.7% of myrcene in the essential oil of *C. sinensis*, obtained in Japan.

When the data of this work are compared with the ones of the literature, in relation to the chemical composition of the essential oil of *C.reticulata* peel (ponkan), they are according to SIMON et al. (2006) which reported as main components the limonene (84.8%), γ-terpinene (5.4%) and myrcene (2.2%).

Table 2 - Chemical composition of essential oils from the pells of five species of the genus *Citrus: C. sinensis* (orange), *C. reticulata* (ponkan), *C. limonia* (lemon lime), *C. aurantifolia* (lime) and *C. medica* (citron).

Compound	IR cal.	IR lit.	OR	PO	LL	LI	CI
			(%)				
α-thujene	924	930	-	0.12	0.28	0.28	1.05
α-pinene	931	939	0.24	0.56	1.25	1.09	2.44
sabinene	970	975	0.18	0.48	1.33	0.93	0.31
β-pinene	976	979	-	0.36	7.75	7.30	2.57
myrcene	988	990	1.48	1.62	1.31	1.02	1.77
octanal	1003	998	0.95	-	-	-	-
α-phellantrene	1005	1002	-	-	-	-	0.04
δ-3-carene	1008	1011	0.23	-	-	-	-
α-terpinene	1016	1017	-	0.11	-	-	0.46
p-cymene	1024	1024	-	0.21	1.59	4.55	8.96
limonene	1031	1029	93.82	84.53	69.42	49.71	39.41
1,8-cineole	1032	1031	-	-	2.61	0.56	0.27
(Z)-β-ocimene	1035	1037	-	-	-	-	0.71
(E)-β-ocimene	1045	1050	-	-	-	-	1.01
γ-terpinene	1059	1059	-	5.90	9.42	7.91	23.43
octanol	1070	1068	0.23	0.28	-	-	-
terpinolene	1084	1088	-	0.29	-	0.37	1.37
linalool	1099	1096	-	-	-	-	0.32
cis-limonene oxide	1131	1136	-	-	-	-	0.05
citronellal	1151	1153	-	-	0.37	-	0.25
terpinen-4-ol	1179	1177	-	0.32	0.71	0.94	0.44
α-terpineol	1194	1188	-	0.50	1.23	2.42	0.63
decanal	1207	1201	-	0.44	-	-	-
citronellol	1223	1225	-	-	-	-	1.07
nerol	1226	1229	-	-	-	-	0.28
thymol methyl ether	1229	1235	-	0.26	-	-	-
neral	1238	1238	-	-	-	-	3.29
geraniol	1250	1252	-	-	-	-	1.61
geranial	1268	1267	-	-	-	4.59	4.76
2-undecanone	1292	1294	-	-	-	-	0.14
neryl acetate	1357	1361	-	-	-	-	0.38
geranyl acetate	1377	1381	-	-	-	-	0.74
(E)-caryophyllene	1418	1419	-	-	-	0.56	0.44
α-trans-bergamotene	1431	1434	-	-	0.40	1.69	0.57
β-bisabolene	1506	1505	-	-	0.77	2.94	0.93
heneicosane	2088	2100	-	-	-	-	0.04
Total (%)			97.13%	95.98%	98.44%	90.16%	99.74%

IR cal. - Kovats index calculated (ADAMS, 2007); IR lit. - retention index of literature; OR - Orange; PO - Ponkan; LL - Lemon lime; LI - Lime; CI - Citron

CAVALCANTI et al. (2004), studying different essential oils with insecticides activity, found the limonene (82.0%), followed by β-pinene (6.6%), as the main components of the essential oil of *C. limonia* peels. The results obtained in this work corroborate with MINH TU et al. (2002), who found as the main components of the essential oil of *C. limonia* peels the limonene (71.0%), γ-terpinene (11.9%) e β-pinene (3.7%).

CHOI et al. (2000), evaluating the antioxidant activity of several species of *Citrus* genus, identified and quantified, by CG/EM, as the main components of essential oils of lime peels the limonene (50.5%), followed by γ-terpinene (17.7%), β-pinene (13.4%), α-pinene (3.6%) geranial (2.1%) and neral (1.0%). According to SAWAMURA (2010), the main components of the essential oils of *C. medica* peels are limonene and γ-terpinene, showing a variation in their composition from 55.0 to 60.0% and 22.0 to 24.0%, respectively. These results confirm the ones obtained in this work.

Principal components analysis (PCA) and hierarchical cluster analysis (HCA)

The analysis of chemical data by the technique of PCA permitted to group chemically the samples in three groups, in a way to express and evidence their similarities and differences. It was observed with the first main component and the third main one, it was possible to describe 95.7% of the data, being 94.3% of the total variance described by the first main component (Figure 1).

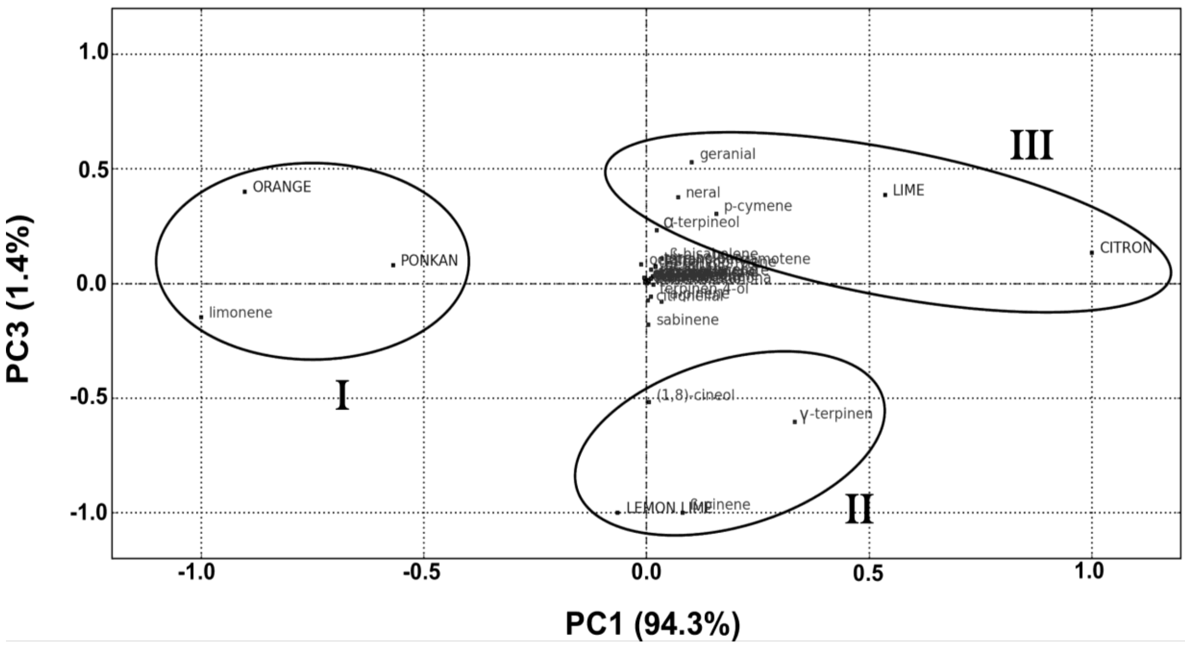

Figure 1 - Biplot graph, PC3 versus PC1, using loadings and scores for different essential oils of *Citrus sinensis* (orange), *C. reticulata* (ponkan), *C. limonia* (lemon lime), *C. aurantifolia* (lime) and *C. medica* (citron), in relation to different concentrations of the chemical constituents.

In the Figure 1, the biplot graphic PC1 x PC3 of the loadings and scores is presented, relating the data of chemical constituents of the essential oils of the five species of *Citrus* genus with their essential oils, in order to correlate them, grouping them in 3 groups. It is observed the samples of the essential oils of *C. sinensis* (orange) and *C. reticulata* (ponkan) (Group I) were closer due to the similarities of the contents of the limonene component. The same way, this similarity between the essential oils of *C. aurantifolia* (lime) and *C. medica* (citron) can be observed (Group III) due to the similarities in relation to the contents of the component neral, geranial, α-terpineol and p-cymene. In relation to the essential oil of *C. limonia* (lemon lime) (Group II) there was a similarity in relation to the content of the component γ-terpinene, (1-8)-cineole and β-pinene. Similar results were presented by VERZERA et al. (2005), who studied the chemical composition of the essential oils of *C. medica*, *Citrus meyerii*, *C. limon* peels and two hybrids (1 and 2) of lemon. These researchers observed the analysis of the main components permitted to group chemically the samples of the essential oils in 3 groups (A, B and C), in order to express and evidence their similarities and differences. It is possible to notice in this work the essential oil of *C. medica* was correlated with the components α-terpinene and geranil acetate (Group B), the essential oils of *C. limon* and hybrid 2 were correlated with the components β-pinene and neryl acetate (Group C) and finally, the essential oils of *C. meyerii* and hybrid 1 were correlated with the component limonene.

ALLEGRONE et al. (2006), studying the chemical composition of the volatile compounds of lemon juice from four cultivars of *C. limon*, observed mainly the presence of monoterpenes, sesquiterpenes and oxygenated monoterpenes (aldehydes, alcohols and esters). These cultivars were obtained in Italy, in the region of Sicíly, and by the multivariate analysis, these researchers also found similar results to the ones obtained from this work. It is possible to observe the volatile components of these cultivars were divided into three groups: limonene (Group I), β-pinene and γ-terpinene (Group II) and neryl acetate, α-terpineol and borneol (Group III). This way, LOTA et al. (2002), analyzing the constituents of the essential oils of the peels from 19 cultivars of *C. limon*, verified through the analysis of main components, which these were divided into three groups: in the first group were the species that grouped due to the concentration of the limonene; in the second one were the species that grouped due to the concentration of linalyl acetate and linalool; and in the third one were the species that grouped due to the concentration of β-pinene and γ-terpinene.

The dendogram of Figure 2, for the essential oils of the five species of *Citrus* genus studied in this work, corroborates with the results previously discussed in Figure 1. The existent grouping between the essential oils of *C. sinensis* (orange) and *C. reticulata* (pokan) (Group I), *C. limonia* (lemon lime) (Group II) and *C. aurantifolia* (lime) and *C. medica* (citron) is evident (Group III). This means the chemical composition of these essential oils grouped is similar.

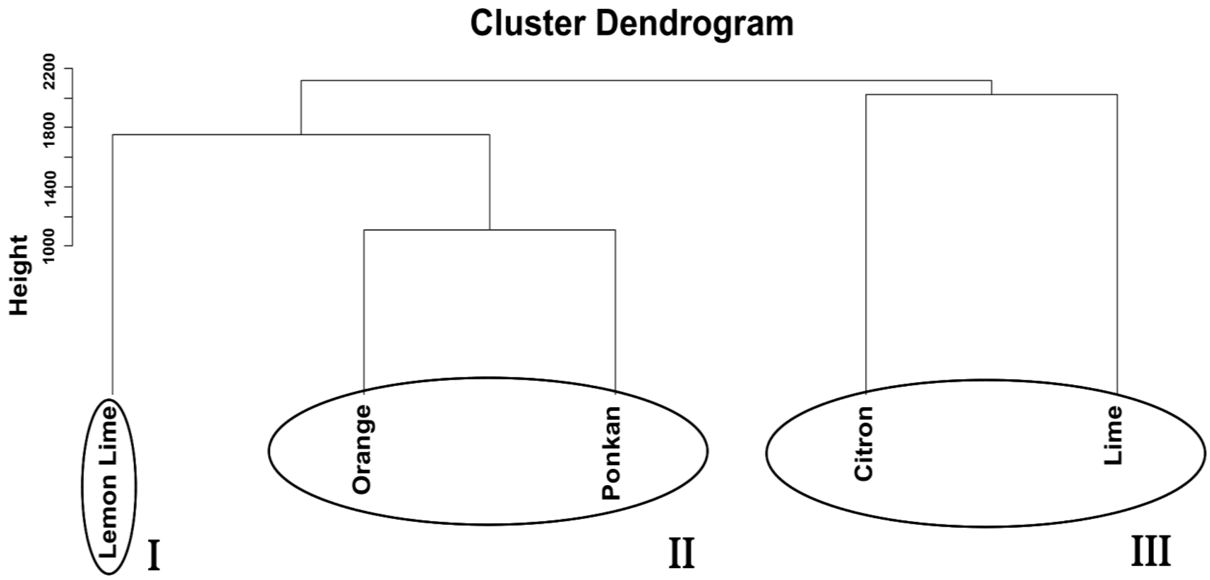

Figure 2 - Dendogram of different essential oils of *Citrus. sinensis* (orange), *C. reticulata* (ponkan), *C. limonia* (lemon lime), *C. aurantifolia* (lime) and *C. medica* (citron), in relation to the quantities of chemical constituents. The circles, I, II and III showed the groups of *Citrus* identified.

Antifungal effect of the essential oils of the five species of Citrus genus

The antifungal activities of the essential oils of *C. sinensis* (orange), *C. reticulata* (ponkan), *C. limonia* (lemon lime), *C. aurantifolia* (lime) and *C. medica* (citron) were studied about the growth and/or mycelial inhibition of the phytopathogens *F. oxysporum, A. alternata and C. musae*.

It was observed in the fumigation test "in vitro" realized about phytopathogens *F. oxysporum*, *A. alternata and C. musae*, which by variance analysis there was a significant difference ($p<0,05$). The five essential oils showed inhibition on mycelial growth of the fungus. Through the regression analysis, it was observed the inhibition increased with the increase of concentrations of the essential oils, in other words, an effect dependent dose was obtained in relation to the treatment tested.

The essential oils of *C. medica* (citron) and *C. aurantifolia* (lime) showed a total inhibition of the mycelial growth of the fungi *F. oxysporum* and *A. alternata* in the concentration of

2000 μg mL^{-1}, while the other essential oils in the same concentration presented a smaller inhibitory effect. On the other hand, for the fungus *C. musae* only the essential oil of citron is that obtained a total inhibition of the mycelial growth in the concentration of 2000 μg mL^{-1}.

The results obtained in this experiment corroborate with the ones obtained by some authors, such as VIUDA-MARTOS et al. (2008) who studying the essential oil of lemon, orange, pummelo and mandarin on the fungi associated with the deterioration of foods, they verified a huge potential antimicrobial. In the same period, similar studies realized by SHARMAN & TRIPATHI (2008), showed the inhibition of the mycelial growth of the fungus *A. niger*, when the essential oil of orange peels was used. The same authors in 2006 reported the essential oil of *Citrus* genus is a mixture of volatile compounds with huge potential antifungal, totally reducing or inhibiting its growth, being it dependent-dose.

It was verified (Figures 3, 4 and 5) (Tables 3, 4 and 5), by the regression analysis of the five essential oils, which EC50 of the essential oil of *C. medica* (citron) was lower in relation to the phytopathogens *F. oxysporum* and *C. musae* (565,9 μg mL^{-1} and 914,2 μg mL^{-1}). On the other hand, for phytopathogen *A. alternata* the essential oil of *C. aurantifolia* (lime) was the one that obtained the lowest EC50 (895,3 μg mL^{-1}).

The effective concentration for inhibition of the mycelial growth in 50% was also estimate by CACCIONIA et al. (1998), who verifying the fungicide activity of the essential oils of the peels of several species of *Citrus* genus, found some results in a similar way. For the six cultivars analyzed of the species *C.sinensis*, they observed the values of EC50, on the fungus *Penicillium digitatum*, and the values of oxygenated monoterpenes quantities were inversely proportional. As the value of EC50 decreased, the quantity of oxygenated monoterpenes increased. The values of EC50 and oxygenated monoterpenes for the six cultivars were 2180; 1594; 1496; 1004; 2245; 2389 μg mL^{-1} and 1.26%; 2.50%; 1.31%; 3.90%; 0.90%; 1.29% respectively. For the essential oils of the three cultivars of *C. limon*, it was observed the higher the content of citral, the more efficient it was against the pathogen. These results corroborated with the ones of this work, in which the most efficient essential oils were *C. medica* (citron) and *C. aurantifolia* (lime), the only ones that contained citral in their composition. Other authors attribute this activity to the presence of the compounds limonene, linalool and citral (SONBOLI et al., 2006). However, some of them describe the minority components have an important role in the antimicrobial activity of the essential oil, resulting from their synergism or antagonism with the major components (DEBA et al., 2008). This can be observed in this work because the component limonene, major in all the essential oils studied, had a lower antifungal action, and when it was combined with the component citral, its action was potentized.

Figure 6 presents the dendogram of EC50 of the five essential oils in relation to the three phytopathogens analyzed, confirming the results previously discussed, showing the existent grouping between lime and citron (Group I), meaning the two essential oils had EC50 much closer.

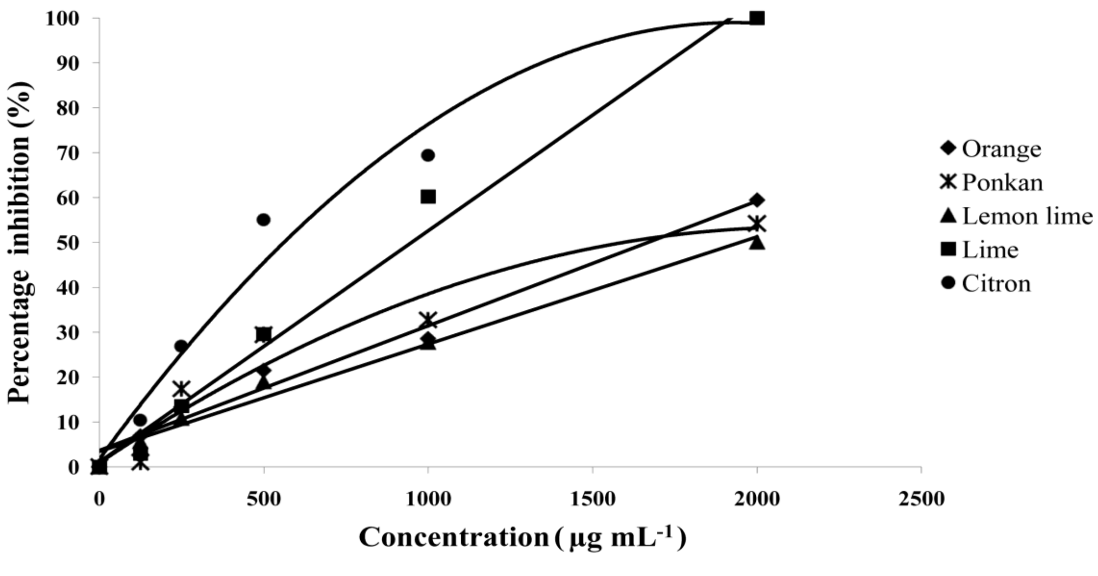

Figure 3 - Antifungal activity of different essential oils of *Citrus. sinensis* (orange), *C. reticulata* (ponkan), *C. limonia* (lemon lime), *C. aurantifolia* (lime) and *C. medica* (citron), on the mycelial growth of the plant pathogen *Fusarium oxysporum* exposed to different concentrations.

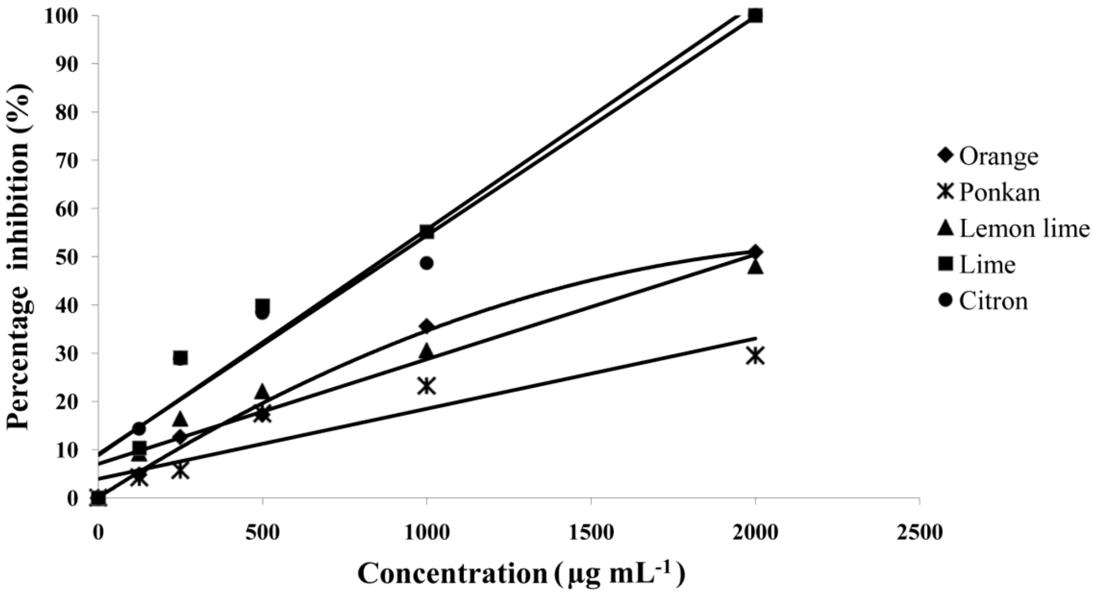

Figure 4 - Antifungal activity of different essential oils of *Citrus sinensis* (orange), *C. reticulata* (ponkan), *C. limonia* (lemon lime), *C. aurantifolia* (lime) and *C. medica* (citron), on the mycelial growth of the plant pathogen *Alternaria alternata* exposed to different concentrations.

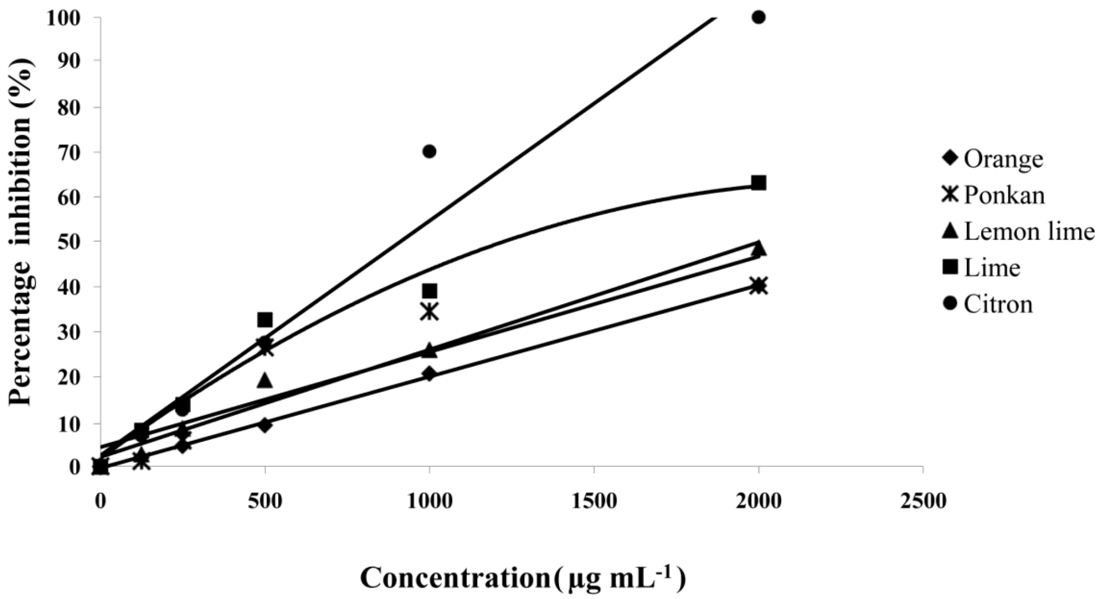

Figure 5 - Antifungal activity of different essential oils of *C. sinensis* (orange), *C. reticulata* (ponkan), *C. limonia* (lemon lime), *C. aurantifolia* (lime) and *C. medica* (citron), on the mycelial growth of the plant pathogen *Colletotrichum musae* exposed to different concentrations.

Table 3 - Regression equations, coefficient of determination and effective concentration for inhibition of mycelial growth by 50% (EC50) of the plant pathogen *Fusarium oxysporum.*

***Citrus* species**	**Regression equation**	**R^2**	**EC50 (μg mL^{-1})**	
C. sinensis (Orange)	$y = 0.027x + 3.648$*	0.98	1716.7 μg mL^{-1}	*F. Oxysporum*
C. reticulata (Pokan)	$y = -1.1x10^{-5} x^2 + 0.049x + 0.918$*	0.94	1521.6 μg mL^{-1}	
C. limonia (Lemon lime)	$y = 0.023x + 3.416$*	0.98	2025.4 μg mL^{-1}	
C. aurantifolia (Lime)	$y = 0.051x + 1.107$*	0.99	958.7 μg mL^{-1}	
C. medica (Citron)	$y = -2.6x10^{-5}x^2 + 0.1x + 1.732$*	0.98	565.9 μg mL^{-1}	

*Significant at 1% probability

Table 4 - Regression equations, coefficient of determination and effective concentration for inhibition of mycelial growth by 50% (EC50) of the plant pathogen *Alternaria alternata.*

Citrus **species**	**Regression equation**	R^2	**EC50 (µg mL^{-1})**	
C. sinensis (Orange)	$y = 9 \times 10^{-6}x^2 + 0.043x + 0.1$*	0.99	1985.0 µg mL^{-1}	*A.alternata*
C. reticulata (Pokan)	$y = 0.014x + 3.933$*	0.86	3290.5 µg mL^{-1}	
C. limonia (Lemon lime)	$y = 0.021x + 7.037$*	0.94	2045.9 µg mL^{-1}	
C. aurantifolia (Lime)	$y = 0.046x + 8.818$*	0.96	895.3 µg mL^{-1}	
C. medica (Citron)	$y = 0.045x + 9.052$*	0.96	909.9 µg mL^{-1}	

*Significant at 1% probability

Table 5 - Regression equations, coefficient of determination and effective concentration for inhibition of mycelial growth by 50% (EC50) of the plant pathogen *Colletotrichum musae.*

Citrus **species**	**Regression equation**	R^2	**EC50 (µg mL^{-1})**	
C. sinensis (Orange)	$y = 0.02x + 0.361$*	0.99	2518.1 µg mL^{-1}	*C. musae*
C. reticulata (Pokan)	$y = 0.021x + 4.302$*	0.80	2176.1 µg mL^{-1}	
C. limonia (Lemon lime)	$y = 0.023x + 2.101$*	0.98	2082.6 µg mL^{-1}	
C. aurantifolia (Lime)	$y = -1.2^{-5}x^2 + 0.05x + 1.834$*	0.97	1279.3 µg mL^{-1}	
C. medica (Citron)	$y = 0.052x + 2.463$*	0.96	914.2 µg mL^{-1}	

*Significant at 1% probability

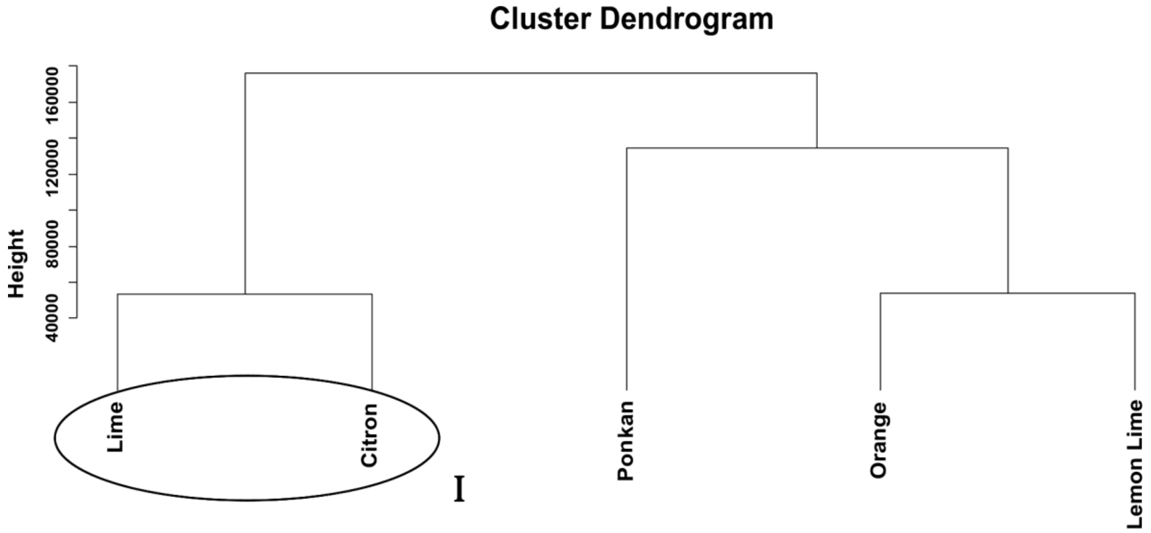

Figure 6 - EC50 dendogram of different essential oils of *C. sinensis* (orange), *C. reticulata* (ponkan), *C. limonia* (lemon lime), *C. aurantifolia* (lime) and *C. medica* (citron). The circle I showed the group of *C. aurantifolia* (lime) and *C. medica* (citron) identified.

Conclusions

The major compound of the essential oils of the five species of *Citrus* genus was the limonene.

Through the Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA), the essential oils were classified in three groups; the first one, *C. sinensis* (orange) and *C. reticulata* (ponkan), due to the similarity of contents of the component limonene; the second one, *C. limonia* (lemon lime), due to the similarity in relation to the content of the component γ-terpinene, (1-8)-cineole and β-pinene; and the third one, *C. medica* (citron) and *C. aurantifolia* (lime), due to the similarity in relation to the contents of the components neral, geranial, α-terpineol and p-cimene.

All the essential oils had antifungal activity on the phytopathogens *F. oxysporum*, *A. alternata* and *C. musae*.

The essential oils of *C. medica* (citron) and *C. aurantifolia* (lime), presented lower EC50 due to the existent synergism among the components limonene, neral and geranial.

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