New Reduction-Oxidation Indices Applied to Mixtures in the Impact on Seasonal and Circadian Rhythm Studies of The Essential Oil From Leaves of Piper Gaudichaudianum Kunth (Piperaceae) – A Folk Medicine and Ritualistic Plant

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**Abstract**

*Piper gaudichaudianum* Kunth (Piperaceae) is widely used in Brazil as medicinal and ritualistic. In this study, chemophenetic patterns were evaluated based on leaves' essential oils (EOs) chemical composition. Several collections were performed to accomplish circadian rhythm and seasonal studies. Besides, a predictive methodology was developed and submitted to Proof of Concept (PoC) to determine the metabolism pattern and evaluate the reduction-oxidation of complex mixtures: Weighted Average Redox Standard ($S_{RO}$) and General Mixture Redox Index ($GM_{RO}$). Fresh leaves EOs obtained by hydrodistillation were analyzed by GC-MS and GC-FID. The main identified compounds were sesquiterpenes. Nineteen terpene skeletons were registered. There was chemical composition variation at different phenological stages. EOs varied more between day and night than seasonally. Nine chemotypes are proposed based on our results and those from literature. $S_{RO}$ and $GM_{RO}$ analyzes highlighted a possible redox balance throughout day and night. Compounds per carbon skeleton diversification in EOs are matched by an increase in compounds $S_{RO}$. We also report for the first time high chemical phenotype plasticity based on EOs analysis and its implications for *P. gaudichaudianum* chemophenetics, chemosystematics and ecology.

**Introduction**

*Piper gaudichaudianum* Kunth (Sin. *Artanthe gaudichaudiana* (Kunth) Miq.; *Piper obscurnum* C.DC.) is a species belonging to the Piperaceae family, native to South America and widely distributed in Brazil, mainly in the Atlantic Forest (Guimarães, 2020; Queiroz; Guimarães, 2020). In terms of ecological importance, this species plays a significant role in its natural habitat as a nutritional source or as member of different biotic interactions (Ramos et al. 2009; Richards et al. 2016; Salazar et al. 2016a). In humid forests, *P. gaudichaudianum* leaves and fruits are an available food resource for different species of insects (Laroca; Lauer; Penz and Araújo, 1990; Figueiredo and Szazima, 2000; Braga et al. 2001; Pereira et al. 2019) and animals (Mikich et al. 2003; Biber et al. 2004; Parrini et al. 2017). In the literature, the correlation of this plant and its importance for feeding, reproduction and population effect of several bat species from the Brazilian Atlantic Forest are described (Marinho-Filho 1991; Mikich 2002; Mikich et al. 2003; Lima and Reis 2004; Almeida 2005; Bianconi et al. 2007; Leiner and Silva 2007; Mello et al. 2008; Bianconi et al. 2010; Barros et al. 2013; Leiser-Miller et al. 2020).

In Brazil *P. gaudichaudianum* is known as “Jaborandi”, “Falso-jaborandi” and “Pariparoba”. It has been described as a medicinal species since the 19th Century (Von Martius 1859). Ethnobotanical surveys show that infusions and fresh leaf chewing are used to dental pain relieve; leaf tea is used as a colagouge and digestive, and against tumors, joint pain and musculoskeletal diseases; tinctures are used to treat diseases of the skin, ears, nose, and oropharynx (Somavilla and Canto-Dorow 1996; Di Stasi et al. 2002; Zuchiwshi et al. 2010; Bolson et al. 2015). Also, this plant is known as an aromatic, and one of the main adulterants of the commercial medicinal species *Pilocarpus jaborandi* Holmes (Rutaceae) (Brandão et al. 2013). In the compendium of "Flora of Saint Germain" the essence of *P. gaudichaudianum* is indicated for "relaxation" and reduction of "mental rigidity" (Margonari 1999). This species is also used as ritualistic, known as “İlyeýe” in Afro-Brazilian religions, in smoked and preparation of baths and fermented drinks to initiate warrior deity “Orixá Xangó” (i.e. Shango) (Rwanda 1954; Guesdes et al. 1985; Barros 2015). Interesting to note that for the rituals of this religion, there is a rigor as to the time and form of harvesting *P. gaudichaudianum* leaves (Barros 2015). For example, for pleasure Xangó, the leaves must be collected between 12 p.m. and 6 p.m. (Rwanda 1954).

Previous phytochemical investigations show this species to be rich in essential oils (EOs), and extracts rich in terpenic alcohols, phytosterols, vitamin E (tocopherols), fatty acids, triterpenes, flavonoids, alkaloids, chromene and prenylated derivatives of benzoic acids (Rorig and Von Poser 1991; Lagos et al. 2004; Peres et al. 2006a; Peres et al. 2006b; Batista-Junior 2008; Lopes et al. 2007; Ramos et al. 2009; Batista et al. 2011). Several studies have demonstrated the antifungal, antibacterial, insecticidal, larvicidal, analgesic, anti-inflammatory, antileishmania and antituberculosis activities for extracts, fractions, and pure compounds from *P. gaudichaudianum* (Parram et al. 1997; Moreira et al. 2001, Di Stasi and Hiruma-Lima 2002; Lago et al. 2004; Morais et al. 2007; Puhl et al. 2011; Bernuci et al. 2016; Chaaban et al. 2018; Finato et al. 2018; Silva et al. 2019; Souza et al. 2020).

The EOs of *P. gaudichaudianum* comprise high percentual amounts of monoterpene, sesquiterpenes and aryipropanoids depending on the collection site (Von Poser et al. 1994; Andrade et al. 1998; Morais et al. 2007; Péres et al. 2009; Sperotto et al. 2013; Krinski and Foerster 2016; Schindler and Heinzmann 2017; Chaaban et al. 2018; Souza et al. 2020). Besides that, there are no approaches in the literature with the purpose of analyzing the chemical phenotypic plasticity for this species. Since *P. gaudichaudianum* has medicinal, ritualistic and ecological importance, as well as, it has a large spatial distribution in Brazil, it is necessary shed light on patterns of chemodiversity and factors that are involved in the chemogeography for this plant. We emphasize that, until now, there are no reports on the chemical variations of EOs from leaves of this species under the influence of the circardian rhythm, and there are no reports of these approaches in the Atlantic Forest in the Rio de Janeiro State (Brazil).

The influence of biotic and abiotic factors on the composition of EOs is well reported in the literature (Sangwan et al. 2001; Defaveri et al. 2011; Ramos et al. 2020; Karagoz et al. 2020). The challenge nowadays is the development of tools/methodologies to evaluate and to interpret phenomena around chemical phenotypic diversity and plasticity due to the influence of biotic and abiotic factors (Brückner and Heethoff 2017; Kessler and Kalske 2018; Zidorn 2019). Among the methods for assessing plasticity and chemotype at different spatial scales, there are: 1) α-chemodiversity indices - Shannon index (Shannon 1948; Gouyon et al. 1986; Mártonfi et al. 1994; Feng et al. 2020), Simpson's diversity index (Simpson 1949; Kfour et al. 2019; Feng et al. 2021), Pielou's uniformity index (Pielou 1966; Feng et al. 2020), and index of Iason's chemodiversity (Iason et al. 2005; Kfour et al. 2019); 2) β-chemodiversity indices - Sorensen index (Sørensen 1948; Feng et al. 2020), Jaccard index (Jaccard 1901; Feng et al. 2020, and Cody index (Cody 1975; Feng et al. 2020); 3) γ-chemodiversity indices - chemical similarity index (Salazar et al. 2016a); and indices of chemical difference in relative abundance - Rao index (Salazar et al. 2016b). All these parameters have a qualitative response to the absence or presence of compounds. It is notable that the equations of the cited indices do not express measures to predict the physicochemical and structural patterns of compounds in a complex mixture to assess the influence of excitatory factors and provide *in situ* phenetic description of the taxa (Zidorn, 2019).
For years, Chemotaxonomy have used data obtained from the chemical structures of micromolecules through evolutionary progress indices to establish evolutionary and phylogenetic trends (Reynolds 2007; Gottlieb et al. 2012). Gottlieb et al. (2012) developed a methodology to correlate the degree of oxidation of secondary metabolites and the biogenetic transformations of their micromolecular skeletons to unravel the functions, biogeography and systematic issues related to compounds for species in the plant Kingdom. Emerenciano et al. (1998) developed an index to assess the oxidative stages of reactions based on terpenoids, from the knowledge about biosynthesis and tools developed by Gottlieb. This index was used to EOs’ compounds by Sayuri et al. (2010). However, although the data provide that these analyzes have a quantitative characteristic, all their processing was based on qualitative data, on the premise of the presence or absence of the variety of compounds in the taxa (Emerenciano et al. 1998). Obviously, this was because at that time (1998) there was not much availability of data and quantification techniques with greater accuracy and precision for secondary metabolites analyzes. Currently, recognizing the importance of the process of reducing oxidation of metabolism for the survival of living organisms (Gottlieb and Kaplan 1993) and the exponential growth of metabolomic analyzes (Pilon et al. 2020), it is necessary the development of an index that can describe the homogeneity and the reduction-oxidation (redox) pattern in the production of the metabolism of a complex mixture for α, β and γ-chemodiversity assessments.

On this thoughts, this work aims to: a) evaluate for the first time the chemical composition, seasonal variation, and circadian rhythm of EOs from leaves of a natural population of *P. gaudichaudianum* in an area of Atlantic Forest in the city of Rio de Janeiro; b) Develop and submit to a Proof of Concept (PoC) a new predictive methodology to assess the redox of complex mixtures of compounds using the Weighted Average Redox Standard (S\textsubscript{WARS}) and the General Mixture Redox Index (GMRI); c) Set the variation and chemophenetic patterns in time and space scales for *P. gaudichaudianum* based on the EO analysis.

**Experimental Section**

**Plant material and experimental design**

Leaves from *Piper gaudichaudianum* Kunth were collected in the Atlantic Forest, in the Tijuca National Park region, Rio de Janeiro - RJ, Brazil (22°58’13” S, 43°14’34” W, Elevation: 452m) from January to December 2017. Authorization for the collection of botanical material was given by the Chico Mendes Institute for Biodiversity Conservation (ICMBio), number 57296-1. Samples of the fertile specimens were collected, identified and deposited with voucher number RB730964 at the Herbarium of the Botanical Garden of Rio de Janeiro (JBRJ), Rio de Janeiro, Brazil. This study was registered with the Genetic Heritage Management Council under identification AE20045. The experimental design consisted of twelve collections of leaves from specimens for the seasonality study and sixteen collections for the circardian rhythm study. For the seasonal study, 100 g of leaves were sampled monthly on the 15th day, at 9 a.m., from January to December 2017. For the study of circadian rhythm, samples were obtained from the same specimen every three hours, with collections performed at 12 p.m., 3 p.m., 6 p.m., 9 p.m., 00 a.m., 03 a.m., 06 a.m. and 09 a.m., in March 14th and October 15th, 2017, respectively. These two sequences of collections are related to the rainy and the dry seasons, respectively. Data on abiotic factors, including average temperature (°C), precipitation (mm), radiation (KJm\textsuperscript{-2}) and humidity (%) of the collection site were obtained from the Brazilian Institute of Metrology and Research (INMET) for the weather station (A652-OMM: 86887) and are shown in the supplementary material Figure S2.

**Essential oils obtaintion and analyses**

The collected leaves were manually crushed and subjected to hydrodistillation for two hours in a modified Clevenger-type apparatus. The EOs were dried over anhydrous sodium sulphate (Na\textsubscript{2}SO\textsubscript{4}, Sigma-Aldrich, Brasil) and the total EO yield was expressed as the percentage value related to fresh plant material (g/100 g) (Oliveira et al. 2013; Ramos and Moreira 2019; Ramos et al. 2020).

EOs were diluted in dichloromethane (1 mg/ mL) [Tedla, Brazil] and submitted to analyzes by Gas Chromatography coupled to Mass Spectrometry (GC-MS) to assist in the identification and GC coupled to a flame ionization detector (GC-FID) to compound quantification.

GC-MS analyzes were performed using the HP - Agilent 6890N gas chromatograph equipped with an automatic GC sampler 120 and coupled to a model 5973 (MS) mass spectrometer. The (5%-phenyl)-methylpolysiloxane capillary column (HP-5MS, 30 m x 0.25 mm I.D., 0.25 µm lm thickness) (Agilent J & W; GC columns, USA) was used for all analyzes. GC-MS conditions were injector temperature of 270°C; injection at 1 µL of the EO solution splitless; oven temperature programming from 60–240°C (3°C / min); Helium as carrier gas (> 99.99%), adjusted at a linear speed of 36.5 cm/ s (1.0 mL/ min); ionization by electron impact at 70 eV in positive mode; ionization source and transfer line temperature of 200 and 250°C, respectively. Mass spectra were obtained by automatic scanning every 0.3 s, with mass fragments in the range of 40 to 600 m/z (Oliveira et al. 2013; Ramos and Moreira 2019; Ramos et al. 2020).

Quantification of volatile constituents was obtained by normalizing the peak area with no correction and using an HP-Agilent 6890 GC Series device, coupled to the FID detector, operated under conditions similar to the GC-MS (Oliveira et al. 2013; Ramos and Moreira 2019). The retention index (RI) was determined from the retention time of a homologous series of n-alkanes (C\textsubscript{6}-C\textsubscript{20}, Sigma-Aldrich) obtained by GC-FID, under the same conditions of EO analysis. The compounds present in the volatile mixture were identified by comparing the fragmentation patterns of the mass spectra with database records (WILEY 7n, NIST) and comparing the calculated RI (Dool and Kratz 1963) with those from literature (Adams, 2017). In addition, co-injection with authentic standard wherever possible as described previously (Oliveira et al. 2013). All analysis were done in triplicate.

**Statistical and chemophenetic analysis**

All data on the percentage of compounds in the EO were reported as mean ± standard deviation for three independent experiments (extraction). For the analysis of circadian, seasonal and chemophenetic variations, the correlation coefficients between climatic and geographic parameters were calculated by yield, chemical classes, main constituents and their carbon skeletons. For correlation analysis, using the Kolmogorov-Smirnov test, the data set with normal distribution was performed by Pearson’s analysis and for those without normal distribution, the Spearman analysis was used. Statistical significance was assessed using the Tukey test (ANOVA by Tukey HSD post hoc test). To evaluate the oxidation state was calculated by the number oxidation (N\textsubscript{ox}) and
oxidative steps (OS) (Emerenciano et al. 1998). In addition to performing the proof of concept in the developed indices of Weighted Average Redox Standard ($S_{RO}$) and General Mixture Redox Index ($GM_{RO}$ or Ramos & Moreira’s index for mixtures). Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were applied to verify the interrelationship in the composition of leaf EO collected at different time and months. For chemotype analysis, information about chemical composition of EO published in the literature for $P$ gaudichaudianum was achieved. It was included in this paper those EO analyzes obtained by hydrodistillation from leaves; a valid number of voucher deposit and analyses by GC-MS and GC-FID. These composition data were applied to the PCA and HCA matrix to determine the chemotypes (Gottlieb et al. 1996; Sadjgrove and Jones 2014). The results were processed by STATISTICA version 10 (StartSoft Inc., Tulsa, USA).

Results And Discussion

The yield and chemical composition of the EOs obtained by hydrodistillation from leaves of specimens of $P$ gaudichaudianum collected in the Tijuca National Park, in Rio de Janeiro/ RJ (Brazil), referring to seasonality studies (January 2017 to December 2017) and circadian rhythm (collections every 3 h in the rainy season - March 2017 - and dry season - October 2017) are shown in Tables 1 and 2, respectively. The results of Pearson’s correlation between environmental abiotic variables, major compounds, chemical classes and calculated $GM_{RO}$ are listed in Table 3.

Essential oil yields

The EOs showed a slightly yellow color and ranged from 0.02–0.23% ($w/w$) considering the seasonal and circadian analyzes (Tables 1 and 2). These values were higher compared to some of the results described in the literature for this species (0.01–0.10%) (Morais et al. 2007; Rodig and Poser 1990). Higher yield values were published for samples from Santa Maria (Rio Grande do Sul, Brazil) that ranged from 1.32 to 1.61% (Schindler et al. 2018). Intermediate values were recorded for samples collected in Atalanta (Santa Catarina, Brazil) (0.24 to 0.46%) (Santos 2009).

For the seasonal study, the highest amounts were registered for those EOs obtained in November (0.11%), December (0.14%), and January (0.12%). The study of circadian rhythm showed the highest EOs content at 6 a.m. (0.23%) in the rainy season (R) and at 12 p.m. (0.16%) in the dry season (D). There was a statistical difference between the averages throughout the day for each season ($p < 0.0001$), as well as between night and day ($p = 0.0351$). However, comparing the average yields between the dry and rainy season there was no significant difference ($p = 0.4833$). In both seasons, the night period (9 a.m. to 6 a.m.) afforded the high values of EO yield.

Pearson’s correlation analyzes showed that there was an inversely proportional correlation in the dry season with relative humidity ($r = -0.887$; $p = 0.003$), temperature ($r = -0.787$; $p = 0.020$) and radiation ($r = -0.862$; $p = 0.006$) in the circadian study. It is known that plant species tend to show different patterns of qualitative plastic responses in an EOs perspective at the level of shading (light intensity), increase in temperature and relative humidity. For example, $P. umbellatum$ showed higher EO’s yields when grown in the shade (Mattana et al. 2010), while Matricaria recutita L. (Asteraceae) under intense light conditions (Saleh 1973). A study correlating the yields of EO of $P. umbellatum$ and photosynthetic activities described that when cyophyte plants, such as $P$ gaudichaudianum, are subjected to high irradiance, chlorosis and necrosis usually occur with the photodegradation of chromopigments, leading to reduced photosynthesis and biomass production (Marchese and Figueira 2005). In addition to this mechanism, there is a decrease in EO accumulation through evaporation provided by increases in gases exchange, temperature, stomatal conductance, and CO$_2$ assimilation rate (Sangwan et al. 2001; Mattana et al. 2006, Mattana et al. 2008; Rehman et al. 2016; Thakur and Kumar 2020).

Chemical profile of the essential oil

Ninety-seven ($n = 97$) constituents were identified by GC-MS, corresponding to an average of 96.3% (91.5–99.8%) and 92.8% (84.5–97.5%) of the EO in the seasonal (S) and circadian (C) studies (Tables 1 and 2), respectively. EOs were found to be rich in non-oxygenated sesquiterpenes (S: 37.9–81.5%, C: 48.3–78.0%), followed by oxygenated sesquiterpenes (S: 10.5–50.5%, C: 16.2–44.8%) and oxygenated monoterpenes (S: 0.0–17.0%; C: 0.0–3.4%). The main identified compounds were Bicyclogermacrene (S: 11.2–23.2%; C: 10.2–28.5%), followed by E-Caryophyllene (S: 3.1–11.2%; C: 1.3–22.7%) and Eudesmadiene (cis-Eudesma-6,11-diene) (S: 2.8–15.3%; C: 1.4–21.7%), in addition to the oxygenated sesquiterpenes ENerolidol (S: 3.8–22.9%; C: 0.3–15.4%), a-Cadinol (S: 1.2–11.2%; C: 0.2–19.4%) and Spathulenol (S: 0.1–3.3%; C: 1.39–15.9%). Bicyclogermacrene has been reported as the main compound in the EO of some $P$ aduncum species, for example, $P. aduncum$ L. (25.1%) (Morandim et al. 2010, 2018); $P. manausense$ (20.9%) (Bernuci et al. 2016), $P. amalago$ L. (27.9%) (Morandim-Giannetti et al. 2010); $P$ arboreum Aubl. (49.5%) (Nivickiene et al. 2006); $P$ cernuum Vell. (25.1%) (Morandim et al. 2010); and $P$ manausense Yunck. (41.0%) (Andrade et al. 2005).

The first study with the EO of $P$ gaudichaudianum was carried out with a sample obtained in the municipality of Sapiranga, Rio Grande do Sul State, South of Brazil, and described a chemical composition rich in $\alpha$-Humulene (37.5%) (Von Poser et al. 1994). For this same Brazilian State, the sesquiterpenes ENerolidol (22.1–22.4%) and $\alpha$-Humulene (16.5–37.5%) were reported for sample from municipality of Riozinho (Péres et al. 2009; Sperotto et al. 2013) and in the municipality of Santa Maria, the predominance was the phenylpropanoid Dillapiole (57.8–70.5%) (Schindler et al. 2017). In the State of Paraná (South of Brazil) in different sites of the municipality of Curtiba, the major identified compounds were Longipinanol (19.1%) and $5$-$epi$-$7$-$epi$-$\alpha$-Eudesmol (13.3%) (Krinski et al. 2016); 1-$epi$-Cubenol (24.2%) and Caladene (33.7%) (Krinski et al. 2018); and $E$-Caryophyllene (17.8%) and $\beta$-Pinene (13.2%) (Krinski et al. 2018). For the municipality of Antonina (Parana State), $\delta$-Cadinene (45.3%) (Bemuci et al. 2016); Germacrene B (21.5%) and $\delta$-Cadinene (9.4%) were the major components (Silva et al. 2019; Silva et al. 2021). In Diamante do Norte (Parana State), the main registered compounds were $E$-Caryophyllene (7.3–7.4%), $\beta$-Pinene (3.8–6.6%) and $\delta$-Cadinene (5.6–7.1%) (Quiqui et al. 2019). In the municipality of Piraquara (Parana State), 1-$epi$-Cubenol (25.1%) and Eudesm-7 (11)-en-4-ol (28.4%) were the majority. In addition, Germacrene B (21.5%) and $\delta$-Cadinene (9.3%) were registered as the main constituents in the municipality of Araquari, State of Santa Catarina, in the South of Brazil (Chaabaa et al. 2018); Viridiflorol (27.5%) and Aromadendrene (15.6%) were identified as major compounds in the municipality of Porto Velho, State of Rondônia, in the North of Brazil (Morais et al. 2007); $\alpha$-Selinene (16.6%) and $\alpha$-Humulene (13.3%) were the main components from samples in the municipality of Sáo Paulo, in the State of São Paulo, Southeast of Brazil (Andrade et al. 1998).
Seasonal variation of the essential oil

In the seasonal study, despite the predominant uniform distribution recorded throughout the year for non-oxygenated sesquiterpenes (Tables 1 and 3), in periods with greater precipitation ($r = 0.701; p = 0.011$) and relative humidity ($r = 0.735; p = 0.006$) there was an increase in non-oxygenated monoterpenes. The non-oxygenated sesquiterpenes showed correlation inversely proportional with precipitation ($r = -0.591, p = 0.043$) and directly to the temperature ($r = 0.625, p = 0.030$). The increase in the average monthly precipitation led to an increase in the concentration of oxygenated sesquiterpenes, a result confirmed by the significant value found in the correlations ($r = -0.828; p = 0.001$) (Table 3). The EOs from the aerial parts of *Peperomia galloidea* Kunth (Piperaceae) showed similar increase in the relative percentages of oxygenated sesquiterpenes in the period of greatest precipitation (Ramos and Moreira, 2019). Some works raise the hypothesis that plant species create mechanisms to control the biosynthetic route from the available resources; in this case, high water content in the environment in a compensatory way to guarantee homeostasis (Cheng et al. 2007; Barros et al. 2009; Bergma et al. 2019).

Bicyclogermacrene, *E*-Caryophyllene, Eudesmiadiene, *E*-Nerolidol and *α*-Cadinol contents showed significant variation throughout the year ($p < 0.01$). The box plot graphic (Fig. 1) presents the variations of the EOs major compounds in the seasonal study. It was possible to observe that, even showing high annual variation, the average of Bicyclogermacrene differs from the other co-majority compounds ($p < 0.01$). The oxygenated sesquiterpene *E*-Nerolidol showed the greatest variation in content among the co-majority (Table 1, Fig. 1). *P. gaudichaudianum* showed its reproductive stage in the period when the average rainfall increases after the dry period, in the months of January (infructescences and inflorescences) to February (infructescences) and early November (inflorescences) to December (inflorescences and inflorescences) of 2017. It is described in the literature that reproductive phenophases occur mainly in the rainy season (Valentin-Silva and Veira 2015). Interestingly, in *P. gaudichaudianum* the relative percentage of *E*-Nerolidol showed to increase up to four times in relation to periods of higher incidence of rain after the dry period. When testing this hypothesis, we observed directly proportional and significant values in Pearson's correlation between the content of *E*-Nerolidol with the precipitation ($r = 0.769; p = 0.003$) and relative humidity ($r = 0.791; p = 0.002$).

The EO components of *P. gaudichaudianum* with relative percentages greater than 5% were submitted to statistical analysis. The Principal Component Analysis (PCA) of the seasonal study showed that the main components PC1 (62.1%) and PC2 (21.5%) explained 83.6% of the total chemical variation between all samples, which were classified into two groups, as shown in Fig. 2. Bicyclogermacrene in PC1 (8.6) showed negative charge and positive charge with low influence in PC2 (+ 0.7). For *E*-Nerolidol, positive charges were observed on PC2 (+ 5.1) for samples collected in January, February, November and December and negative charges on PC1 (-1.7) for samples collected from March to October. Compounds Eudesmiadiene, *α*-Cadinol and *E*-Caryophyllene justify the variations in smaller scales, which showed moderate to low negative charges in PC axes. The samples collected in the months of April, March, and October, considered transition months between seasons in the South Hemisphere, showed more positive charges influenced by the concentrations of *E*-Nerolidol. That said, Group I (January, February, November, and December) was composed with EOs constituents rich in Bicyclogermacrene and *E*-Nerolidol and Group II (March to October) resulted from the grouping of samples rich in Bicyclogermacrene followed by *E*-Caryophyllene > Eudesmiadiene > *α*-Cadinol.

The Hierarchical Cluster Analysis (HCA) for the seasonal study is shown in Fig. 3. The clusters in the dendogram are formed from the branches corresponding to the euclidean distances of the samples in relation to the closest samples. Corroborating the results found in the PCA analysis, the samples were grouped into two clusters (Groups I and II), and it can be said that each sample group had a different chemical composition from each other. Interestingly, this separation respected the months (Group I) when the species was in the reproductive phase and reflected the importance of *E*-Nerolidol in this process.

Chemical variations in the EOs from leaves of *P. gaudichaudianum* due to possible phenological influence have already been reported for several species (Farhat et al. 2016; Daghbouche et al. 2020; Hazrati et al. 2020; Ramos et al. 2020). It is known that resource allocation patterns are established seasonally to respond to the different physiological demands associated with growth, defense and/ or reproduction (Gomes et al. 2019; Ramos et al. 2020). *Piper mollicomum* Kunth, for example, in the vegetative period showed high amounts of the oxygenated monoterpene Linalool. Once the reproductive period was established, the biosynthesis production of the oxygenated monoterpene 1,8-Cineole increased (Ramos et al. 2020).

*E*-Nerolidol is one of the main components of nocturnal floral bouquets called "white olfactory images", in addition to playing an important role in the protection against herbivores. In the latter case, this compound catalyzed by terpene synthase, and the subsequent oxidative degradation of alcohol by a cytochrome P-450 monoxygenase, through the intermediate route, produces 4,8-Dimethylone-1,3,7-triene (DMNT), principal homoterpene responsible for attracting parasitoids and herbivorous predators (Balao et al. 2011). In addition, there is a premise in the literature that herbivore-induced volatile emissions would be facilitated by the ability to accurately manipulate the quantity and composition of volatiles through altered expression of genes that encode stages in their biosynthesis (Pichersky and Gershenzon 2002). For example, a study with *Cucumis sativus* L. (Cucurbitaceae) demonstrated that attacks by constitutive herbivores lead to the activation of genes for the decoding of *E*-Nerolidol synthase for the intermediate production of DMNT (Bouwmeester et al. 1999).

Another point to be highlighted refers to a study that evaluated the variations of *Piper* herbivoria by *Eois* (Hübner, 1818) (specialized herbivores) in different forest patterns (dry and wet) and variations in abiotic factors. It was observed that the incidence of *Eois* parasitism increased significantly with the increase of precipitation, mainly in humid forest (Connahs et al. 2009). This leads to the hypothesis that *P. gaudichaudianum*, throughout its evolutionary history, has adapted in order to acquire this chemical phenotypic plasticity (increase of *E*-Nerolidol) as a response mechanism to environmental issues arising from the ecological pressure exerted by herbivory, as the one caused by *Eois*.

On the other hand, it is also described in the literature that the recognition of the homoterpene emission leads to a reduction in the pollinator's preference or in the pollen transfer efficiency (Chauta et al. 2017). Another issue that strengthens the argument proposed to *P. gaudichaudianum* is that the volatiles of leaves and inflorescences can be different but act synergistically to attract visitors. Differences in the chemistry of leaves and inflorescences are not unexpected, as plants are under selection to attract pollinators to flowers, besides leaf herbivores defense (Parachnowitsch and Manson 2015). In study with *Nicotiana attenuata* Torr. ex S. Watson and *Datura wrightii* Regel (Solanaceae) it has been demonstrated that the presence of leaf odor further increases the attraction
for the mixture of flowers pollinated by moths. This interaction of mixtures of flowers and leaves can, therefore, be seen as a strategy to optimize the olfactory message and, thus, improve the orientation of the food source based on odors more safely and without risk of mistaken attraction (Karpati et al. 2013).

Circadian rhythm variation in the essential oil

In the circadian study, a significant variation ($p < 0.05$) was observed in the contents of the main compounds Bicyclogermacrene (R: $13.3-19.7\%$; D: $10.2-28.6\%$), $E$-Caryophyllene (R: $1.3-22.7\%$; D: $4.2-20.2\%$), Eudesmadiene (R: $1.5-21.7\%$; D: $2.3-12.7\%$), $ENerolidol$ (R: $0.3-14.2\%$; D: $1.2-15.3\%$), $\alpha$-Cadinol (R: $0.2-15.4\%$; D: $1.9-19.4\%$) and Spathulenol (R: $3.3-10.9\%$; D: $1.4-15.9\%$) (Fig. 4). The average relative percentages in the driest period were higher than in the rainy season. However, the effects between the dry and rainy periods under the composition showed no significant difference ($p > 0.05$) (Fig. 4).

The PCA and HCA studies were applied to the compounds of the EOs from the rainy and dry periods of the circadian study and are presented in Figs. 5 and 6. The PCA showed a total variance of 90.8% and the main components PC1 and PC2 presented proportional values between themselves, 45.8% and 41.1%, respectively. The two-dimensional axial system generated by the PCA (Fig. 5) clearly showed the discrimination of two groups due to chemical variability: Group I - rich in Bicyclogermacrene, Eudesmadiene, $\alpha$-Cadinol and Spathulenol; and Group II - rich in Bicyclogermacrene, $E$-Nerolidol and $E$-Caryophyllene. The HCA analysis corroborated also again with the PCA analysis, demonstrating the formation of these two groups (euclidean distance of 51.0), correlating this difference between the day (9 a.m. to 6 p.m.) and the night (9 a.m. to 6 a.m.) (Fig. 6). Analyses of the variation in a smaller euclidean distance (26.1), showed that at dusk there was a distinction between the rainy (R) and dry (D) periods, increasing the Eudesmadiene content in the dry period.

Differences were observed in the variance between day and night (paired ANOVA, $F_{11,77} = 25.22, p < 0.001$) when testing the hypothesis observed in the multivariate analysis. The set of factors temperature, humidity, and radiation which define the day and night parameters, had more influence on the chemical composition of $P$. gaudichaudianum EOs than the variations between the dry and rainy seasons. Analysis of all major compounds separated, showed to follow this logic (day vs night), as well as when compared to each other ($p < 0.001$). From this premise, patterns can be observed (Fig. 7). In both seasons, the compound Bicyclogermacrene registered constant relative percentage, with low amplitude of variation throughout the day or night, but different between the two one. It was noticeable that the period of the day increases the average content (~ 21%) of Bicyclogermacrene (Table 2; Fig. 3). The compounds Eudesmadiene, $\alpha$-Cadinol and Spathulenol showed, at night, an increase of up to four times in relation to the content found during the day and with a directly proportional relation to each other (Table S2 - Supplementary Material), (Table 2; Fig. 3). During the day, the compounds $E$-Caryophyllene and $E$-Nerolidol have their percentages marked, in contrast to the nocturnal pattern. Interestingly, $E$-Nerolidol registered its maximum content at 12 p.m. (Table 2; Fig. 7).

Pearson’s correlation analysis (Table 3) demonstrated high values of significant direct correlations and inversely proportional to radiation in both periods with the main compounds mentioned above. The oxygenated sesquiterpene $E$-Nerolidol deserves special attention, as it presented an outstanding significant correlation with radiation, temperature, and humidity ($p < 0.01$). In the literature it is reported that most plants emit spikes of volatile terpenoids at noon or in the early afternoon, regulated by light or the internal circadian clock (Dudareva et al. 2003). The increase in radiation provides an increase in the levels and emission of the stimulus by elicitation in genes related to the sesquiterpene biosynthesis. For example, the content of $E$-Nerolidol increased according to the UV-B creep rate in young and mature leaves of *Vitis vinifera* L. Vitaceae (Gil et al. 2012). This fact reinforces the hypothesis of the role of $E$-Nerolidol in the protection of *P. gaudichaudianum* against herbivores or parasites. In addition, terpenoids have been recognized for their protective role in high temperature conditions and other environmental stresses (Behnke et al. 2010; Loreto et al. 2014; Srivastava et al. 2020).

Considering our results, it is reported for the first-time substantial evidence of the formation of a possible chronotype for the essential oil from leaves of *P. gaudichaudianum* (Granshaw et al., 2003). The chronotype is associated with the preference obtained or observed from certain synchronic physiological pattern, mainly, differentiated by the periods of the days and nights. The chronotype is also associated with differences in time between the various physiological events at the different spatiotemporal scales (Apostol, 2011; Shawa et al., 2018).

Biosynthetic considerations and reduction-oxidation impact

In the seasonal (S) and circadian (C) study, the compounds identified and their respective percentages in the *P. gaudichaudianum* EOs were grouped according to their respective carbon skeletons (Bülow and König 2000; Sayuri et al. 2010; Verma et al. 2019). The results are shown in Tables 4 and 5, respectively. It was possible to find a total of 19 carbon skeletons (S: 19; C: 15), being four for monoterpenes and fifteen for Sesquiterpenes. The main carbon skeletons (C-skeletons) were Bicyclogermacrone (S: 11.2–23.2%; C: 10.2–28.6%) > Aromadendrane (S: 2.1–19.3%; C: 5.0-19.3%) > Eudesmane (S: 5.8–16.2%; C: 2.3–25.4%) > Cadinane (S: 2.2–14.6%; C: 1.7–27.8%) and > Famesane (S: 3.3–22.9%; C: 0.0-16.1%). The C-skeletons with greater diversification (greater number of compounds) were Cadinane (S: 22; C: 18) > Eudesmane (S: 12; C: 9) > Aromadendrane (S: 8; C: 6) > Caryophyllene (S: 8; C: 4).

Table 1

Results of the seasonal analysis of the essential oils obtained from leaves of *Piper gaudichaudianum* Kunth (Piperaceae) collected from January to December 2017. Yields and General Mixture Redox Index (GM$_{RO}$) are also presented.
| C-Skeleton | Compounds* | R<sub>calc</sub> | R<sub>lit</sub> | Relative peak area (%) ± SD |
|------------|------------|-----------------|-----------------|-----------------------------|
|            |            |                 |                 | Jan  | Feb  | Mar  | Apr  | May  | Jun  | Jul  |
| Hexane     | 3E-Hexenol | 844             | 844             |      | tr   |      |      |      |      |      |
| Pinane     | α-Pinene   | 931             | 932             | 0.2±0.0 | 0.2±0.0 | 0.3±0.0 |       |       |       |       |
| Camphane   | Camphene   | 956             | 954             |      |      |      |      |      |      |      |
| Pinane     | β-Pinene   | 975             | 979             | 0.6±0.2 | 0.5±0.0 | 0.5±0.0 | 0.1±0.0 |       |       |       |
| Myrcane    | Myrcene    | 985             | 988             |      |      |      |      | 0.3±0.0 |       |       |
| Menthan    | Limonene   | 1022            | 1024            |      |      |      |      |      |      |      |
| Myrcane    | Z-Linalool oxide | 1064 | 1067 | 0.8±0.1 | 1.2±0.2 |       |       |       |       |       |
| Myrcane    | Linalool   | 1093            | 1095            | 5.4±0.0 | 1.2±0.0 | 4.3±0.0 | 1.2±0.0 |       |       |       |
|            | Undefined m/z 154 | 1095 | | | 0.4±0.0 |       |       |       |       |       |
| Nonane     | α-Nonanal  | 1100            | 1100            | 0.1±0.0 |       |       |       |       |       |       |
| Menthan    | 1-Terpinene | 1132          | 1130            | 0.3±0.0 |       |       |       |       |       |       |
| Camphane   | Camphor    | 1142            | 1141            | 0.1±0.0 | 0.1±0.0 | 4.6±0.0 | 3.5±0.0 | 4.8±0.2 |       |       |
| Camphane   | Camphene hydrate | 1144 | 1145 | 0.4±0.0 | 1.4±0.0 | 0.3±0.1 |       |       |       |       |
| Menthan    | α-Terpinene | 1182           | 1186            | 0.2±0.0 | 1.2±0.0 | 6.3±0.0 | 2.1±0.0 | tr   |       |       |
| Camphane   | Borneol    | 1162            | 1165            |      |      |      |      |      |      |      |
| Camphane   | Bornyl acetate | 1282          | 1285            |      | tr   | 0.3±0.0 |       |       |       |       |
| Undecane   | Undecanal  | 1303            | 1305            |      |      |      |      |      |      |      |
| Elemane    | Bicycloelemene | 1322         | 1329            | 0.7±0.0 | tr   | 0.5±0.0 | 0.3±0.1 | 2.3±0.2 |       |       |
| Elemane    | δ-Elemene  | 1332            | 1335            | 0.5±0.0 | 3.2±0.0 | 3.2±0.0 | 5.7±0.0 | 4.6±0.0 | 3.5±0.2 |       |
| Cubebane   | α-Cubebene | 1345            | 1348            | 0.3±0.0 | tr   | 1.2±0.0 |       |       |       |       |
| Myrcane    | Neryl acetate | 1356           | 1359            |      |      | 1.3±0.1 |       |       |       |       |
| Copaane    | α-Ylangene | 1372            | 1373            | 0.3±0.0 |       |       |       |       |       |       |
| Copaane    | α-Copaene  | 1375            | 1374            | 0.4±0.0 | 1.6±0.0 | 3.8±0.1 | 6.3±0.0 |       |       |       |
|            | undefined m/z 202 | 1376 | | 1.2±0.2 |       |       |       |       |       |       |
| Myrcane    | Geranyl acetate | 1376          | 1379            | 0.3±0.0 | tr   | 0.4±0.0 |       |       |       |       |
| Bourbon    | β-Bourbonene | 1386           | 1387            | 0.1±0.0 | 0.1±0.0 | tr   |       |       |       |       |
|            | undefined m/z 206 | 1387 | | 0.3±0.0 |       | 1.2±0.0 |       |       |       |       |
| Elemane    | β-Elemene  | 1388            | 1389            | 1.7±0.2 | 0.7±0.0 | 4.6±0.1 | 2.3±0.0 | 2.3±0.0 | 5.7±0.2 | 4.5±0.2 |       |
| Aromadendrane | α-Gurjunene | 1409          | 1409            | 0.2±0.0 |       |       |       |       |       | 2.3±0.0 |       |
| Caryophyllane | iso-Caryophyllene | 1411 | 1409 | tr   | 0.3±0.0 | 1.2±0.0 |       |       |       |       |
| Caryophyllane | ECaryophyllene | 1417 | 1419 | 3.3±0.2 | 8.7±0.1 | 9.0±0.8 | 6.9±0.3 | 7.6±0.5 | 10.2±0.7 | 11.2±1.8 |       |
| Copaane    | β-Copaene  | 1428            | 1430            | 1.2±0.1 |       | 1.9±0.0 | 3.2±0.0 |       |       |       |
| Aromadendrane | β-Gurjunene | 1431          | 1434            | 0.9±0.0 |       |       |       |       |       |       |
| Humulane   | β-Humulene | 1433            | 1436            |       |       |       | 2.3±0.2 |       |       |       |
| Elemane    | γ-Elemene  | 1436            | 1437            | 0.8±0.0 | 0.2±0.0 | 1.2±0.0 | 1.8±0.0 |       |       |       |
| Aromadendrane | Aromadendrene | 1437 | 1438 | 1.7±0.1 | 1.5±0.2 | 2.4±0.2 | 4.2±0.5 | 1.9±0.0 | 1.6±0.0 | 2.1±0.0 |       |
| Famesane   | Z-β-Famesene | 1439          | 1440            |       |       |       |       |       |       |       |       |
| Humulane   | α-Humulene | 1450            | 1452            | 1.2±0.1 | 4.0±0.0 | 7.2±0.2 | 3.9±0.0 | 2.3±0.0 | 6.4±0.3 | 4.3±0.4 |       |
| Famesane   | E-β-Famesene | 1453          | 1454            |       |       |       |       |       |       |       |       |
| Aromadendrane | al- Aromadendrene | 1457 | 1458 | 0.4±0.0 | 0.8±0.0 | 0.2±0.0 |       |       |       |       |       |
| Aromadendrane | dehydro- | 1459            | 1460            |       |       | 2.3±0.0 |       |       |       |       |       |
| Aromadendrane | Z-Cadina-1(6),4-diene | 1461 | 1461 | 1.2±0.0 |
| ---------------|------------------------|------|------|---------|
| Caryophyllane   | 9-epi-E-Caryophyllene | 1462 | 1464 |         |
|                 | γ-Gurjunene            | 1472 | 1475 | 0.2±0.0 | 0.5±0.0 |
| Cadinane        | γ-Murolene             | 1477 | 1478 | 1.3±0.0 |         |
| Cadinane        | Amorpha-4,7(11)-diene | 1479 | 1479 | 1.2±0.0 | 0.2±0.0 | 0.1±0.0 |
| Germacrane      | Germacrene D           | 1481 | 1480 | 0.5±0.0 | 7.5±0.0 | 4.7±0.2 | 5.7±0.2 | 5.3±0.2 | 2.3±0.3 | 7.8±0.5 |
| Cadinane        | α-Amorphene            | 1482 | 1483 | 1.9±0.0 | 0.1±0.0 |         | 0.3±0.0 |
| Eremophilane    | Aristolochene          | 1485 | 1487 |         |         |         |         |         |
| Eudesmane       | Z-Eudesma-6,11-diene   | 1488 | 1489 | 3.1±0.1 | 4.7±0.2 | 8.4±0.5 | 10.2±0.4 | 14.3±0.7 | 15.3±0.9 | 11.2±0.5 |
| Eudesmane       | β-Selinen              | 1493 | 1492 | 0.7±0.0 | 1.9±0.0 |         | 1.5±0.0 |         |         |
| Cadinane        | γ-Amorphene            | 1494 | 1495 | 0.4±0.0 |         |         | 1.0±0.0 |         |         |
| Eremophilane    | Valencene              | 1496 | 1496 | 2.8±0.0 |         |         | 0.4±0.0 |         |         |
| Eudesmane       | α-Selinene             | 1498 | 1498 |         |         |         | 0.3±0.0 | 0.9±0.0 |         |
| Bicyclogermacrene | Bicyclogermacrene     | 1499 | 1500 | 12.2±0.9 | 17.0±1.2 | 16.9±0.3 | 18.1±0.4 | 20.3±0.9 | 19.3±1.3 | 15.3±0.2 |
| Cadinane        | α-Murolene             | 1502 | 1500 | 0.5±0.0 | 0.1±0.0 |         |         |         |         |
| Famesane        | E,E-α-Famesene         | 1504 | 1505 |         |         |         |         |         |
| Bisabolene      | β-Bisabolene           | 1506 | 1505 | 0.2±0.0 | 0.9±0.0 |         |         |         |
| Cadinane        | γ-Cadinene             | 1512 | 1513 | 0.5±0.0 | 1.0±0.0 | 1.0±0.0 | 0.1±0.0 |         |         |
| Eudesmane       | 7-epi-α-Selinen        | 1518 | 1520 |         |         |         |         |
| Cadinane        | δ-Cadinene             | 1521 | 1522 | 1.6±0.0 | 1.2±0.0 | 0.2±0.0 | 0.1±0.0 |         |         |
| Cadinane        | Zonarene               | 1528 | 1528 |         |         |         |         |
| Cadinane        | Z-Cadina-1,4-diene     | 1533 | 1533 | 1.2±0.0 | 0.3±0.0 |         |         |         |
| Cadinane        | α-Cadinene             | 1537 | 1537 | 2.3±0.0 |         |         |         |
| Eudesmane       | Selina-3,7(11)-diene   | 1545 | 1545 | 0.1±0.0 |         |         |         |
| Elemane         | Elemol                 | 1548 | 1548 | 0.4±0.0 |         |         | 0.3±0.2 |         |
| Germacrane      | Germacrene B           | 1557 | 1559 | 2.1±0.0 | 1.2±0.0 | 2.3±0.2 | 5.6±0.0 | 1.2±0.4 | 1.2±0.3 |
| Cadinane        | β-Calacorene           | 1564 | 1564 |         |         |         |         |
| Famesane        | Nerolidol              | 1561 | 1561 | 17.6±0.4 | 22.9±0.7 | 6.3±0.2 | 5.8±0.2 | 4.3±0.2 | 4.2±0.3 | 3.8±0.2 |
| Famesane        | Z-Nerolidol            | 1531 | 1531 | 0.3±0.0 |         |         |         |         |
| Aromadendrane   | Spathulenol            | 1576 | 1577 | 1.4±0.0 | 1.0±0.0 | 3.3±0.1 |         | 2.3±0.2 | 1.2±0.4 |
| Caryophyllane   | Caryophyllene oxide    | 1582 | 1582 | 1.4±0.0 | 1.5±0.0 | 1.1±0.0 |         | 1.2±0.1 |         |
| Aromadendrane   | Viridiflorol           | 1592 | 1592 | 1.8±0.0 | 1.2±0.0 | 3.2±0.0 | 4.4±0.0 | 5.8±0.4 |         |
| Eudesmane       | Rosifoliol             | 1602 | 1600 | 1.8±0.0 |         | 0.4±0.0 |         |         |
| Aromadendrane   | Ledol                  | 1601 | 1602 | 5.3±0.2 | 0.3±0.0 | 4.0±0.2 | 1.2±0.0 | 3.5±0.0 |         |
| Eudesmane       | 5-epi-7-epi-α-Eudesmol | 1606 | 1607 | 0.3±0.0 |         |         |         |
| Humulane        | Humulene epoxide II    | 1608 | 1608 | 3.9±0.0 | 0.2±0.0 |         | 0.1±0.0 |         |
| Cadinane        | 1,10-di-epi-Cubenol    | 1618 | 1618 | 1.0±0.0 |         |         | 0.1±0.0 |         |
| Cadinane        | α-Corocalene           | 1620 | 1622 | 2.4±0.0 |         |         |         |
| Eudesmane       | 10-epi-γ-Eudesmol      | 1622 | 1622 |         |         |         |         |         |
| Compound Type | Compound Name | Relative Percentage (%) | Yields (%) | Other Compounds | Identified Compounds in Numbers | Identified Compounds in relative percentage (%) | GM<sub>RO</sub><sup>b</sup> | GM<sub>RO</sub><sup>b</sup> | GM<sub>RO</sub><sup>b</sup> | GM<sub>RO</sub><sup>b</sup> | GM<sub>RO</sub><sup>b</sup> | GM<sub>RO</sub><sup>b</sup> |
|---------------|---------------|--------------------------|------------|----------------|-------------------------------|-----------------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Non-Oxygenated Monoterpenes | | | | | | | | | | | | | |
| C-Skeleton | Compounds                  | IR<sub>calc</sub> | IR<sub>lit</sub> | Relative peak area (%) ± SD |
|------------|----------------------------|-------------------|-----------------|-----------------------------|
|            |                            |                   |                 | Rainy season (March)         |
|            |                            |                   |                 | 6 a.m.  | 9 a.m.  | 12 p.m. | 3 p.m. | 6 p.m. | 9 p.m. | 12 a.m. |
| Myrcane    | Linalool                   | 1093              | 1095            | tr      | tr      |          |         |         |         | 0       |
| Menthanes  | Limonene                   | 1021              | 1024            | tr      | tr      |          |         |         |         | 0       |
| Mentham     | Camphor                    | 1140              | 1141            | 0.1±0.0 | 0.1±0.0 | 0.2±0.0  | 0.1±0.0 | 0       |
| Mentham     | α-Terpineol                | 1183              | 1186            | 0.1±0.0 | 1.0±0.0 | 1.4±0.0  | 0.1±0.0 | 0       |
| Elemene     | δ-Elemene                  | 1331              | 1335            | 0.6±0.0 | 3.7±0.2 | 3.5±0.1  | 3.1±0.1 | 0.5±0.0 | 0.3±0.0 | 6       |
| Cubebane    | α-Cubebene                 | 1344              | 1348            | tr      | 0.3±0.0 | 0.3±0.0  | 0.2±0.0 | 0.7±0.0 | 0.1±0.0 | 0       |
| Myrcane     | Neryl acetate              | 1353              | 1359            | 1.6±0.2 | 2.3±0.1 | 3.4±0.0  | 0.4±0.0 | 0       |
| Copane     | α-Copaene                  | 1372              | 1374            | 2.2±0.2 | 1.6±0.1 | 2.1±0.1  | 4.1±0.2 | 4.4±0.1 | 1.3±0.1 | 1.6±0.2 | 4       |
|            | Undefined m/z 202          | 1379              | tr              | 2.1±0.0 | 1.2±0.0 | 0.2±0.0  | 0       |
| Elemene     | β-Elemene                  | 1387              | 1389            | 2.1±0.2 | 1.2±0.1 | 0.3±0.0  | 0.3±0.0 | 0.9±0.1 | 0.6±0.0 | 1       |
| Caryophyllane| iso-Caryophyllene        | 1406              | 1409            | 0.2±0.0 | 0.3±0.0 | 0.4±0.0  | 1.2±0.0 | 1.5±0.0 | 0.1±0.0 | 0       |
| Caryophyllane| E-Caryophyllene           | 1416              | 1419            | 4.2±0.7 | 9.1±1.0 | 13.3±0.4 | 12.2±1.3 | 22.7±3.4| 4.7±1.21| 1.3±0.3 | 3       |
| Copane     | β-Copaene                  | 1428              | 1430            | 0.7±0.0 | 0.3±0.0 | 0.1±0.0  | 0.1±0.0 | 0.7±0.1 | 0.8±0.1 | 1       |
| Aromadendrane| β-Gurjunene                | 1431              | 1434            | 0.5±0.1 | 0.3±0.0 | 0.1±0.0  | 2       |
| Humulane    | β-Humulene                 | 1435              | 1436            | 1.2±0.2 | 2.3±0.0 | 3.2±0.1  | 3.9±0.1 | 4.2±0.0 | 0.1±0.0 | tr      |
| Elemene     | γ-Elemene                  | 1436              | 1437            | 1.2±0.0 | 1.0±0.1 | 0.1±0.0  | 0.1±0.0 | 0.4±0.0 | 0.4±0.0 | 0       |
| Aromadendrane| Aromadendrene             | 1438              | 1438            | 2.3±0.1 | 1.3±0.2 | 1.6±0.2  | 2.3±0.3 | 3.5±0.2 | 1.5±0.2 | 0.1±0.0 | tr      |
| Humulane    | α-Humulene                 | 1450              | 1452            | 4.0±0.4 | 5.2±0.5 | 5.0±0.1  | 4.7±0.1 | 5.4±0.3 | 1.3±0.2 | 0.6±0.8 | 3       |
| Farnesane   | Eβ-Farnesene               | 1452              | 1454            | 1       |
| Aromadendrane| allo-Aromadendrene       | 1457              | 1458            | 1.9±0.0 | 0.3±0.0 | 0.2±0.0  | 0.5±0.1 | 0.4±0.1 | 1.2±0.1 | 1.3±0.0 | 1       |
| Cadinane    | Amorpha-4,7(11)-diene      | 1476              | 1479            | 0.3±0.0 | 0       |
| Germane     | Germacrene D               | 1481              | 1480            | 1.5±0.0 | 5.6±0.1 | 6.0±0.2  | 5.3±0.4 | 6.7±0.5 | 1.1±0.0 | 0.5±0.1 | 2       |
| Cadinane    | α-Amorphene                | 1482              | 1483            | 1       |
| Eudesmane   | cis-Eudesma-6,11-diene     | 1486              | 1489            | 18.5±4.1| 4.9±0.1 | 1.5±0.2  | 3.4±0.5 | 4.8±0.1 | 19.3±3.2| 21.7±4.3| 1       |
| Eudesmane   | β-Selinene                 | 1490              | 1492            | 0.2±0.0 | 0.3±0.0 | 0.2±0.0  | 0       |
| Cadinane    | γ-Amorphene                | 1493              | 1495            | 0.4±0.0 | tr      | 0.1±0.0  | 0       |
| Eudesmane   | α-Selinene                 | 1498              | 1498            | 0.2±0.0 | 0       |
| Bicyclogermacrane| Bicyclogermacrene| 1498              | 1500            | 15.7±1.3| 19.6±2.4| 19.4±1.3 | 19.7±1.4| 19.1±1.5| 13.3±1.2| 14.0±2.1| 1       |
| Cadinane    | α-Muurolene                | 1504              | 1500            | 0       |
| Cadinane    | γ-Cadinene                 | 1510              | 1513            | tr      | tr      | 0.1±0.0  | 0       |
| Eudesmane   | 7-epi-α-Selinene           | 1518              | 1520            | 0.1±0.0 | 0.8±0.1 | 0.3±0.0  | 0.1±0.0 | tr      |
| Cadinane    | δ-Cadinene                 | 1523              | 1522            | 2.3±0.2 | 0.2±0.1 | 0.3±0.0  | 3.2±0.6 | 2.2±0.2 | 1.0±0.1 | 1.4±0.2 | 6       |
| Cadinane    | Zonarene                   | 1526              | 1528            | 0.5±0.0 | 0.2±0.0 | 0       |
| Eremophilane| γ-Vetivenene               | 1530              | 1531            | tr      | tr      | 0       |
| Cadinane    | E-Cadina-1,4-diene         | 1532              | 1533            | 0       |
| Cadinane    | α-Cadinene                 | 1535              | 1537            | 0       |
| Eudesmane   | Selina-3,7(11)-diene       | 1542              | 1545            | 0       |
| Germacrane  | Germacrone B               | 1557              | 1559            | 0.7±0.1 | 0.2±0.0 | 0       |
|     | Compound                      | RI<sub>calc</sub> | RI<sub>lit</sub> | RI<sub>calc</sub> - RI<sub>lit</sub> | GM<sub>RO</sub><sup>b</sup> |
|-----|-------------------------------|-------------------|-----------------|-----------------------------------|-----------------|
|     |                               |                   |                 |                                   |                 |
|     | Farnesane                      | 1560              | 1561            | 0.6±0.0                           | 0.3±0.2         |
| Cadinane | β-Calacorene                  | 1563              | 1564            | tr                                | 0               |
| Farnesane | Z'-Nerolidol                  | 1531              | 1531            | tr                                | 0               |
| Aromadendrane | Spathulenol                  | 1574              | 1577            | 7.1±0.2                           | 3.3±0.1         |
| Caryophyllane | Caryophyllene oxide          | 1579              | 1582            | 2.7±0.2                           | 1.1±0.1         |
| Aromadendrane | Viridiflorol                  | 1588              | 1592            | 4.3±0.1                           | 1.7±0.2         |
| Eudesmane | Rosifoliol                    | 1598              | 1600            | tr                                | 1.0±0.1         |
| Aromadendrane | Ledol                        | 1601              | 1602            | 1.4±0.1                           | 0.6±0.0         |
| Eudesmane | 5-epi-7-epi-α-Eudesmol         | 1605              | 1607            | 0.6±0.0                           | 1               |
| Humulane | Humulene epoxide II           | 1609              | 1608            | tr                                | 2.3±0.2         |
| Cadinane | 1,10-di-epi-Cubenol            | 1615              | 1618            | 1.3±0.0                           | 1.5±0.0         |
| Cadinane | α-Corocalene                  | 1623              | 1622            | 0.3±0.1                           | 0.1±0.0         |
| Cadinane | epi-α-Muurolol                 | 1638              | 1640            | 0.4±0.0                           | 0.3±0.0         |
| Cadinane | α-Muurolol                    | 1642              | 1644            | 1.0±0.2                           | 0.9±0.1         |
| Eudesmane | β-Eudesmol                    | 1648              | 1650            | 1.3±0.2                           | 3.0±0.1         |
| Eudesmane | α-Eudesmol                    | 1651              | 1652            | 0.6±0.0                           | 1.0±0.1         |
| Cadinane | α-Cadinol                     | 1653              | 1652            | 12.1±0.1                          | 2.3±0.1         |
| Cadinane | Z'-Calamenen-10-ol             | 1660              | 1660            | tr                                | 0.1±0.0         |
| Caryophyllane | 14-hydroxy-Z-Caryophyllene   | 1664              | 1666            | 1.9±0.1                           | 1.6±0.1         |
| Cadinane | Cadalene                      | 1672              | 1675            | 0.2±0.0                           | 2.0±0.0         |
| Cadinane | Amorpha-4,9-dien-2-ol          | 1697              | 1700            | 0.3±0.0                           | 2.2±0.0         |
| Undefined m/z 220 |                       | 1718              | 1.2±0.0         | 1.1±0.0                           | 0.3±0.0         |

Non-Oxygenated Monoterpenes

|          |                   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   |

Oxygenated monoterpenes

|          |                   | 0.2   | 1.6   | 2.3   | 3.4   | 2.4   | 1.8   | 0.4   | 0.0   | 0.0   |

Non-Oxygenated Sesquiterpenes

|          |                   | 60.1  | 57.7  | 57.9  | 63.2  | 78.1  | 48.3  | 48.7  | 6     |       |

Oxygenated sesquiterpenes

|          |                   | 33.1  | 22.1  | 28.2  | 24.5  | 16.2  | 40.5  | 44.8  | 3     |       |

Other compounds

|          |                   | 4.0   | 28.0  | 33.0  | 34    | 43    | 38    | 38    | 4     |       |

Identified compounds in numbers

|          |                   | 93.9  | 84.5  | 91.9  | 94.3  | 97.0  | 90.9  | 93.9  | 9     |       |

Identified compounds in relative percentage (%)

|          |                   | 0.23±0.03 | 0.9±0.02 | 0.1±0.01 | 0.14±0.02 | 0.13±0.02 | 0.17±0.01 | 0.12±0.02 | 0     |       |

Yields (%)

|          |                   | -3.9  | -4.8  | -4.5  | -4.5  | -3.6  | -3.7  | -3.9  |       |       |

GM<sub>RO</sub><sup>b</sup>

|          |                   |       |       |       |       |       |       |       |       |       |

<sup>a</sup> All compounds were identified by MS and IR in accordance with experimental. <sup>b</sup> General Mixture Redox Index. tr - Trace (relative percentage value less than 0.05%).

Table 3.

Pearson's correlation analysis between environmental abiotic variables, major compounds, chemical classes and calculated General Mixture Redox Index (GM<sub>RO</sub>) of the essential oils obtained from leaves of <i>Piper gaudichaudianum</i> Kunth (Piperaceae), collected from January to December 2017 (seasonality), and in the rainy (March 2017) and dry season (October 2017) (circadian rhythm).
### Table 4

Percentages of the carbon skeletons of the essential oils from leaves of *Piper gaudichaudianum* Kunth (Piperaceae) in the seasonality study for the period of 12 months (January to December 2017).

| C-Skeleton       | Percentages (%) | Jan | Feb | Mar | Apr | May | Jun | Jul | Aug | Sept | Oct | Nov | Dec |
|------------------|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|------|-----|-----|-----|
| Aromadendrane    |                 | 10.9| 6.4 | 12.9| 12.1| 2.1 | 13.1| 5.7 | 14.8| 10.1 | 9.0 | 13.3| 19.3|
| Bicyclogermacrene|                 | 12.2| 17.0| 16.9| 18.1| 20.3| 19.3| 15.3| 12.3| 11.2 | 20.2| 22.1| 12.1|
| Bisabolane       |                 | -   | 0.2 | 0.9 | -   | -   | -   | -   | tr  | -    | tr  | -   | 0.1 |
| Bourbonane       |                 | -   | 0.1 | 0.1 | tr  | -   | -   | -   | -   | 0.1  | -   | -   | -   |
| Cadinane         |                 | 18.5| 11.5| 7.2 | 2.2 | 7.0 | 7.0 | 10.0| 15.3| 19.7 | 12.1| 11.4| 6.5 |
| Caryophyllane    |                 | 6.1 | 10.8| 10.1| 6.9 | 7.7 | 11.8| 12.5| 5.6 | 8.7  | 10.4| 7.0 | 5.5 |
| Camphane         |                 | 0.1 | 0.1 | 5.0 | 5.2 | 5.1 | -   | -   | -   | -    | -   | -   | -   |
| Copaene          |                 | -   | 1.9 | 1.6 | -   | 5.7 | -   | 9.6 | 8.5 | 7.2  | 8.2 | 2.7 | 5.3 |
| Cubebane         |                 | -   | 0.2 | tr  | -   | 1.2 | -   | 0.4 | 5.6 | 3.4  | 1.2 | 2.9 | -   |
| Elemene          |                 | 3.6 | 1.3 | 9.0 | 5.6 | 10.2| 10.5| 10.6| 12.4| 5.4  | 3.2 | 6.3 | 9.9 |
| Eremophilane     |                 | Tr  | 2.8 | tr  | 0.4 | -   | tr  | tr  | tr  | tr   | -   | -   | -   |
| Eudesmane        |                 | 11.9| 7.6 | 10.8| 13.7| 16.1| 15.4| 12.1| 14.5| 16.2 | 8.6 | 5.8 | 11.0|
| Farnesane        |                 | 17.9| 22.9| 6.3 | 5.8 | 4.3 | 4.2 | 3.8 | 4.6 | 4.3  | 5.3 | 10.3| 17.3|
| Germacrane       |                 | 0.5 | 9.9 | 5.9 | 8.01| 11.0| 3.6 | 9.0 | 4.2 | 3.4  | 11.6| 6.7 | 6.5 |
| Guaine           |                 | 0.2 | 0.5 | -   | -   | -   | -   | -   | 1.6 | -    | -   | 2.1 | 3.2 |
| Humulane         |                 | 5.1 | 4.2 | 7.2 | 3.9 | 2.3 | 6.5 | 6.6 | 4.4 | 5.5  | 7.6 | 1.4 | 0.8 |
| Menthan          |                 | 0.6 | 1.2 | 6.3 | 2.1 | tr  | -   | -   | -   | -    | -   | tr  | -   |
| Mrycane          |                 | 6.3 | 1.2 | 5.8 | 2.8 | tr  | 0.5 | -   | -   | -    | 0.3 | -   | -   |
| Pinane           |                 | 0.6 | 0.7 | 0.7 | 0.4 | -   | -   | -   | -   | -    | -   | -   | -   |

tr - Trace (percentage value less than 0.05%).

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*Significant at $p < 0.05$ | **Significant at $p < 0.01$
In the dry and rainy seasons it was possible to find 12 and 15 C-skeletons, respectively. Geranyl pyrophosphate derivatives were favored in the rainy season. Geranyl pyrophosphate derivatives were favored in the rainy season. Geranyl pyrophosphate derivatives were favored in the rainy season.

humidity, intensity and quality of light (Zeng et al. 2017; Liebelt et al. 2019). Our study showed that the internal circadian clock also regulates the production of precursors and in the configuration of the C-skeletons of the EOs components. A study on the expression of the genes associated with the Metileritritol-phosphate (MEP) and Mevalonate (MEV) pathways has been previously mentioned, which indicates that the internal circadian clock also regulates the production of precursors and in the configuration of the C-skeletons (Table 5).

In the circadian study the dynamics related to the production of the compounds was marked by the same pattern between day and night in both seasons (Table 5, Scheme 1). It was registered that in some periods there was an increase in the compound percentage related to the increase in abiotic factors, as sugars for pollination reward, since these metabolites formed from Farnesane, with acyclic skeletons, demand less energy expenditure in their production than cyclic compounds. This evidence reinforces the hypotheses previously raised for the role of sugars for pollination reward, since these metabolites formed from Farnesane, with acyclic skeletons, demand less energy expenditure in their production than cyclic compounds. This evidence reinforces the hypotheses previously raised for the role of Enerololid (acylic) in P. gaudichaudianum as a participant in the attractiveness of pollinators.

Comparing the percentages between C-skeletons it was found that the contents of compounds with Bicyclogermacrane were in high percentages during the reproductive period (January, February, November and December), as well as in high precipitation season (January, March, and April). A targeting for the formation of compounds with C-skeletons from routes linked to Farnesyl Pyrophosphate, the sesquiterpenes precursor, was marked. Geranyl pyrophosphate derivatives were present in high relative percentages, suggesting that Farnesyl Pyrophosphate drives to form compounds with the central Germacrane skeleton, in relation to the carbon skeleton that differs from the precursor skeleton of Farnesane. Despite noting this fact, in the reproductive period (January, February, November and December), the biosynthetic route drives to the precursor Farnesane. This result in reproductive phase suggests the hypothesis that the metabolism is directed towards the production of basal metabolites, such as aromadendrene skeleton, whose precursor is Bicyclogermacrane, is conditioned to displacement due to consumption, almost total, of substrates with Germacrane skeleton. Bicyclogermacrane route was reduced in August and September when the Cadinane route was favored. However, Bicyclogermacrane route harms the Cadinane route by up to two times. The C-skeleton percentages of Caryophyllane were low compared to the variation of the other sesquiterpene C-skeletons. The increase in the Humulane skeleton is conditioned by the increase in the Caryophyllane skeleton, plainly by the fact that these two compounds are in a sequence of chemical transformation. Compounds with Elemane skeletons were favored in the months of May to August (intermediate rain rates) (Scheme 1).

In the dry and rainy seasons it was possible to find 12 and 15 C-skeletons, respectively. Geranyl pyrophosphate derivatives were favored in the rainy season. Also, in this season, only the predecessor compounds of Myrcane's C-skeleton were favored overnight. On the other hand, in the rainy season Myrcane was favored for the daytime period, because at dusk there was a deviation from the biosynthetic route to produce Menthe and Camphane C-skeletons.

Table 5
Percentages of the carbon skeletons of the essential oil components from leaves of Piper gaudichaudianum Kunth (Piperaceae) in the circadian study, during the Rainy (R, March) and Dry (D, October) season, from 12 a.m. to 12 p.m.
During the day (dry or rainy), Farnesyl Pyrophosphate derivatives were higher from 9 a.m. to 3 p.m. for the compounds with C-skeleton equal to the precursor (Farnesene), which demands less energy for the plant. Again, we emphasize that this period is the one with the highest luminous incidence, reinforcing the hypothesis that there has been a completely change in metabolism for the routes involved with photosynthesis (basal metabolism), consequently, reducing the energy effort for specialized metabolism. During this same period, the displacement for production of most Bicyclogermacrane is constant and relatively greater in the dry season, a route that is commonly active in the plant. From 3 p.m. to 18 p.m., there was exclusive drive to produce the Caryophyllane C-skeleton (including under most of the Bicyclogermacrane C-skeleton).

At night from 9 p.m. to 6 a.m. there was a deviation from the Germacrane route, favoring the routes of Cadinane and Eudesmane C-skeletons. In addition, the displacement of Bicyclogermacrane to produce C-skeleton compounds from Aromadendrane was favored. These two changes, described previously, justify the significant reduction in the values of the majority in the EO composition.

Based on our results for *P. gaudichaudianum* EO, it is possible to postulate that there was a diurnal propensity for acyclic and monocyclic C-skeletons, which requires lower energy costs for construction and structural specialization for production. At night, the opposite pattern was observed, favoring the production of bicyclic and tricyclic C-skeletons, which demand greater expenditure on energy in construction and structural specialization for production. These findings point out for the possibilities of changes in biological activities and the indications of popular uses of *P. gaudichaudianum* already reported. In other words, biological properties of this plant are markedly related to the collection time. From an ecological point of view, this evidence sheds light on the concern to collect samples to obtain EOs used in the construction of experimental models of baits for bats (Mikich et al. 2003; Bianconi et al. 2007; Bianconi et al. 2012; Leiser-Miller et al. 2020). So far, it is known that the emission of volatile compounds is important for these animals (Bianconi et al. 2012). These compounds act as allelochemicals (caironomas, alomornas or sinomonas) that guarantee interspecific interactions. It is known that the structural characteristics (branching pattern of carbon chains, charges, unsaturated double bonds, and others) and the functional groups (carboxylic acids, alcohols and aldehydes) significantly alter the perceptible odor quality (Uchida 2000; Gadziola et al. 2020; Murata 2020). For example, the elongation of carbon chains leads to the emergence of pungent odors (Murata 2020). In addition, it is known that small enantiomeric and structural changes can lead to sexual isolation and contribute to the speciation process (Wicker-Thomas et al. 2011; Hembry and Weber 2020). Besides, it has been reported that two populations of moths (*Ostrinia nubilalis* (Hübner, 1796)) living in the same region are being sexually isolated by a modification in a desaturase enzyme involved in the pheromone biosynthesis (Wicker-Thomas et al. 2011).

In circadian and seasonal studies of EOs from *P. gaudichaudianum*, the compounds were analyzed in relation to their oxidation number (NOX), following Hendrickson-Cram-Hammond (Hendrickson et al. 1970) rules on the sum of the oxidation states of each atom of the molecule and oxidative steps (OS) (Emerenciano et al., 1998). As mentioned by Sayuri et al. (2010), Nox does not allow the comparison between different chemical classes, since the number of carbon atoms between chemical classes is usually different. To facilitate this comparison, the OS calculation was obtained by subtracting the NOX from the compounds of interest by the NOX from the common biosynthetic precursor of that C-skeleton, a result divided by two according to Eq. (1) (Emerenciano et al., 1998).

\[
OS = \frac{(N_{OX_{molecule}} - N_{OX_{precursor}})}{2} \quad (Eq. 1)
\]

The EO compounds from *P. gaudichaudianum* showed NOX ranging from −10 to −38 (Figure S1 - Supplementary Material). The monoterpens’ NOX were between −16 and −10. Only the monoterpen bicyclic Camphor showed the highest NOX of −10, among the results of circadian and seasonal studies. For sesquiterpenes, the NOX values were determined between −24 and −16. The highest NOX was found for Cadalene (-16). However, most terpene compounds with higher NOX values are found in the EO at much lower relative percentages.

In this static analysis model based on NOX and OS, for the two studies (Seasonal and Circadian), when comparing the values with the precursor of the respective chemical class or tepene type, it was observed that the generated compounds kept the numbers of OS (0 to 2) constant (Figure S1 - Supplementary Material). Although the unspecific activity of terpene synthases is widely reported and the great diversification in C-skeletons of terpenic compounds, the latter did not occur due to the increased degree of oxidation of the produced chemical constituents (Emerenciano et al. 2006; Vattekkatte and Boland 2020). This finding has already been reported in a Chemotaxonomy study with terpenoids in Asteraceae and in the analysis of temporal patterns of skeletal production in essential oils of *Baccharis microdonta* Steud. ex Baker and *B. elaegnoides* DC. (Sayuri et al. 2010).

Previously reported studies on this kind of chemosystematic analysis, based on evolutionary correlation, postulate that the production and diversity of specialized metabolites reach the maximum in more advanced taxa. However, in groups that present these diversifications in metabolites, evolution tends to find oxidative balance, always maintaining one or two unoxidized classes. For example, there are Flavonoids and Terpenoids (Emerenciano et al., 2006); Iridoids (Das at al., 1987) and Limonoids (Kaplan; Gottlieb, 1982). In other groups, the variations may be in the diversity of the produced skeletons. In general, even at a lower hierarchical level than family, this fact may have occurred with *P. gaudichaudianum*. But an in-depth chemosystematic analysis is missing to comprove it.

Furthermore, as already mentioned, this analysis based on NOX and OS is a static approach that points out only the absence and presence of compounds in the mixture. The Redox Theory developed by Gottlieb (1989) and studied for decades, demonstrated that the evolution of micromolecules occurs through the oxidation of highly oxidized compounds. It is postulated that oxidative pathways in plants occur in parallel with a protective mechanism against oxidative degradation, reflected directly in the role of atmospheric oxygen (Gottlieb and Kaplan, 1993). These findings led to the development and application of static quantitative methodologies (Chemosystematic) to assess the evolutionary advances of species based on micromolecules patterns (Gottlieb 1982; Gottlieb and Borin 2012; Feitoza and Lima 2020). It is well known that redox reactions are part of the metabolic cycle of plants, mainly in the physiological processes of plastic responses to seasonal variations and under the command of the circadian clock (Dietz and Pfammschmidt 2011). This process normally involves complex chemical mechanisms. Assessing these molecular oxidation patterns of a mixture is necessary to really understand the redox mechanism on a fluid
Aromadendrane-based skeleton. Consequently, the latter showed a higher pattern of oxidative and structural diversification than Bicyclogermacrane in a most reducing state in the mixtures by decreasing the $S_{OR}$ related to issues of biosynthetic route. For example, Bicyclogermacrane has only one member compound and the weighted quantitative variations lead to the proportional correlation between the tested parameters, suggesting that the diversification of the carbon skeleton is followed by an increase in the $S_{OR}$ test), so Spearman's nonparametric test was applied to correlate them (Fig. 9) and to answer the question. It was possible to observe a significant inversely compounds in each carbon skeleton and the $S_{OR}$ and circadian study) lead to an increase in oxidation or a reduction in the compounds of the EOs from leaves of $P. gaudichaudianum$ (Gottlieb and Kaplan 1993). This index (GM$_{RO}$ or EM$_{RO}$), conceptually, can be applied in the large areas of Ecology, pure chemistry and product development. For example, in chemical ecology, at the level of $\alpha$-chemodiversity, it can predict and explain pattern about changes in metabolism induced by abiotic and ontogenic factors as well as interactions in ecological niches. At the level of $\beta$-chemodiversity EM$_{RO}$ can correlate and explain phenomena related to adaptive fluctuations of the special metabolism of specimens in different sites. Finally, at the level of $\gamma$-chemodiversity, this index supports to understand changes in interspecies oxidation-reduction patterns throughout ecological succession (Kessler and Kalske 2018).

The results of the GM$_{RO}$ or EM$_{RO}$ calculations from the seasonal and circadian studies of EOs of $P. gaudichaudianum$ are presented in Tables 1 and 2. It was possible to register variation between $-6.4$ to $-3.6$ in the studies (S: $-5.6$ to $-3.6$; C: $-6.4$ to $-3.6$).

In the annual variation (January to December 2017) it was registered averages of GM$_{RO}$ with significant difference ($p<0.01$). Note that the reproductive period (January, February, November, and December) coincided with the highest values of GM$_{RO}$ (greatest oxidation). After periods with high rainfall, the GM$_{RO}$ values show a decrease (greater reduction). In the same period, there were decrease in the diversity of substances present in the EOs. However, the results of Pearson's correlation for the annual variation did not show significant values, but they predict adaptive tendencies of the specimen in space.

Figure 8 (Radar graph) shows the GM$_{RO}$ variation values obtained for the EOs of the circadian study. Results demonstrated that the average values showed a significant difference during the days (October and March) and between the periods ($p = 0.05$). The average in the rainy season (March) (R: $-4.8$ to $-3.7$; D: $-6.4$ to $-4.2$) and greater oxidation at night (R: $-3.9$ to $-3.7$; D: $-4.3$ to $-3.6$). Pearson's correlation showed significant correlations between relative humidity, precipitation, and radiation. These results describe the natural metabolic movement that leads to a possible redox balance throughout the day. In addition, our GM$_{RO}$ results reinforce the protection hypothesis that specialized metabolites exert under stress conditions to minimize the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). All these variations aim to guarantee the full functioning and maintenance of plant physiology (Dietz and Pfannschmidt 2011).

A study comparing the effects of adaptation and damage to vine leaves showed that the metabolism of isoprenoids was modulated according to UV-B rates. In addition, this study associated the damages caused to the generation of ROS with the increase of the excitation energy (Gil et al. 2012). In the literature it is described that volatile terpenoids (Monoterpenes and Sesquiterpenes) are quickly combined with ROS and that these reactions are stimulated by changes in light and temperature conditions (Gil et al. 2012; Jaiswal et al. 2020). Likewise, the data obtained from GM$_{RO}$ at the macro metabolic level corroborate the Redox Theory with a quantitative parameter, which postulates that at the oxidation level, the specialized metabolism requires the existence of binary antioxidant systems: meaning that there will be a balance to guarantee a proportion of different classes of compounds in the redox system. Therefore, compounds may vary in quantity (abundance) or in reducing power (high potential), to achieve “general reducing power”, considered a metabolic homeostasis (Gottlieb and Kaplan 1993).

This theoretical statement led to the question: does the diversification of the number of compounds by carbon skeletons during different periods (seasonal and circadian study) lead to an increase in oxidation or a reduction in the compounds of the EOs from leaves of $P. gaudichaudianum$? The number of compounds in each carbon skeleton and the $S_{RO}$ values obtained in seasonal and circadian studies did not have normal distributions (Kolmogorov-Smirnov test), so Spearman's nonparametric test was applied to correlate them (Fig. 9) and to answer the question. It was possible to observe a significant inversely proportional correlation between the tested parameters, suggesting that the diversification of the carbon skeleton is followed by an increase in the $S_{RO}$ of the compounds. However, the scatterplot (Fig. 9) clearly shows that the diversification and the increase in the reduction do not occur homogeneously. This fact is related to issues of biosynthetic route. For example, Bicyclogermacrane has only one member compound and the weighted quantitative variations lead to the most reducing state in the mixtures by decreasing the $S_{RO}$. In fact, this compound is a biogenic intermediate for the formation of compounds with Aromadendrane-based skeleton. Consequently, the latter showed a higher pattern of oxidative and structural diversification than Bicyclogermacrane in a
biogenic compensatory way. The same fact was observed for Germacrane in relation to Cadinane and Eudesmane. Based on Gottlieb's redox theory, it is possible to formulate the hypothesis that the diversification of carbon skeletons in the biosynthetic routes leads to an increase in the level of weighted average oxidation (S\text{\alpha}) as a biosynthetic control. However, the quantitative percentage increase of the main intermediate metabolites (precursors) are responsible for guaranteeing the retraction of skeletal diversifications and, consequently, of the generalized oxidation of \textit{P. gaudichaudianum} EO compounds. This evidence demonstrates that static models, such as OS analysis, do not clearly reflect biosynthetic movements at different time scales, so we suggest the new S\text{\alpha} and GM\text{\alpha} indices. Thus, we make a statement of PoC related to the two new indices (S\text{\alpha} and GM\text{\alpha}) that can be applied to study redox of complex mixtures.

### Chemophenetic aspects in \textit{Piper gaudichaudianum}

Based on data from this study and those from literature, sixty (n = 60) EOs chemical composition (Table S3 - Supplementary Material) from \textit{P. gaudichaudianum} leaves were compiled. The data were processed and analyzed by PCA and HCA and are shown in Figs. 10 and 11, respectively.

For better results considering the data set, PCA was built in three axes, with a total variance of 69.6%, being PC1 (32.0%), PC2 (26.3%) and PC3 (11.3%). From these data it was possible to observe the initial separation between two groups (Fig. 10): **Group I** - With less variability, with a predominance of the shikimate pathway, and with positive charge on the PC1 and PC3 axis and negative separation on PC2 (Dillapiole); and **Group II** - With greater variability, with a predominance of acetate-levonate (MEV) and metiliritritol-phosphate (MEP) routes, and with loads distributed in opposition to the previous group (\textalpha-Humulene, \textit{E}-Caryophyllene, \textit{\textalpha}-Cadinene, 1-\textit{epi}-Cubenol, Longipinanol, Viridiflorol, Germacrane B and Bicyclogermacrene). The compounds that most contributed to the separation of the groups in PC1 and PC2 with negative charge were Bicyclogermacrene (20.3) and Dillapiole (23.4), respectively. PC3 was responsible for the smallest variations between samples. The values found were remarkably close to each other. HAA (Fig. 11) showed higher rates of similarity but confirming the separation of those groups assigned in the PCA. From the combined analysis of PCA and HAA, it was possible to define nine (n = 9) possible different chemotypes for \textit{P. gaudichaudianum}: **chemotype \textit{\textalpha}-Cadinene**, with one sample (PR2) (Bemuci et al. 2016); **chemotype \textit{\textalpha}-epi-Cubenol**, with two samples (PR3-1; PR6) (Krinski et al. 2018; Souza et al. 2020); **chemotype Longipinanol**, with one sample (PR2) (Krinski et al. 2016); **chemotype Viridiflorol**, with one sample (RO) (Morais et al. 2007); **chemotype \textit{\textalpha}-Humulene**, with three samples (SP1-1 to 2 and RS2) (Von Poser et al. 1994; Andrade et al. 1998); **chemotype \textit{E}-Caryophyllene**, with three samples (PR3-2 and PR5-1 to 2) (Krinski et al. 2018; Quiqui et al. 2019); **chemotype Germacrane B**, with three samples (PR4, PR7 and SC) (Chaabán et al. 2018; Silva et al. 2019; Silva et al. 2021); **chemotype Dillapiole**, with sixteen samples (RS1-1 to 16) (Schindler et al. 2017); and **chemotype Bicyclogermacrene**, with thirty samples (RS3, RS4 and this study – RJ1 1 to 28). The RS3 and RS4 samples showed high percentage of \textit{\textalpha}-Nerolidol (22.6–24.4%) \textit{\textalpha}-Humulene (21.3–21.3%) and lower percentage of Bicyclogermacrene (7.4–13.2%). The percentage content of this last compound led to the grouping RS3 and RS4 on chemotype Bicyclogermacrene. Although we can not rule out negative biases around plant collection errors (schedules, season), transportation, identification, quantification, or detection of compounds, they are unlikely to significantly affect the Dillapiole, Bicyclogermacrene, Germacrane B and \textit{E}-Caryophyllene cluster to be identified by different research groups and different specimens. These results clearly show the plastic chemical response capacity observed by \textit{P. gaudichaudianum} EO to edaphoclimatic and biotic factors.

The identified chemotypes were distributed on the Brazilian map, regarding to a chemogeographic approach (Fig. 12). The species areas of occurrence were highlighted in green according to the Flora of Brasil 2020 Project (Flora do Brasil 2020). It was observed that the samples found and analyzed were grouped mainly in the South and Southern regions of Brazil (Rio de Janeiro, São Paulo, Paraná, and Rio Grande do Sul States), except for a sample colleted in Rondônia State (North region). The samples showed high levels of non-oxygenated sesquiterpenes (8.3–81.5%) in relation to the other compounds. Only a few samples in the State of Paraná showed significant amounts (7.1–22.8%) of Monoterpenes. We highlight, considering the analyzed data set, that the production of Monoterpenes from Geranyl Pyrophosphate is not favored in \textit{P. gaudichaudianum} EOs, but it is of sesquiterpenes by Farnesyl Pyrophosphate. Some samples showed higher amounts of compounds from the shikimate pathway in Southern Brazil: Dillapiole (70.5–57.8% - RS1-1 to 16) and Myristicin (15.2% - PR6). So, we emphasize more in-depth evaluations at different time scales for all samples in the State of Paraná, since this great chemical plasticity may suggest not only a chemotypic variation, but a possible formation of geotypes. However, the PCA and HAA data set evidenced in this study for the same population (State of Paraná) that forms a chemotype has been described in the literature for other plant species (Gouyon et al.1986; Guo et al. 2008; Nielsen et al. 2013).

Results for EO from \textit{P. gaudichaudianum} registered in the literature thirty-one (n = 31) types of carbon skeletons (Scheme S1 and Table S3 - Supplementary Material). One (n = 1) C\textsubscript{6}C\textsubscript{6}C\textsubscript{3} derivative (Miristinic, Eugenol and Dillapiole) (Alkylbenzene); one (n = 1) derived from C\textsubscript{6}C\textsubscript{6} benzoic acid (benzyl benzoate); one (n = 1) chromene (Eupatoriumchromene); and twenty-eight (n = 28) from the MEP and MEV pathways, mainly Farnesyl Pyrophosphate precursors.

The C-skeletons via MEP and MEV with the highest qualitative occurrence (presence and absence) were Caryophyllane (n = 58), Aromadendrane (n = 56), Humulane (n = 56) and Germacrane (n = 55). The propensity for routes in the production of compounds with Humulane and Caryophyllane C-skeletons is found qualitatively and quantitatively in the samples (n = 59). Exception for PR1, in which the production of the Longipinane C-skeleton was favored, a tricyclic compound structurally more complex than Humulane, that is the Longipinane precursor.

Correlating the relative percentage of the compounds by the C-skeleton and the latitude (Lt) and longitude (Lg) (data from literature and from our study), it was observed that there was a significant (p < 0.05) and directly proportional increase of geographic position with the quantitative percentage of the compounds Germacrane (Lt: r = 0.563; Lg: r = 0.578), Bicyclogermacrene (Lt: r = 0.572; Lg: r = 0.793) and Aromadandarnate (Lt: r = 0.532; Lg: r = 0.508). So, these data suggest a longitudinal and latitudinal quantitative biosynthetic gradient from the Tropic of Capricorn to the Equator for the formation of compounds with Germacrane carbon skeletons towards Aromadandarnate. It was also found that the formation of possible chemotypes showed greater chemical structural (skeleton) diversification and and did not present spatial homogeneity in the distribution of chemical phenotypes (chemical compound) in relation to their longitudinal and latitudinal occurrence. Most chemotypes showed diversification in skeletons centered on biogenetic derivatives or compounds with a Germacrane or Humulane skeleton, following the biosynthetic path of Germacrane (PR4; PR7 and SC); Cadinane (PR2) and Cubebane (PR6 and PR3-1) or Germacrane; Bicyclogermacrene (RJ1 to RJ28, RS3 and RS4) and Aromadandarnate (RO). However, when the precursor was Humulane (RS2, SP) it followed the
biosynthetic pathway for the formation of Caryophyllane (PR3-1) or Longipinane (PR1). Thyme (Thymus vulgaris L.), a pioneer and invasive species in several countries, showed phenotypic chemical modulations in the terpenes present in the EO in different geographical positions and under evaluation in the edge effect. It was reported that the chemical response of plasticity was mainly related to environmental factors and that the most important mechanism for successful plant invasion at the forest edge is associated with the presence of the carvacrol type chemotype (Nielsen et al. 2013). This is in favor of the argument of the structural (skeleton) spatial diversification of the chemotypes present in P. gaudichaudianum, which also has pioneering characteristics.

Conclusions

P. gaudichaudianum EOs content as well as the relative percentage of the compounds are influenced by the circadian rhythm and season. The highest content was achieved in the months of December to February, and at 6 a.m. in the rainy season and at 12 p.m. in the dry season. The major identified compound was Bicyclogermacrene, with variations of E-Caryophyllene, Eudesmadiene, ENerolidol, α-Cadinol and Spathulenol. We report for the first time the high chemical phenotype plasticity presented by P. gaudichaudianum in a different time scale. It was possible to correlate changes in chemical composition at different phenological stages and under different abiotic factors. The variation between the dry and rainy periods did not strongly influence in the chemical composition, however, there were significant variations in the volatiles between day and night. More complex terpenes (bicyclic and tricyclic) were biosynthesized during the nighttime. That said, a possible chronotype based on the chemical composition of EOs is described for the first time in the genus Piper. We demonstrated that C-skeleton types are an important tool for chemophenetic analyzes and their percentage of occurrence showed trends of significant variation in the biosynthetic routes throughout the seasonal and circadian rhythm. Static models of chemosystematic analysis (considering oxidative steps) are not enough to determine oxidation patterns during temporal variations of terpenoids. Thus, for the first time and using P. gaudichaudianum as a model and considering the compound quantification of its EO, it was possible to develop and make a proof of concept of a new approach based on “Weighted Average Redox Standard” (SRO) and the “General Mixture Redox Index” (GMRO). These calculations led to correlate the production of EO compounds to the general metabolism of the species, demonstrating that there is a direction for a possible redox balance throughout the day. It was also possible to demonstrate that the diversification in the number of compounds per carbon skeleton in the P. gaudichaudianum EO is followed by an increase in the SRO of the compounds. These oxidative diversifications have as their main control point the quantitative increase in biogenetic precursors. Also, chemophenetic approach of P. gaudichaudianum allowed to determine nine possible chemotypes. Considering carbon skeletons, it was demonstrated that most chemotype diversifications are centered on biogenetic derivatives or compounds with a Germacrane or Humulane skeleton. Despite the diversification of the skeletons of the chemotypes, the data analysis did not corroborate the existence of homogeneous spatial occurrence in the compounds expressed by the chemical phenotypes in a gradient with latitude and longitude. All data together, provide evidence of ecological, chemosystematic and chemophenetic significance for the management and conservation of this medicinal and ritualistic species used by the Brazilian population.

Declarations

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Authors’ contributions

Designed and conducted the entire experiment (Ramos, Y. J. and Moreira, D. L.). Collections, species identification, data analysis, laboratory analysis (Ramos, Y. J.; Candido-Fonseca, I.; Costa-Oliveira, C.; Queiroz, G. A.; Guimarães, E. F. and Defaveri, A. C. A.). Statistical analysis (Ramos, Y. J.) and manuscript preparation and revision (Ramos, Y. J.; Costa-Oliveira, C.; Defaveri, A. C. A. and Moreira, D. L.). All authors have read and approved the final manuscript.

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Figures

**Figure 1**
Box plot analyses of the major compounds (%) registered in the essential oils from leaves of Piper gaudichaudianum Kunth (Piperaceae) collected monthly for the seasonality study (January to December 2017). Means followed by different letters are significantly different according to Tukey test (p < 0.05).

**Figure 2**
Biplot (Principal Component Analysis - PCA) resulting from the analysis of the essential oils obtained from leaves of Piper gaudichaudianum Kunth (Piperaceae) collected for the seasonality study monthly, from January to December 2017.
Figure 3

Dendrogram representing the similarity relation of the essential oils composition from leaves Piper gaudichaudianum Kunth (Piperaceae) collected for the seasonality study monthly, from January to December 2017.

Figure 4

Box plot analyses of the major compounds (%) present in the essential oils from leaves of Piper gaudichaudianum Kunth (Piperaceae) in the circadian rhythm study from 12 a.m. to 12 p.m., during Rainy (March) and Dry (October) seasons. Means followed by different letters are significantly different using Tukey test (p < 0.05).
Biplot (Principal Component Analysis - PCA) resulting from the analysis of the essential oils from leaves of *Piper gaudichaudianum* Kunth (Piperaceae) in the circadian study, during the Rainy (R, March) and Dry (D, October) seasons, from 12 a.m. to 12 p.m.

Dendrogram representing the similarity relation of the essential oils from leaves of *Piper gaudichaudianum* Kunth (Piperaceae) in the circadian study, during the Rainy (R, March) and Dry (D, October) seasons, from 12 a.m. to 12 p.m.
Figure 7
Box plot analyses of the major compounds (%) present in the essential oils from leaves of Piper gaudichaudianum Kunth (Piperaceae) in the circadian rhythm study from days (9 a.m. to 6 p.m.) and nights (9 p.m. to 6 a.m.), during March (Mar, rainy season) and October (Oct, dry season). Means followed by different letters are significantly different using Tukey test (p < 0.05).

Figure 8
Radar plot representation of the General Mixture Redox Index obtained from essential oils from leaves of Piper gaudichaudianum Kunth (Piperaceae) in the circadian rhythm study from 12 a.m. to 12 p.m., during March (Mar, rainy season) and October (Oct, dry season).
Figure 9

Correlation between compound numbers for carbon skeleton and Weighted Average Redox Standard (S_{40}) sums of compounds for the compounds identified in the essential oils from leaves of Piper gaudichaudianum Kunth (Piperaceae) in the seasonality and circadian rhythm studies.

Figure 10

Triplot (Principal Component Analysis - PCA) resulting from the analysis of the 60 essential oils composition from leaves of Piper gaudichaudianum Kunth obtained in this study (seasonality study - RJ1-12; circadian study - RJ13-29) and from literature database RO (Morais et al. 2007); RS1 - 1 to 16 (Schindler et al. 2017); RS2 (Von Poser et al. 1994); RS3 (Péres et al. 2009); RS4 (Sperotto et al. 2013); SC (Chaaban et al. 2018); SP1 - 1 and 2 (Andrade et al. 1998); PR1 (Krinski et al. 2016); PR2 (Bernuci et al. 2016); PR3 - 1 and 2 (Krinski et al. 2018); PR4 (Silva et al. 2019); PR5 - 1 and 2 (Quiqui et al. 2019); PR6 (Souza et al. 2020) and; PR7 (Souza et al. 2020).
Figure 11

Dendrogram representing the similarity relation of the 60 essential oils composition from leaves of Piper gaudichaudianum Kunth obtained in this study (seasonality study - RJ1-12; circadian study - RJ13-29) and from literature database RO (Morais et al. 2007); RS1 - 1 to 16 (Schindler et al. 2017); RS2 (Von Poser et al. 1994); RS3 (Péres et al. 2009); RS4 (Sperotto et al. 2013); SC (Chaaban et al. 2018); SP1- 1 and 2 (Andrade et al. 1998); PR1 (Krinski et al. 2016); PR2 (Bernuci et al. 2016); PR3 - 1 and 2 (Krinski et al. 2018); PR4 (Silva et al. 2019); PR5 - 1 and 2 (Quiqui et al. 2019); PR6 (Souza et al. 2020) and; PR7 (Souza et al. 2020).

Figure 12

Spatial distribution of Piper gaudichaudianum Kunth chemotypes in Brazil in accordance with this study (seasonality study - RJ1 to 12; circadian study - RJ13 to 29) and from literature database RO (Morais et al. 2007); RS1 - 1 to 16 (Schindler et al. 2017); RS2 (Von Poser et al. 1994); RS3 (Péres et al. 2009); RS4
(Sperotto et al. 2013); SC (Chaaban et al. 2018); SP1- 1 and 2 (Andrade et al. 1998); PR1 (Krinski et al. 2016); PR2 (Bernuci et al. 2016); PR3 - 1 and 2 (Krinski et al. 2018); PR4 (Silva et al. 2019); PR5 - 1 and 2 (Quiqui et al. 2019); PR6 (Souza et al. 2020) and; PR7 (Souza et al. 2020).

Supplementary Files

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- Scheme01.png
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