Study of seasonality and location effects on the chemical composition of essential oils from *Eugenia uniflora* leaves

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Received 3 May, 2021; Accepted 22 July, 2021

The essential oils of *Eugenia uniflora* leave possess several biological activities but a great variability in their chemical composition is observed. In the present study, gas chromatography and mass spectrometry were applied to examine the essential oil from leaves of four different specimens over the seasons of the year, two of which are located in a habitat of a Brazilian metropolis, and the other two in a natural reserve. The collected data allowed identifying twenty-nine compounds; an aliphatic ketone, sesquiterpenes, fatty acids, hydrocarbons, and phthalate derivatives. Sesquiterpene hydrocarbons and oxygenated sesquiterpenes were the chemical classes prevailing in most samples. The curzerene was observed in higher content (10.5-53.4%) in all samples in which the presence of sesquiterpenes class was confirmed. Phthalate derivatives were identified for the first time in the essential oil of *E. uniflora* leaves. The occurrence of a common chemical marker for four specimens was not observed. Besides, no compound was observed in the same specimen throughout the seasons, and specimens of the same habitat exhibited different essential oil chemical profiles. According to the multivariate analysis applied to the chemical profile for all individuals in different seasons, four different clusters were identified without dependence on season or location.

**Key word:** *Eugenia uniflora*, seasonality, location, essential oil, chemical composition.

INTRODUCTION

The Myrtaceae family is comprised of more than 140 genera and about 5744 species distributed in different regions of the world (The Plant List, 2013a). *Eugenia* is the largest genus containing about 1011 species, of which 391 occur in Brazilian territory (COPPETEC-UFRJ, 2020; The Plant List, 2013b). *Eugenia uniflora* (‘pitanga’), *Eugenia involucrata* (‘cherry’), and *Eugenia caryophyllata* (‘clove’) are the most well-known species due to their...
importance in gastronomy and cosmetics. However, interesting pharmaceutical properties have been also reported involving their essential oils leaves. Specifically, *E. uniflora* is widely and popularly used due to its medicinal properties (Sobeh et al., 2019). Several pharmacological activities are reported for the essential oils from *E. uniflora* leaves such as cytotoxic, antibacterial (Figueiredo et al., 2019; Sobeh et al., 2016), antifungal (Siega, 2018; Souza et al., 2018), leishmanicidal (da Silva et al., 2018), molluscicide (Pinheiro et al., 2017), analgesic (Savegnago et al., 2015), antioxidant (Victoria et al., 2012), larvicide (Leite et al., 2009), repellent (Lobo et al., 2019), antiproliferative (Gomes et al., 2020), anti-inflammatory (Falcão et al., 2018), antinociceptive and hypothermic (Amorim et al., 2009). Some of these activities were associated with the leaf oil compounds, such as selina-1,3,7(11)-trien-8-one (36.37 %) and selina-1,3,7(11)-trien-8-one epoxide (27.32%) (dos Santos et al., 2018). The essential oil of *E. uniflora* leaf with red fruit, which inhibited the yeast form of *P. brasiliensis* at a concentration of 62.5 μg/ml, showed as major constituents curzerene (42.0-43.2%), germacrene D (8.7-9.0%), and germacrene A (5.9-8.9%) (Costa et al., 2010). The sesquiterpenes selina-1,3-7(11)-trien-8-one and oxidoselina-1,3-7(11-trien-8-one isolated from the essential oil of *E. uniflora* leaves showed pro-oxidative activity and citoxicity in relation to the IMR90 cell line (Ascari et al., 2021).

Some studies have addressed the effect of the location or the leaves collection period on the chemical profile. For example, in the essential oil of *E. uniflora* leaves collected in Nigeria, was observed the presence of caryophyllene (5.7%), germacrene B (5.8%), seline oxide-1,3,7 (11)-trien-8-one (14%), seline-1,3,7 (11)-trien-8-one (17%) and furanandene (24%) (Weyerstahl et al., 1988). On the other hand, the essential oil leaves collected in the northeast region of Brazil showed seline-1,3,7 (11)-trien-8-one oxide (17.3%) and seline-1,3,5 (11)-trien (48.5%) as the main components (de Morais et al., 1996). Moreover, the constitution of essential oil leaves collected at Belém (PA, Brazil) presented germacrene B (15.6%), germacrene and curzerene (30%) (Maia et al., 1999). According to Amorim et al. (2009), the essential oil also collected at Rio de Janeiro showed mainly the presence of atrac tylone, 3-furanandesemene, spathuleno, β-elemene, γ-elemene, globulol, ledene, and β-caryophyllene. The other side, the essential oil collected at Rio de Janeiro too (Brazil), presented as major compounds (+/-seline-1,3,7 (11)-trien-8-one and (+/-)-seline-1,3,7 (11)-trien-8-one epoxide (Marques et al., 2018). Due to the great variability in the essential oil chemical composition in different locations, some studies involving the search for understanding this problem can be found in the literature. However, the multivariate data analysis, specifically, Principal Component Analysis (PCA) has been extensively used to establish similarities and differences within groups of complex mixtures such as essential oils (Stashenko et al., 2010). Considering the use and the potential of *E. uniflora* essential oils in cosmetics area and or for ethnomedicinal purposes, it is important to understand the secondary metabolites production for the pharmaceutical and therapeutic industry benefits. In this sense, this work aims to evaluate the chemical profile of oils leaves over a year and try to establish correlations between different habitats of individuals.

**MATERIALS AND METHODS**

**Plant material**

*E. uniflora* leaves (about 60-70 g) from four specimens were collected on the tenth day after the beginning of each season 2018, two of them occurring near the Ayton Senna highway in the metropolitan city of Sao Paulo, adjacent to the School of Arts, Sciences and Humanities, the University of Sao Paulo (EACH-USP), according to the geographical coordinates: Lat: 23°29’09” S, Long: 46°30’306” O and Alt: 734 m for Eu1 (voucher SPFE 711) and Lat: 23°29’09” S, Long: 46°30’306” O (voucher SPFE 711) and Alt: 734 m for Eu3 (voucher SPFE 712). The other two selected specimens are located in a preserved region in the Municipal Park of Mogi das Cruzes (SP), according to the coordinates: Lat: 23°29’29” S, Long: 46°11’683” O and Alt: 867m for Eu2 (voucher SPFE 713) and Lat: 23°29’286” S, Long: 46°11’684” O and Alt: 848 m for Eu4 (voucher SPFE 714). The botanical identification was carried out by Dra. Fabiana Pioker (EACH, USP- East Campus), and dried samples of *E. uniflora* are deposited at the SPF Herbarium of the School of Arts, Science, and Humanities.

**Leaf essential oils extraction**

The hydrodistillation technique was applied to obtain the volatile oil from the fresh leaves using a Cleveenger-type apparatus. To extract the essential oils from each sample studied, 50 g of fresh leaves were ground and mixed with 1 L of water. The mixture was transferred to a 3 L round-bottom flask placed on a heating mantle connected to a condenser. After 4 h of heating, the essential oil was collected and dried over anhydrous sodium sulfate (ca. 1 g) (Morais et al., 2019). All these processes were performed once a time with each leaf sample. The essential oils were maintained in sealed vials in a freezer at - 27°C to preserve the chemical constituents.

**Chromatographic analysis**

The chromatographic analyses of the leaf essential oils were performed in a chromatographic system coupled to Shimadzu and Model QP 2020 mass spectrometer. The optimized operating conditions for these analyses were: ZBSHT capillary chromatographic column (30 × 0.25 mm × 0.25), mobile phase flow rate (He): 2.5 ml/min; injector Split ratio 1/25, and the injection volume of 3 μl. The operating temperatures of the equipment are as follows: injector at 260°C, detector at 280°C, and column at programmed temperature starting at 60°C for 1 min, 3°C/min rise to 220°C, later rise from 10°C/min to 280°C and remaining for 1.67 min. The conditions of the mass spectrometer employed were scan detector 1,000; scanning interval of 0.50 fragments and fragments detected in the range of 40 to 550 Da. To identify the chemical composition of the essential oil, two NIST107 and NIST21 libraries were used to compare the mass spectra data. The identification of each peak was assigned only when the similarity was above 80%. The retention index was calculated by reference to a solution of
the C8-C22 homologous series by the Van den Dool and Kratz equation (van Den Dool and Dec. Kratz, 1963). To obtain the relative percentages (%) by peak area normalization of the identified compounds, all samples were analyzed in gas chromatography with a flame ionization detector (GC-FID) using an Agilent CG 6850 system. GC was equipped with the same chromatographic conditions used in the CG-MS analysis.

Principal components analysis (PCA) and hierarchical cluster analysis (HCA) for identified chemical compounds in extracts

PCA and HCA from chemical profile (% w/w) of all essential oil obtained in different seasons and locations (Table 1) were performed. Both analyses aimed to identify similarities and differences among the samples, as well as the chemical reasons for samples' grouping. Scaling and mean-centering to the data were applied to the data before PCA. Ward's method was used for HCA. The multivariate data analyses were executed in SIMCA 16 software (Trial version, Sartorius Stedim Data Analytics AB Umeå, Sweden).

RESULTS AND DISCUSSION

Descriptive analysis of primary data

To evaluate the chemical profile of essential oil leaves among individuals of the same habitat, the identified chemical constituents and their contents are presented in Table 1. Thirty compounds present in the essential oil of specimens Eu1, Eu2, Eu3, and Eu4 were identified in the four seasons of the year by CG-MS analysis. The essential oils predominantly contain sesquiterpenes, phthalate derivatives, hydrocarbons, fatty acids, and some of their methyl esters. According to the chemical constituents’ data, the essential oils of E. uniflora leaves indicate the expressive presence of sesquiterpenes, while the Wi3 and Wi4 samples showed the identification of only hydrocarbons and/or phthalate derivatives. The Sp1 oil is comprised 38.1% of sesquiterpene hydrocarbons and 49.7% of oxygenated sesquiterpenoids. Nine components in total were identified in the Sp2, which were equal to 61.0% of its content. The chemical profile of the Sp2 oil was constituted mostly by 51.3% of oxygenated sesquiterpenes and 12.2% by oxygenated sesquiterpenoids. The Sp3 oil consisted mainly of 36.5% curzerene, followed by α-guaiene (7.7%) and δ-maillene (2.7%). The sesquiterpene hydrocarbons (49.8%) were the major components in the Su1 essential oil composition and 32.0% of oxygenated sesquiterpenes, mainly as β-elemene (35.6%), spathulenol (16.3%), germacr-3,7 (11),9-trien-6-one (15.7%). In the Su2 oil only two components were identified: eudesma-4(14),11-diene (8.4%), and α-selinene (3.6%). Oxygenated sesquiterpenes (51.6%) were the major components in the Su3 oil and sesquiterpene hydrocarbons (36.1%). Curzerene (26.2%) was the major constituent in this oil, followed by germacr-3,7 (11),9-trien-6-one (23.4%) and β-elemene (21.8%). In Su4 oil, oxygenated sesquiterpenes (50.5%) and sesquiterpene hydrocarbons (22.5%) were observed. The predominant component in this oil was curzerene (46.6%), followed by caryophyllene (8.4%) and β-elemene (8.2%). Sesquiterpene hydrocarbons (22.4%) were the major components in the Au1 oil composition, followed by 12.2% by oxygenated sesquiterpenes. This oil composition was marked by the presence of germacr-3,7 (11),9-trien-6-one (21.7%), β-elemene (16.3%), spathulenol (4.9%), viridiflor (4.6%), β-elemene (4.1%) and caryophyllene (2.0%). The chemical composition of the au2 oil showed 53.1% of oxygenated sesquiterpenes and 9.9% of sesquiterpene hydrocarbons. The oxygenated sesquiterpene curzerene was the major component in this oil (46.2%). In the Au3 was identified only the bis(2-ethylhexyl) phthalate (58.2%). Only two oxygenated sesquiterpenes, caryophyllene oxide (9.7%) and spathulenol (6.3%) were identified in the Au4 oil. The chemical composition of Wi1 oil showed 38.8% of oxygenated sesquiterpenes and 7.7% of sesquiterpene hydrocarbons. The oxygenated sesquiterpene curzerene was the major component (20.5%). The curzerene is the main component (53.4%) identified in the Wi2 oil. A typical chemical composition was observed to the Wi3 oil which showed mainly the presence of fatty acid derivatives (29.7%), phthalate derivatives (29.0%), hydrocarbons (26.2%), and only one oxygenated sesquiterpene (1.9%). The literature reports the importance of fatty acids as an important source of the reserve of energy and the essentiality on the membrane lipids composition of all living organisms, like the plants. Specifically, the role presence of fatty acids in plants involves metabolic pathways to pathogen defence or as a biosynthetic precursor for cuticular components (Kachroo and Kachroo, 2009). Thus, in this case, individuals 2 and 3 can undergo a possible attack from a predator and or the specimens(s) need to store energy during the drought in the winter period.

Another atypical chemical profile was observed in the Wi4 oil, since only phthalate and fatty acids derivatives were identified. Phthalate derivatives were also detected in Eu3 during fall and winter. Phthalate derivatives have been recognized as contaminants to be utilized as plasticizers and environmental hazards, particularly those with reduced molecular weight, and can be observed in the essential oil constitution (Manayi et al., 2014; Roy, 2020). According to Chen (2004), the difference between the natural and synthetic phthalate derivatives is the abundance of 14C. Phthalic acid diethyl ester (30) was isolated from Nigella glandulifera and Eichhornia crassipes (Nguyen et al., 2007; Shanab et al., 2010); while bis(2-ethylhexyl) phthalate (29) was isolated from N. glandulifera (Nguyen et al., 2007). There are various bioactivities of these natural phthalates derivatives such as antimicrobial, antioxidant, antitumor, larvicidal, and others (Roy, 2020).
Table 1. Individual compounds and main chemical classes identified in essential oil from *E. uniflora* in different seasons by CG-MS analysis and their percentage (% relative area).

| Compounds* coding | Name of the identified compound | RI (library) | RI (experimental) | Individuals* |
|--------------------|----------------------------------|--------------|------------------|--------------|
| 1                  | 2-Butanone                       | 313          | NC               | Sp1 0.0     |
| 2                  | δ-Maeline                        | 1380         | 1377             | Sp2 0.0     |
| 3                  | β-Elemene                        | 1387         | 1391             | Sp3 5.8     |
| 4                  | Caryophyllene                    | 1420         | 1415             | Sp4 2.0     |
| 5                  | α-Guaiene                        | 1439         | 1440             | Su1 0.0     |
| 6                  | g-Elemene                        | 1445         | 1431             | Su2 0.0     |
| 7                  | 7-Isopropenyl-1-methyl-4-        |              |                  | Su3 0.0     |
|                    | methylene decahydrulozulene       |              |                  | Au1 0.0     |
| 8                  | Eudesma-4(14),11-diene           | 1476         | 1480             | Au2 0.0     |
| 9                  | Germacrene D                     | 1482         | 1476             | Au3 0.0     |
| 10                 | α-Selinene                       | 1494         | 1489             | Au4 0.0     |
| 11                 | Curzerene                        | 1495         | 1498             | Wi1 0.0     |
| 12                 | Guai-1(10),11-diene              | 1500         | 1508             | Wi2 0.0     |
| 13                 | Selin-3(7)[11]-diene             | 1538         | 1541             | Wi3 0.0     |
| 14                 | Germacrene B                     | 1557         | 1551             | Wi4 0.0     |
| 15                 | Spathulenc                      | 1578         | 1579             |              |
| 16                 | Caryophyllene oxide               | 1581         | 1579             |              |
| 17                 | Viridiflorol                     | 1595         | 1593             |              |
| 18                 | β-Elemene                        | 1597         | 1600             |              |
| 19                 | Atractylon                       | 1652         | 1657             |              |
| 20                 | Germaca-3,7(11),9-trien-6-one     | 1683         | 1695             |              |
| 21                 | Myristic acid                    | 1768         | 1770             |              |
| 22                 | Palmitic methyl ester            | 1934         | 1931             |              |
| 23                 | Palmitic acid                    | 1970         | 1973             |              |
| 24                 | Margaric acid methyl ester       | 2008         | 2011             |              |
| 25                 | Phthalic acid, 2,4-Dimethylpent-3-| 2055         | 2050             |              |
|                    | yl isobutyl ester               |              |                  |              |
| 26                 | Heneicosane                      | 2100         | 2108             |              |
| 27                 | Stearic acid                     | 2158         | 2156             |              |
| 28                 | n-Pentacosane                    | 2500         | 2486             |              |
| 29                 | Bis(2-ethylhexyl) phthalate      | 2552         | 2555             |              |
| 30                 | Phthalic acid diocyl ester       | 2741         | NC               |              |

Total identified compounds (%) 89.8 64.7 56.2 46.9 81.5 12.0 87.7 74.2 53.6 63.0 58.2 16.0 46.5 53.4 84.9 61.7

Class of compounds

Sesquiterpene hydrocarbons (Sr. Nº. 2-10, 12-14) 25.7 13.4 4.1 10.4 12.6 12.0 14.3 22.5 6.1 9.9 - - 7.7 - - 3.0
In our case, the phthalate derivatives in some samples exhibited a high percentage, even higher than the sesquiterpene present in the essential oil. According to Table 1, these compounds’ class were the majority in the Wi3 and Wi4 samples. The results obtained from the essential oil analysis of four different individuals, indicate the absence of a biomarker compound, even in individuals from the same habitat. According to Costa et al. (2009), the seasonal study of the essential oils of E. uniflora leaves showed the presence of spathulenol and caryophyllene oxide in dry seasons and seline 1,3,7 (11)-trine-8-one as the major compound in rainy seasons. However, our data showed the occurrence curzerene in all individuals during spring, but not in other seasons. Moreover, it was the major metabolite or one of it in the composition of the Sp1, Sp2, Sp3 Sp4, Su3, Su4, Au2, Wi1, and Wi2 oils (9.7- 53.4%). The individual Eu3 showed a considerable increase in curzerene content from spring to summer (10.5-26.2%) and this was not observed in the other seasons. The highest content was observed during the winter (53.4%) for specimen 2.

The data presented in Table 1, it confirmed the predominance of several sesquiterpenes in different seasons for the studied individuals, except in winter for specimens 3 and 4, and autumn for 3. In the winter it was observed the presence of fatty acid derivatives. This is the first report of phthalate derivatives occurrence in the essential oil of E. uniflora leaf. The literature reports the presence of these compounds in the essential oil of other plants such as Silybum marianum seeds, Rizophora flowers (Saranya et al., 2015), and Calycotome villosa subsp. intermedia (Chikhi et al., 2014). There was no similarity between the chemical compositions of specimens from the same habitat. According to De Morais et al. (1996), the chemical composition of essential oils may differ depending on the necessity of the plant to adapt to its habitat and due to chemotypes.

### Principal components analysis (PCA) and hierarchical cluster analysis (HCA) for identified chemical compounds in essentials oils

The five more atypical samples were Wi3 (Group 1), Wi4 (Group 1), Su1 (Group 2), Sp1 (Group 3), and Su3 (Group 3). The remaining samples showed a similar chemical profile (Figure 1). This finding is also confirmed by the scores plot (Figure 2A). Overlapping score and loading plots (Figure 2A and B) with support of biplot graph for the first two principal components (Figure 3) is possible to identify that the Group 1 samples, which are from different locations in winter, were relatively plenty of 4 common compounds myristic acid (21), palmitic acid (23), stearic acid (27) and phthalic acid diocyl ester (30). However, the Wi3 sample was also abundant in phthalic acid 2,4-dimethylpent-3-yl isobutyl ester (25), heneicosane (26), and n-pentacosane (28), while the Wi4 sample was relatively rich in palmitic methyl ester (22) and margaric acid methyl ester (24). Su1 (Group 2) was comprised mainly by 2-butanone (1), guaia-1(10),11-diene (12), spathulenol (15), β-elemene (18), and germacr-3,7(11),9-triene-6-one (20). Group 3 samples from the same location and different seasons (spring and summer) were rich in γ-elemene (6), germacrene B (14) and viridiflorol (17). A significant number of samples had a high amount of curzerene (Table 1). This volatile oxygenated sesquiterpene has been identified as one of the major constituents in the essential oils of E. uniflora leaves, especially, when are derived from bright red fruit specimens (Costa et al., 2010). According to the literature, plants can exhibit changes on their chemical composition by the high CO2 levels, such as the increase monoterpane concentrations (Idso and Idso, 2000). However, the essential oils from individuals 1 and 3, located in area with high concentration of CO2 by vehicles emission, were not affected the secondary metabolites production. The PCA and
Figure 1. Dendrogram corresponding to HCA for the identified compounds by relative area percentage (%) of CG-MS analysis.

Figure 2. Score scatter (A) and loading (B) 3D plots corresponding to PCA for the identified compounds in the essential oil samples using relative peak area (%). The four main groups categorized by HCA were also identified using the same color scale (Figure 1).

HCA data showed the season and location are not the major factors to influence the chemical variability of essential oil composition from *E. uniflora* leaves. This chemical variability can be associated to the ecosystem differences, not only by the biotic and abiotic factors. The secondary metabolites production can depends on the climate changes, which can influence on the soil microflora, all pollinisers and other insects affecting the plant antogeny, adaptation, and including phytochemicals productivities (Thakur et al., 2019).

This should be taking into consideration for commercial applications, specifically in perfumery, where the
chemical composition of this feedstock has a paramount impact on final product quality attributes (Gallucci et al., 2010). Also, the essential oil of E. uniflora leaves is strongly recommended because of its powerful industrial or pharmaceutical properties, the high variability of essential oils chemistry from different specimens, should be considered during the chemical synthesis or biotechnological products manufacturing (Ochoa-Villarreal et al., 2016). Besides, none of the groups found by the multivariate techniques matched the chemotypes reported for this species (Costa et al., 2009).

**Conclusion**

In all essential oil samples from *E. uniflora* leaves, sesquiterpene hydrocarbons and oxygenated sesquiterpenes were prevalent, except for two specimens during the winter and autumn seasons. The curzerene, an oxygenated sesquiterpene, was the most abundant compound in samples with sesquiterpenes. The chemical compositions of specimens from the same habitat are different and without correlations, and there are no direct influence of metabolites production according to the type habitat. Considering the chemical profile variability of essential oils among specimens by principal components analysis, commercial applications should be manufactured using other approaches.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

**ACKNOWLEDGEMENT**

This work was supported by the Fundação de Amparo do Estado de São Paulo (Fapesp No 2016/05369-0).

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