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ORIGINAL PAPER

Chemical variability and evaluation of physical parameters of the essential oil of the leaves of *Casearia sylvestris* varieties and morphoanatomical characterization of the leaves

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Abstract

Casearia sylvestris Swartz is a traditional medicinal plant classified into the '*sylvestris*' and '*lingua*' varieties. The essential oil (EO) of the leaves showed anti-inflammatory, antiulcerogenic, cytotoxicity, antimicrobial, and antileishmanial activities. Studying the chemical variability of this EO is important to establish its quality specifications and standardization. Here, we evaluated the population, seasonal, and circadian chemical composition of the EO of *C. sylvestris* varieties, the morphoanatomical characteristics of *C. sylvestris* leaves, and the physical parameters of the EO. Gas chromatography–mass spectrometry (GC–MS) and principal component analysis (PCA) were used to assess the metabolic profile of the EO of *C. sylvestris* varieties. The main compounds in the EO were β-elemene, α-humulene (**2**), germacrene D (**3**), bicyclogermacrene (**4**), spathulenol (**5**), caryophyllene oxide, and humulene epoxide II. Population, intrapopulation, seasonal, and circadian chemical variability was verified. Higher contents of germacrene D (**3**), α-muurolol, and α-cadinol differentiated the EO of the '*lingua*' variety from the EO of the '*sylvestris*' variety. The optical rotation of the EO of the '*lingua*' and '*sylvestris*' varieties ranged from -99.5 to $-98.7°$ and from $+82.3$ to $+190.1°$, respectively, whilst the EO of these varieties had the same refractive index (1.500) and density (0.922 g/mL). The '*lingua*' and '*sylvestris*' varieties presented trunk fissures of 5.0 and 0.5 cm, respectively. The palisade index was 2.9 for '*lingua*' and 3.9–4.3 for '*sylvestris*'. The leaves were amphistomatic in '*lingua*' and hypostomatic in '*sylvestris*'.

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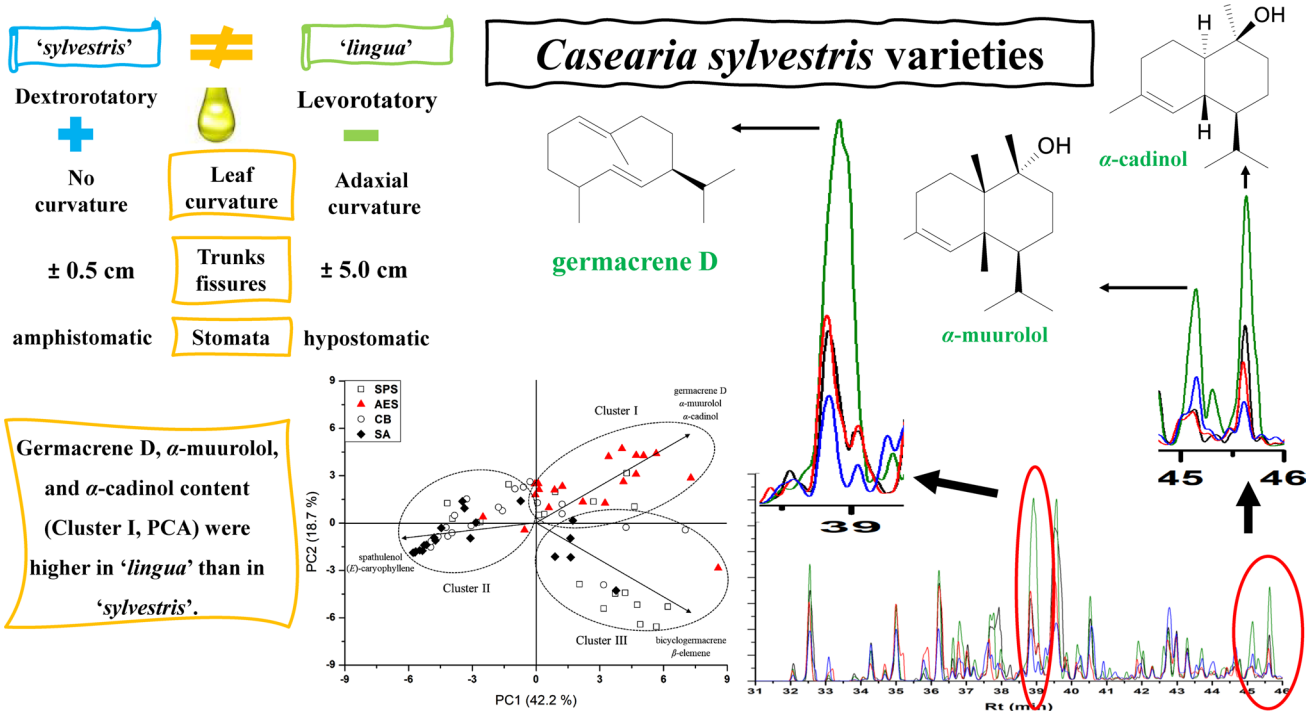
Graphical abstract



Keywords Chemical variability · Circadian · Palisade index · Principal component analysis · Seasonal analysis · Sesquiterpenes

Introduction

Casearia sylvestris Swartz (Salicaceae), commonly known as guaçatonga in Brazil, is a medicinal plant found in Latin America and other tropical and subtropical regions (Ferreira et al. 2011; Xia et al. 2015). Due to morphological differences, it is classified into two varieties: *C. sylvestris* '*sylvestris*' is a tree with larger leaves, typical of dense and humid forests such as the Atlantic Forest, whereas *C. sylvestris* '*lingua*' is a shrub with smaller leaves, typical of hot and dry habitats like the Brazilian Cerrado. Moreover, the literature describes intermediate specimens between both varieties (Cavallari et al. 2010; Claudino et al. 2013; Bueno et al. 2015). Chemically, the differences between the varieties are the predominance of glycosylated flavonoids in *C. sylvestris* '*lingua*' and casearin-like diterpenes in *C. sylvestris* '*sylvestris*' (Bueno et al. 2015, 2021; Carvalho et al. 2022). The major volatile compounds (sesquiterpenes) identified in the essential oil of the leaves (EO) of these two varieties are also different (Carvalho et al. 2021a, b). Other secondary metabolites identified in *C. sylvestris* were neolignans, triterpenes, *nor*-isoprenoids, gallic acid derivatives, and phenylpropanoids (Xia et al. 2015).

In Brazilian folk medicine, *C. sylvestris* leaves are used in the treatment of gastric disorders, anti-inflammatory, antiophidic, wound healing, anti-thermic, topical anesthetic, and antiseptic (Ferreira et al. 2011). *C. sylvestris* EO has interesting pharmacological activities including anti-inflammatory and antiulcerogenic effects observed in vivo (rats), cytotoxicity to tumor cell lines (A549, HeLa, and HT-59), antimicrobial activity against Gram-positive and Gram-negative bacteria (including *Helicobacter pylori*), antileishmanial activity against *Leishmania amazonensis* (promastigote and amastigote forms), antiviral (against herpes simplex virus type 1), and antifungal activity against *Candida glabrata*, *C. krusei*, *C. albicans*, and *Saccharomyces cerevisiae* (Esteves et al. 2005; Silva et al. 2008; Bou et al. 2013; Pereira et al. 2016, 2017; Carvalho et al. 2018; Moreira et al. 2019; Spósito et al. 2019). Nonetheless, pharmacological studies on the EO from *C. sylvestris* did not specify the botanical variety.

Environmental factors like temperature, rainfall, location, altitude, latitude, relative humidity (RH), ultraviolet radiation (UVR), soil composition, seasonality, and the circadian cycle may modify the composition and yield of *C. sylvestris* EO, directly interfering with its pharmacological activity. These factors highlight the importance

of location, period, and time of plant collection (Gobbo-Neto and Lopes 2007; Sarrazin et al. 2015; Kiazolu et al. 2016; Evergetis et al. 2016).

Recent studies on the volatile chemical profile of *C. sylvestris* EO have shown that the chemical composition of the EO of the leaves *in natura* differs from the chemical composition of the EO of fresh and dried leaves. In addition, there are chemical differences between the varieties, and the chemical composition of different populations of '*sylvestris*' varieties (Carvalho et al. 2021a, b). Here, we have evaluated the chemical composition population, seasonal, and circadian of *C. sylvestris* EO varieties through multivariate statistical analysis, as well as the correlation with edaphic-climatic factors, besides morphoanatomical evaluations of the leaves and determined the EO physical parameters.

Experimental

Plant material

Casearia sylvestris Swartz (Salicaceae) leaves were collected from 10 specimens of four different populations in the state of São Paulo, Brazil, as follows: SPS population (Cerrado biome): Medicinal Botanical Garden of the School of Pharmaceutical Sciences of Unesp, Araraquara (21° 48′ 88″ 3–21° 48′ 98″ 9 S, 48° 11′ 86″ 1–48° 12′ 13″ 3 W); AES population (Cerrado biome): Araraquara Experimental Station of Instituto Florestal de São Paulo, Araraquara (21° 44′ 14″ 6–21° 44′ 63″ 4 S, 48° 10′ 40″ 1–48° 10′ 81″ 3 W); CB population (Atlantic Forest biome): Carlos Botelho State Park, São Miguel Arcanjo (24° 3′ 42″ 8–24° 3′ 84″ 0 S, 47° 59′ 45″ 4–47° 59′ 80″ 5 W); and SA population (Cerrado biome): campus of the School of Agriculture of Unesp, Botucatu (22° 50′ 22″ 5–22° 50′ 94″ 8 S, 48° 25′ 50″ 6–48° 25′ 63″ 7 W). The population collections were conducted in Jul and Dec/2016; the seasonal collections were performed from Jun/2016 to May/2018; and the circadian collections were made in Feb and Aug/2017. The collected specimens were identified by Dr. Luis V. S. Sacramento (School of Pharmaceutical Sciences, Unesp, Araraquara), and voucher specimens were deposited at Herbarium "D. Bento Pickel" (AES population: SPSF 51.207–51.215 and 51.818, CB population: 51.816–51.825) and Herbarium Maria E. P. K. Fidalgo of the Botanical Institute of São Paulo (SPS population: FAC 101–110, and SA population: 301 310). This study was registered under the code AEFB157 of the Brazilian National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen).

Morphological and anatomical analysis

The length × width of the leaves (Digital Caliper, Model 6150 Lee Tools®), the estimated height, the diameter at breast height (DBH), and the trunk shape were determined for the *C. sylvestris* specimens. Anatomical analysis was performed with leaves from the middle region of the branches of selected specimens (SPS 101, 102, 107; AES 201, 206, 209; CB 403, 406, 407; and SA 302, 305, and 310). The paradermal sections of the middle region of the leaves were cut with a razor blade, discolored with 2.0% sodium hypochlorite for 5 min, washed with deionized water, stained with Astra blue for 5 min, and fixed in a glass blade. The palisade cells in five groups of four epidermal cells were counted, and the stomata were analysed on the adaxial and abaxial surfaces by using a Leica® Microscopic (Wetzlar, Germany), DMLB AxioCam Icc1 40 x (Johansen 1940; Evans 2002).

Extraction and gas chromatography analysis of the essential oil

The leaves of each *C. sylvestris* specimen were separately dried in an air circulating oven at 40 °C for 72 h, and storage at − 4 °C. The dried leaves (30 g) were extracted by hydrodistillation in a Clevenger-type apparatus over 4 h (Brasil 2010). The EO of 10 specimens of each population was solubilized in hexane (3.0 µL/mL), mixed, and analyzed by gas chromatography–mass spectrometry (GC–MS) on a Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autosampler and fitted with an Rtx-5MS capillary column (5% diphenyl and 95% polydimethylsiloxane; 30 m × 0.25 mm, 0.25-µm film thickness). Helium (99.9999%) was used as carrier gas (1.0 mL/min). The injection volume was 1.0 µL (split mode, 1:10). The injector and ion source temperatures were 240 and 280 °C, respectively. The oven temperature was programmed to rise from 60 to 250 °C (3 °C/min, 80 min). The electron ionization source operated at 70 eV. The mass spectra were registered with scan intervals of 0.5 s, in the m/z range between 45 and 600 Da. The relative areas were calculated by the chromatogram peak area normalized method. The compounds in the EO were computer-matched with spectra of the mass spectral libraries Wiley 7, NIST 08, and FFNSC 1.2. They were identified by comparison of their retention indices relative to the series of *n*-alkanes (C8–C40 Sigma-Aldrich®) (Van Den Dool and Kratz 1963) and by comparison with the corresponding retention index in the literature (Adams 2007). The identities of (*E*)-caryophyllene (**1**), *α*-humulene (**2**), and caryophyllene oxide were further confirmed by injection with authentic standard compounds purchased from Sigma-Aldrich®.

Chemometric methods

The data matrixes were organized into samples (columns) and variables (rows). The initial data were the 9120 chromatographic (GC–MS) absolute intensity (mAU) variables (t_R: 0–80 min) × samples (80, 480, and 160 depending on the analysis), exported in ASCII format, and organized in Microsoft Excel® 97-2003 (Microsoft®, USA). The chromatographic peaks were aligned by using Matlab® R2021a (MathWorks Inc., Natick, USA) with an implemented COW (Correlation Optimized Warping) algorithm http://www.models.kvl.dk/users/rasmus/.

A data matrix (X) with the samples (rows) and the variables (columns) was organized with the selected mean-centered, auto-scaled preprocessing data, and principal component analysis (PCA) was performed by using Pirouette® v. 4.5 rev. 1 (Infometrix Inc., Bothell, USA). The population collections (SPS, AES, CB, and SA) were mean-centered by using matrixes with 179 variables × 80 samples. The seasonal collections (SPS and AES) were auto-scaled by using matrixes with 187 variables × 478 samples. The circadian collections (SPS and AES) were auto-scaled by using matrixes with 165 variables × 40 samples.

Physical parameters of the essential oil

The density of the EO was determined on a pycnometer (1.0 mL) at 20 °C. A Carl Zeiss® G refractometer (Jena, Germany) (20 µL) fitted with a monochromatic light at 589 nm was used to measure the refractive index at 25 °C. The optical rotation of the EO was measured in a Perkin Elmer® 341 LC digital polarimeter (Shelton, USA) (1.0 mg/mL, hexane) at 34 °C in a 1.0-mL bucket with 1.0-dm optical path. Monochromatic light at 589 nm was employed (Brazil 2010).

Infrared spectroscopy of the essential oil

The IR spectra of the EO were recorded on an Alpha Platinum ATR FT-IR Brucker® spectrometer (Karlsruhe, Germany) (20 µL), from 4000 to 500 cm^{-1}, with 64 scans and 4-cm^{-1} resolution. Each EO sample represented the mixture of EO from the leaves of 10 specimens.

Soil analysis

The soil samples (500 g) were collected between 10 and 20 cm below the surface of each specimen (Freitas et al. 2014) and homogenized. The chemical and physical properties were determined according to Embrapa (2013). To this end, pH, organic matter (OM), phosphorous (P resin), potassium (K), calcium (Ca), magnesium (Mg), H + Al (potential acidity), the sum of bases (SB), cation exchange capacity (CEC), base saturation (BS), sulfur (S), aluminum (Al), sodium (Na), and particle size were analysed.

Statistical analysis

One-way ANOVA and Tukey's statistical analyses were performed for the yield and chemical composition of the EO and the morphological, anatomical, and physical analyses of the leaves; $p > 0.05$ was considered significant.

Results and discussion

Morphological analysis

First, we focused our efforts on the differentiation between '*lingua*' and '*sylvestris*' varieties of *C. sylvestris* based on morphological criteria. According to Vieira et al. (2014), a DBH of 1.3 ± 0.2 cm is typical of the early stage of *C. sylvestris*, while DBH higher than 12.9 ± 2.0 cm characterizes an adult stage. Moreover, Vieira et al. (2014) observed heights of 1.3 ± 0.2 m for the early stage and 10.3 ± 0.6 m for the adult stage of *C. sylvestris*. Literature data also point out that *C. sylvestris* has been found as a sub-shrub or tree with height between 1.5 and 15.0(18.0) m (Torres and Yamamoto 1986; Klein and Sleumer 1984). In this study, the mean DBH of *C. sylvestris* was 16.8 ± 9.3, 29.0 ± 13.6, 30.2 ± 7.5, and 21.1 ± 9.3 cm for the SPS, AES, CB, and SA population, respectively, without significant statistical difference ($p > 0.05$). The *C. sylvestris* specimens showed an estimated height of 4.0, 3.6, 4.5, and 3.6 m (SPS, AES, CB, and SA populations, respectively. These results indicated that all the specimens were characterized in the adult stage.

Thick trunks like the trunk of the '*lingua*' variety (typical of Cerrado) are more resistant to hot and dry habitats (Klein and Sleumer 1984; Sleumer 1980; Claudino et al. 2013; Bueno et al. 2016). It was observed that the AES population ('*lingua*') presented trunks with maximum fissures of 5.0 cm (Fig. S1a and S1b), which was more significant than the maximum fissures of 0.5 cm observed in the trunks of the SPS, CB, and SA populations ('*sylvestris*') (Fig. S1c and S1d). Therefore, the trunk morphology could be used to differentiate the *C. sylvestris* varieties visually. Based on this morphology criteria, the specimens of the AES population (except 4 and 8) could be classified as '*lingua*', whereas the specimens of the SPS population (except 4, 8, and 9) and CB population could be classified as '*sylvestris*'. However, we were not able to classify the SA population according to trunk morphology.

Literature reports *C. sylvestris* leaves measuring around (4.0−)5.0–12.0(−14.0) × (1.0−)2.0–3.5(−4.0) cm (Torres and Yamamoto 1986; Marquete 2001).

Our results were similar to those from literature and revealed that the length × width of *C. sylvestris* leaves ranged from 9.0 ± 1.7 × 2.8 ± 0.4 cm (SPS Population), 7.1 ± 0.7 × 2.3 ± 0.3 cm (AES Population), 8.7 ± 1.4 × 2.8 ± 0.3 cm (CB Population), and 9.6 ± 1.2 × 2.7 ± 0.5 cm (SA Population). The length of the leaves of the SPS, AES, CB and SA populations did not differ significantly ($p > 0.05$). However, the width of '*sylvestris*' leaves (SPS, CB, and SA populations) was statistically different ($p > 0.05$) from the width of '*lingua*' leaves (AES population).

Klein and Sleumer (1984) classified '*sylvestris*' leaves as oblong, whereas which is similar to the leaves of the SPS, CB, and SA populations, and '*lingua*' leaves as more or less ovate to oblong-ovate shapes, which was the case of the leaves of the AES population, with adaxial curvature.

For the SPS and AES populations, we observed the flowering stage from May to July and from July to October, respectively. Concerning fructification, it occurred from August to October in both populations, in accordance with literature data (Torres and Yamamoto 1986; Klein and Sleumer 1984).

Palisade index and stomata analysis

The palisade index (PI) of the SPS, CB, and SA populations were 4.3 ± 0.5, 4.2 ± 0.4, and 3.9 ± 0.5, respectively, representing polygonal epidermal cell walls. The AES population had PI of 2.9 ± 0.4 (with a significant statistical difference from others, $p > 0.05$) with rounded epidermal cells walls. Based on the studies of Claudino et al. (2013), who related PI of 3.9 ± 0.2 to '*sylvestris*' and 2.8 ± 0.4 to '*lingua*', the specimens of the SPS, CB, and SA populations were considered to belong to the '*sylvestris*' variety, whereas the specimens of the AES population belonged to the '*lingua*' variety, as verified by Claudino et al. (2013) for *C. sylvestris* varieties.

The stomata of the specimens of the SPS, AES, CB, and SA populations did not present statistically different indices of the abaxial surface (Fig. S2). However, the adaxial surfaces (Fig. S3) distinguished between the varieties through the presence and absence of paracitic stomata in the '*lingua*' and '*sylvestris*' varieties, respectively (Claudino et al. 2013). Therefore, stomatal cells on the adaxial surface indicated that the leaves of the AES population, classified as '*lingua*', were amphistomatic, whereas the absence of stomatal cells on the adaxial surface indicated that the leaves of the SPS, CB, and SA populations, classified as '*sylvestris*', were hypostomatic.

EO chemical variability

Chemical profile of the EO of the populations

After identifying the varieties of the *C. sylvestris* populations SPS, AES, CB, and SA based on morphological criteria, we investigated the chemical composition of the dried leaves EOs of these populations. Table 1 displayed the chemical composition of the EO from SPS, AES, CB, and SA as determined by GC–MS, expressed as the mean of 10 specimens for each population, % ± SD. Other data used for the identification of the EO compounds (e.g., retention time, experimental retention index, retention index from literature, and similarity %) are given in Tables S1–S4 and Fig. S4).

The main compounds identified in the EO of *C. sylvestris* '*sylvestris*' were a) SPS population: bicyclogermacrene (**4**) (15.5%) and *α*-humulene (**2**) (10.7%); b) CB population: bicyclogermacrene (**4**) (10.2%) and (*E*)-caryophyllene (**1**) (10.0%); and c) SA population: spathulenol (**5**) (10.5%). The main compounds identified (Fig. 1) in the EO of *C. sylvestris* '*lingua*' were d) AES population: germacrene D (**3**) (17.7%) and bicyclogermacrene (**4**) (17.1%). Although these compounds have been detected in the EO of *C. sylvestris* by other authors (Esteves et al. 2005; Schneider et al. 2006; Tininis et al. 2006; Sousa et al. 2007; Bou et al. 2013; Amaral et al. 2017; Pereira et al. 2017; Carvalho et al. 2018, 2021a, b; Moreira et al. 2019; Spósito et al. 2019), differences between the EO composition of '*sylvestris*' and '*lingua*' variabilities has not been previously reported.

Sesquiterpenes were the only compounds identified in the EO of the dried leaves of all the *C. sylvestris* populations (SPS, AES, CB, and SA). Sesquiterpene hydrocarbons were the main compounds in the EO of *C. sylvestris* ranging from 54.1 to 74.7%, Analysis of the chemical profile of the EO of different *C. sylvestris* populations (SPS, AES, CB, and SA) indicated higher contents of *δ*-elemene, germacrene D (**3**), bicyclogermacrene (**4**), *δ*-cadinene, *α*-muurolol, and *α*-cadinol (Fig. 2 and Fig. S5) in the '*lingua*' variety (AES population) as compared to the '*sylvestris*' variety (SPS, CB, and SA populations).

EO population variability of the specimens from SPS, AES, CB, and SA

Associated with chemometric techniques (Gutierrez et al. 2012), the chromatographic profile of the EO of *C. sylvestris* can be used as a standard reference for controlling the quality, analyzing adulterants, and identifying the origin of the sample (Liang et al. 2004; Lu et al. 2012; Salcedo et al. 2016; Wang et al. 2019). For this purpose, analysis of many specimens under different environmental conditions and of distinct genetic origins is required.

Table 1 Chemical composition of the EO of the dried leaves of *C. sylvestris* '*sylvestris*' (SPS, CB, and SA populations) and '*lingua*' (AES population) from specimens collected in July/2016

Compound	Population (%, RA)*			
	SPS	AES	CB	SA
δ-elemene	6.0 ± 1.4	5.5 ± 1.3	5.8 ± 1.5	2.8 ± 0.8
α-cubebene	0.2 ± 0.1	–	0.1 ± 0.1	1.2 ± 0.4
α-copaene	1.7 ± 0.7	1.1 ± 0.6	1.1 ± 0.3	1.9 ± 0.7
β-elemene	3.6 ± 0.5	3.7 ± 1.1	8.2 ± 3.5	2.8 ± 1.4
α-gurjunene	0.9 ± 0.5	–	3.9 ± 1.4	1.3 ± 0.4
(*E*)-caryophyllene	8.6 ± 1.4	9.5 ± 1.1	10.0 ± 2.9	3.6 ± 1.2
β-copaene	1.0 ± 0.5	–	1.1 ± 0.4	1.9 ± 1.1
γ-elemene	1.0 ± 0.6	–	2.5 ± 1.3	1.9 ± 0.4
aromadendrene	1.8 ± 0.7	0.5 ± 0.2	2.0 ± 1.3	1.1 ± 0.5
alloaromadendrene	2.7 ± 0.8	0.8 ± 0.2	0.4 ± 0.1	2.2 ± 0.7
(*E*)-muurola-3,5-diene	–	–	–	2.5 ± 1.2
α-humulene	10.7 ± 1.8	4.1 ± 2.7	2.7 ± 0.5	3.6 ± 1.5
9-*epi*-(*E*)-caryophyllene	1.1 ± 0.8	0.9 ± 0.4	1.1 ± 0.3	1.0 ± 0.4
γ-gurjunene	1.1 ± 0.4	–	0.3 ± 0.2	–
γ-muurolene	0.5 ± 0.2	0.6 ± 0.3	1.2 ± 0.4	0.7 ± 0.3
germacrene D	8.5 ± 1.4	17.7 ± 3.0	8.9 ± 1.3	5.9 ± 1.6
β-selinene	1.8 ± 0.6	0.5 ± 0.3	2.3 ± 1.3	1.5 ± 0.9
(*E*)-*β*-guaiene	1.1 ± 0.8	–	–	2.1 ± 1.5
valencene	–	–	0.8 ± 0.4	–
bicyclogermacrene	15.5 ± 1.2	17.1 ± 1.4	10.2 ± 1.2	7.3 ± 1.4
(*Z*)-*γ*-bisabolene	1.1 ± 0.9	–	2.0 ± 0.9	1.1 ± 0.5
α-muurolene	–	0.9 ± 0.3	0.4 ± 0.3	–
γ-cadinene	0.6 ± 0.4	0.9 ± 0.4	1.0 ± 0.5	0.6 ± 0.3
δ-cadinene	5.0 ± 1.7	5.6 ± 1.1	1.8 ± 0.5	4.5 ± 1.7
(*E*)-cadina-1,4-diene	0.2 ± 0.1	–	–	1.5 ± 0.8
germacrene B	–	–	1.3 ± 0.7	1.1 ± 0.7
Sesquiterpene hydrocarbon Content	**74.7 ± 0.8**	**69.4 ± 1.0**	**69.1 ± 0.9**	**54.1 ± 0.9**
maaliol	1.0 ± 0.6	1.5 ± 1.1	–	0.5 ± 0.3
(*E*)-nerolidol	–	1.4 ± 0.5	–	–
palustrol	–	1.1 ± 0.6	0.6 ± 0.4	0.7 ± 0.3
spathulenol	6.0 ± 1.7	3.8 ± 1.0	2.6 ± 0.4	10.5 ± 1.6
caryophyllene oxide	–	1.4 ± 0.4	–	–
globulol	2.6 ± 0.8	–	3.0 ± 0.6	5.2 ± 2.7
rosifoliol	–	2.1 ± 1.0	–	–
viridiflorol	1.8 ± 0.6	–	2.0 ± 0.8	2.3 ± 1.1
guaiol	–	–	1.1 ± 0.4	–
humulene epoxide II	–	1.0 ± 0.3	–	–
5-*epi*-7-*epi*-*α*-eudesmol	–	1.6 ± 1.1	–	1.7 ± 0.8
cubenol	1.1 ± 0.8	3.3 ± 1.8	1.3 ± 0.3	1.7 ± 0.4
α-muurolol	1.1 ± 0.7	1.1 ± 0.4	0.5 ± 0.2	–
α-cadinol	2.6 ± 1.0	5.0 ± 0.6	2.2 ± 1.1	1.0 ± 0.6
Oxygenated sesquiterpenes Content	**16.2 ± 0.9**	**23.3 ± 0.8**	**13.3 ± 0.5**	**23.6 ± 1.0**
Identified compounds Content	**90.9 ± 0.8**	**92.7 ± 0.9**	**82.4 ± 0.7**	**77.7 ± 0.9**

Bold values were expressed as mean (%) +/- SD. These values refer to the sum of the total compounds by classes of terpenes of 10 specimens

*RA: relative area (%) in the GC–MS chromatogram, expressed as the mean of 10 specimens for each population

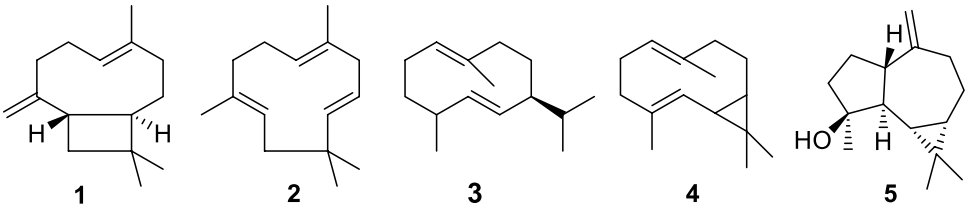


Fig. 1 Chemical structures of the main compounds in the EO of *C. sylvestris*

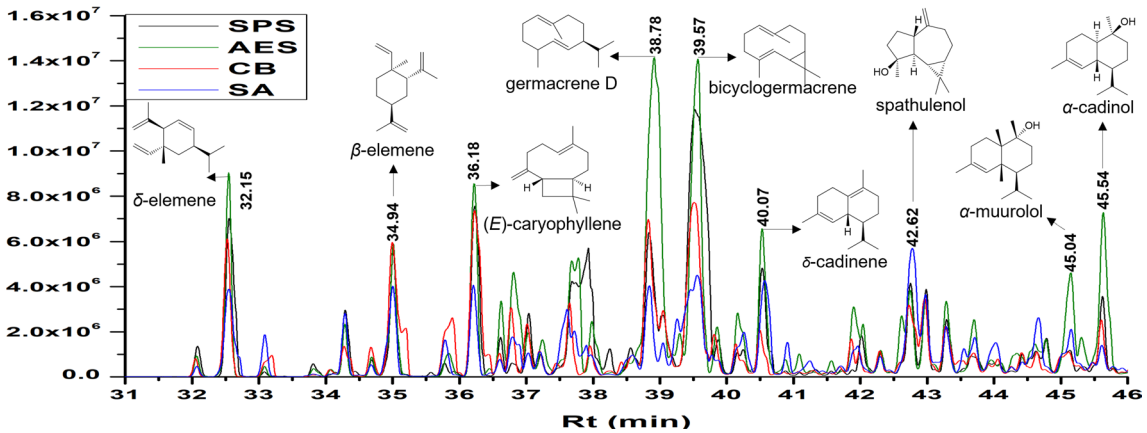


Fig. 2 Overlapped chromatograms (TIC) of the EO of the SPS, AES, CB, and SA populations of *C. sylvestris*. Each line represents the mean of 10 specimens for each population (Jul/16)

Here, we aligned the chromatographic peaks before analyzing the chemical variability of the EO of *C. sylvestris* specimens from different populations (Fig. S6). By using the COW algorithm, we aligned and adjusted the retention time (Rt) of the chromatographic peaks to correct the displacements of the Rt and peak intensities (Nederkassel et al. 2006). According to Casale et al. (2010), Martins et al. (2011), and Sherman et al. (2018), minor variations in temperature, column age, spine bleed, noise, and contaminants may shift the Rt and peak intensities, which can be minimized by peak alignment.

Analysis of the chemical data of the specimens from *C. sylvestris* varieties ('*sylvestris*' and '*lingua*') populations (SPS, AES, CB, and SA) revealed three clusters (I, II, and III, Fig. 3). Cluster I included most specimens ('*lingua*') from the AES population and a few specimens from the SPS population ('*sylvestris*'). These two populations were collected at a region of transition between the Cerrado and Atlantic Forest biomes (Araraquara city), where intermediate specimens between both varieties may be found (Cavallari et al. 2010; Claudino et al. 2013; Bueno et al. 2015). The SPS population specimens presented in cluster I were collected in brighter and less humid places, which are characteristics similar to the conditions of the Cerrado biome. Although the SPS population specimens did not present typical botanical characteristics of the '*lingua*', like trunks with more significant fissures or leaves with adaxial curvature, the chemical composition of their EO was similar to the chemical composition of the EO of the '*lingua*' (AES population). Thus, these specimens may be considered intermediate between '*sylvestris*' and '*lingua*' varieties. The main compounds in cluster I were germacrene D (**3**), *α*-muurolol, and *α*-cadinol.

Cluster II included most CB and SA specimens ('*sylvestris*') and a few specimens of the SPS ('*sylvestris*') and AES ('*lingua*') populations. Although the CB population belongs to the Atlantic Forest biome and typically presents fungi in its leaves, the chemical composition of its EO and the morphology of its leaves resembled those of the SA population, which occurs in the Cerrado biome. Interestingly, the two AES specimens in cluster II were in a shaded place, which is typical of the Atlantic Forest. The main compounds in cluster II were (*E*)-caryophyllene (**1**) and spathulenol (**5**). Cluster III included only '*sylvestris*' specimens, mainly of the SPS and SA populations, and the main compounds were *β*-elemene and bicyclogermacrene (**4**).

Higher contents of germacrene D (**3**), *α*-muurolol, and *α*-cadinol (cluster I) differentiated the '*lingua*' from the '*sylvestris*' populations. The SPS ('*sylvestris*') and AES ('*lingua*') populations were from the same region (similar environment), indicating that genetic differences may influence the quantitative chemical differences between these populations.

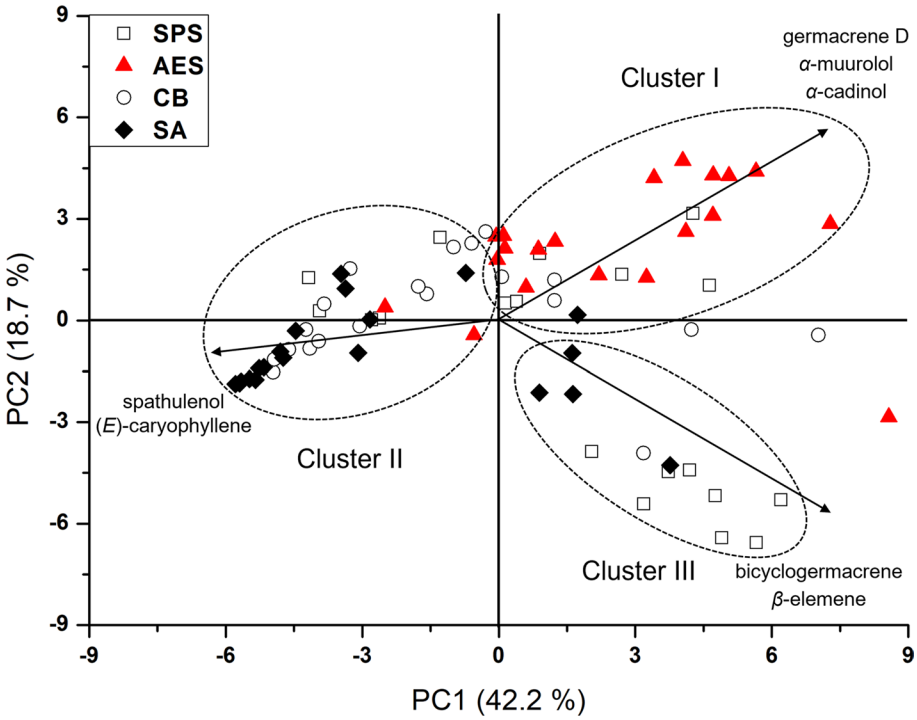


Fig. 3 Scatterplot of the population PCA of the EO of *C. sylvestris* revealing chemical clusters I, II, and III. Each population is composed of 10 specimens (Jul/2016)

EO seasonal variability of the specimens from SPS and AES populations

Seasonal chemometric analysis (Fig. 4) demonstrated that the chemical composition of the EO of the SPS population ('*sylvestris*') did not show seasonal variability because all the specimens belonged to cluster I. Pereira et al. (2020) did not observe seasonal variation for the EO of the '*sylvestris*' variety either, which corroborates our data. Seasonal chemometric analysis of the EO of the AES population ('*lingua*') (Fig. 4) showed two clusters (I and II) and a few non-clustered specimens, indicating that seasonal factors influenced the chemical composition of this EO. Most of the '*lingua*' specimens were in cluster I. Meanwhile, some EO from

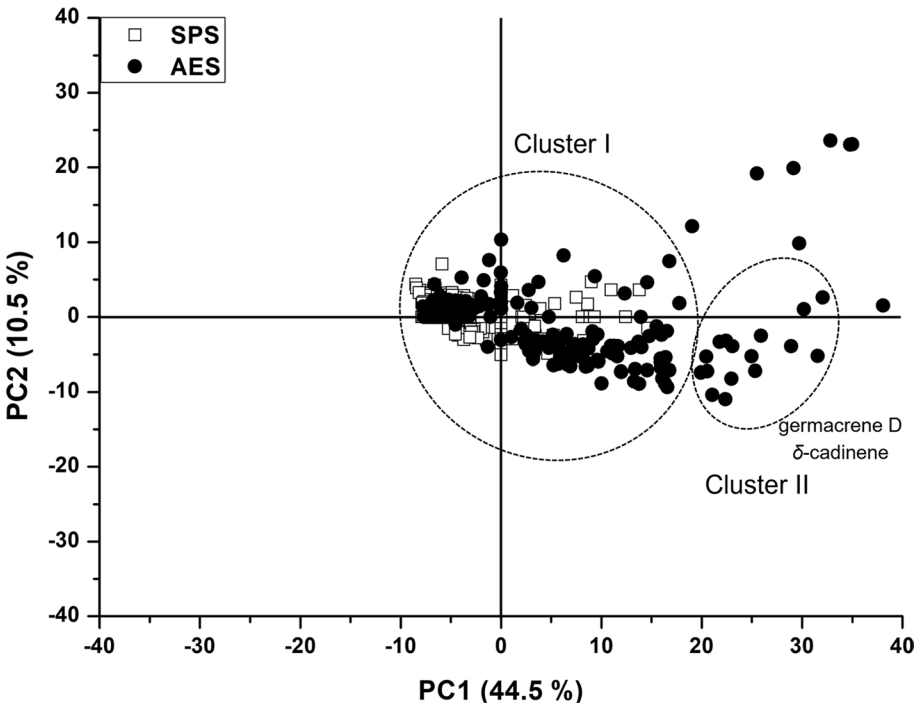


Fig. 4 Scatterplot of the seasonal PCA of the EO of *C. sylvestris* showing chemical clusters I and II

'*lingua*' (AES population) specimens (cluster II) presented the highest contents of germacrene D (**3**) and *δ*-cadinene from November to March. This variation could be related to the higher temperature and rainfall in this period.

Analysis of the seasonal variability of the EO also allowed to differentiate between the '*lingua*' and '*sylvestris*' varieties: germacrene D (**3**), *α*-muurolol, and *α*-cadinol presented higher peak areas in the '*lingua*' variety (Fig. 5).

EO circadian variability of the specimens from SPS and AES populations

We observed that the harvest time during the circadian cycle influenced the EO composition of the '*sylvestris*' variety (SPS population) just in terms of the content of some compounds, mainly *δ*-elemene, *α*-humulene (**2**), and spathulenol (**5**) (Fig. 6). The circadian cycle did not affect the qualitative compositions of the EO. The scatterplot of the circadian PCA in February and August/2017 showed that most of the '*sylvestris*' specimens were in a single cluster (Fig. S7 and S8). However, Tininis et al. (2006) verified circadian chemical variability between 7:00 a.m. and 3:00 p.m. when they analyzed the EO of the leaves of a CB population ('*sylvestris*'), which may be associated with the different biomes.

The EO of the '*lingua*' variety (AES population) showed lower contents of *δ*-elemene, *β*-elemene, (*E*)-caryophyllene (**1**), germacrene D (**3**), bicyclogermacrene (**4**), and *δ*-cadinene at 9:00 a.m. (Fig. 7). In contrast, the content of spathulenol (**5**) was higher at 9:00 a.m., indicating that the harvest time influenced the composition of the EO of the '*lingua*' variety.

Chemometric analysis of the EO of the '*lingua*' variety during the circadian cycle showed two clusters in Feb and Aug/17 (Figs. 8 and 9, respectively): specimens collected at

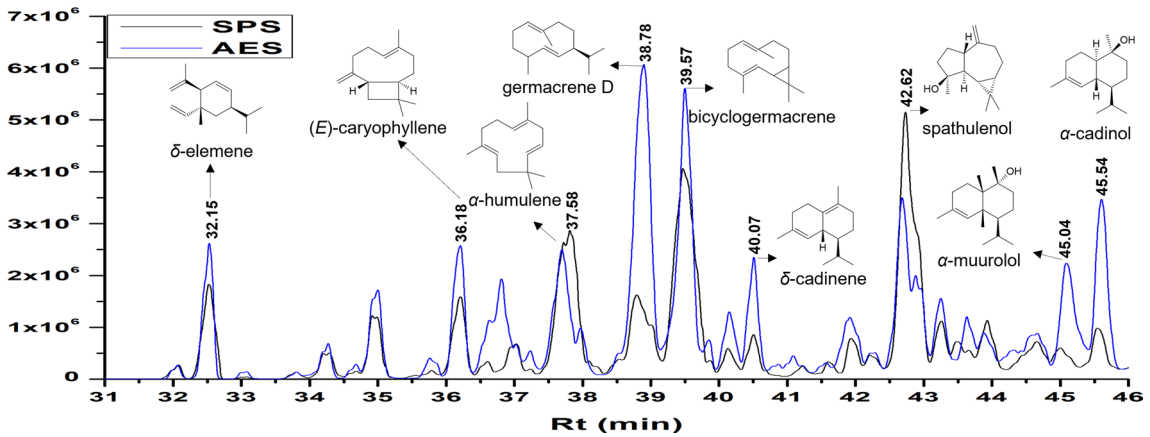


Fig. 5 Overlapped seasonal chromatograms (TIC) of the EO of the SPS and AES populations of *C. sylvestris*. Each line represents 240 specimens (Jul/16 to May/18)

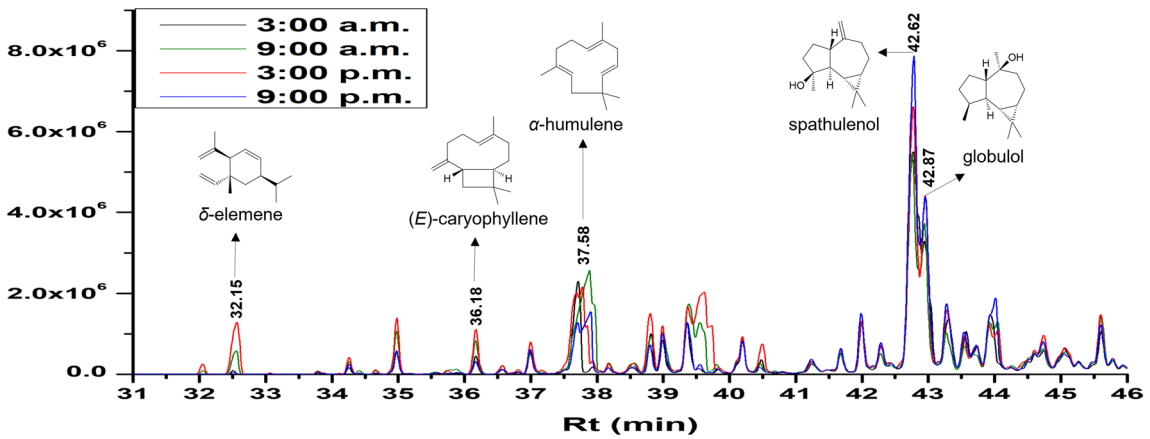


Fig. 6 Overlapped circadian chromatograms (TIC) of the EO of the SPS population of *C. sylvestris*. Each line represents 20 specimens (Feb and Aug/17)

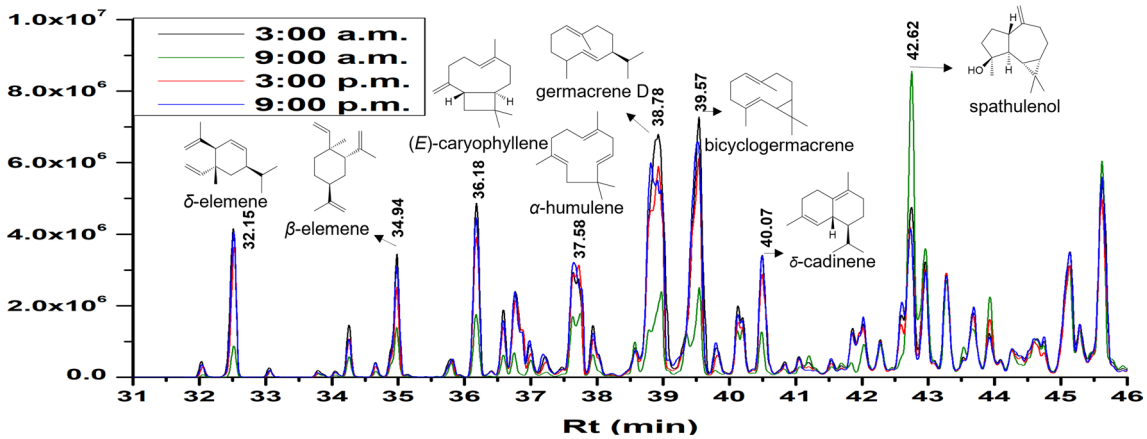


Fig. 7 Overlapped circadian chromatograms (TIC) of the EO of the AES population of *C. sylvestris*. Each line represents 20 specimens (Feb and Aug/17)

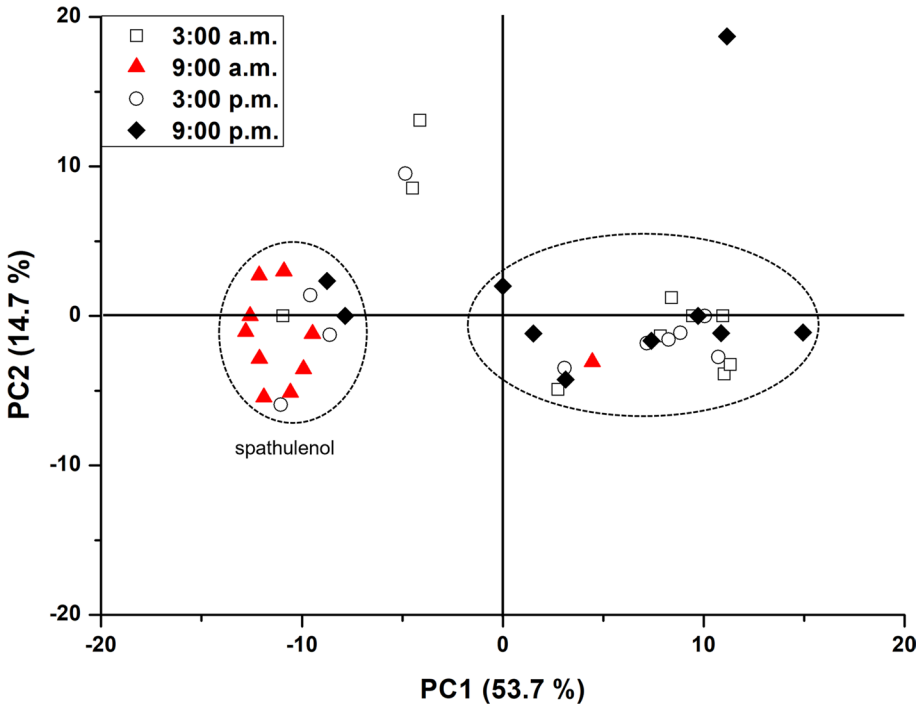


Fig. 8 Scatterplot of the circadian PCA in February of the EO of the AES population ('*lingua*') of *C. sylvestris* showing the chemical clusters according to harvest time

9:00 a.m. predominated in one cluster, and specimens collected at 3:00 a.m., 3:00 p.m., and 9:00 p.m. predominated in another cluster.

EO content of *C. sylvestris*

Population EO content (SPS, AES, CB, and SA populations)

The EO content (v/w) of the dried leaves of the SPS, AES, CB, and SA populations (Fig. S9) ranged from 0.6 to 1.2%. These contents were similar to literature data (0.3 to 2.5%) for dried leaves (Silva and Bauer 1970; Scavone et al. 1979; Esteves et al. 2005; Tininis et al. 2006; Schneider et al. 2006; Castellani et al. 2006; Sousa et al. 2007; Silva et al. 2008; Bou et al. 2013; Amaral et al. 2017; Pereira et al. 2017; Carvalho et al. 2018, 2021a, b; Moreira et al. 2019; Spósito et al. 2019). The EO content of the leaves of the CB population, which occurs in a typical Atlantic Forest region, was statistically higher than the EO content of the leaves of the other populations.

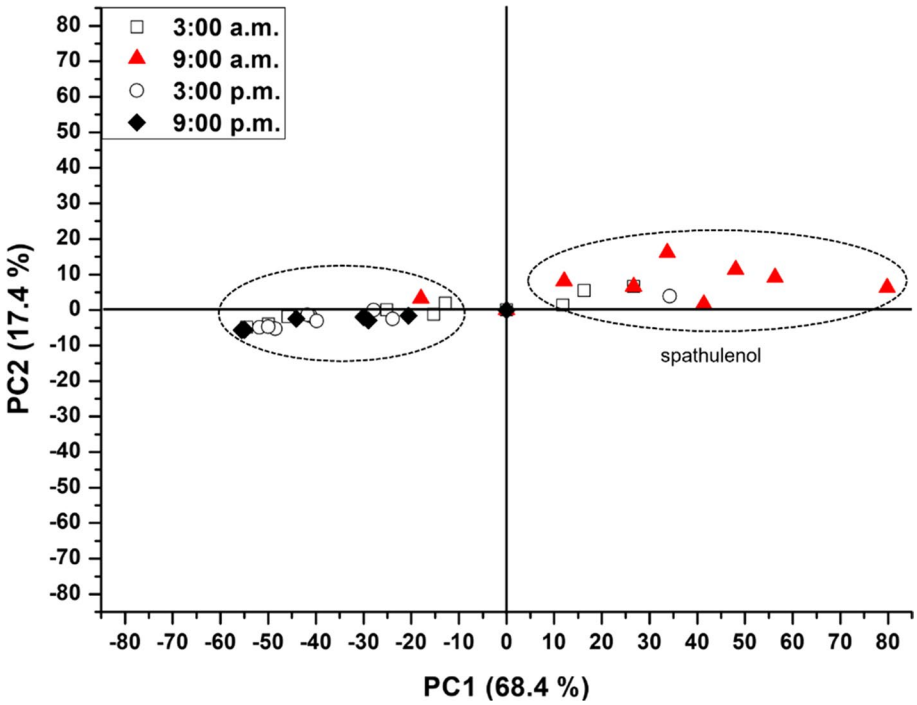


Fig. 9 Scatterplot of the circadian PCA in August of the EO of the AES population ('*lingua*') of *C. sylvestris* showing the chemical clusters according to harvest time

Seasonal essential oil content (SPS and AES populations)

The mean seasonal EO content of the leaves of the SPS population ('*sylvestris*') was 1.1 ± 0.2%. We observed lower content in June and July in both years. We determined the EO content along 24 months and verified statistical difference only in two months (Fig. S10). The monthly statistical difference between Jul/16 and Jan/18 could be related to the average climatic factors: UVR, which ranged from 6 (Jul/16) to 1 (Jan/18); RH, which ranged from 68 (Jul/16) to 89% (Jan/18); and temperature, which ranged from 18.1 (Jul/16) to 26.0 °C (Jan/18). In both years, July corresponded to flowering and January corresponded to the non-reproductive stage. Climatic factors (Fig. S11), as the UVR, RH, and temperature may be responsible for changing the content of secondary metabolites, according to literature data (Blank et al. 2005; Gobbo-Neto and Lopes 2007; Figueiredo et al. 2008). The lower EO content observed in June and July could be related to the fact that the plants grow more slowly during the winter, so decreasing the biosynthesis of secondary metabolites, whereas higher temperatures, RH, and rainfall increase the biosynthesis of EO components (Soni et al. 2015).

The mean seasonal EO content of the leaves of the AES population was 0.8 ± 0.3%. The EO content did not show statistically different values along the months (Fig. S12), indicating that rainfall, temperature, RH, and UVR did not impact the EO content (Fig. S13). Similarly, the phenological stage did not affect the EO content of the AES population. Nevertheless, Castellani et al. 2006 correlated the presence of fruits to an increase in the *C. sylvestris* EO content.

According to Castellani et al. 2006, *C. sylvestris* leaves have lower EO content in spring/summer (higher rainfall index and temperature). In this study, for the SPS population, we also observed that a higher rainfall index in spring/summer may be related to a decrease in the EO content of *C. sylvestris* leaves.

The mean of the seasonal EO content was 1.1 ± 0.3% in the SPS population (Fig. S14) and 0.8 ± 0.3% in the AES population (Fig. S15), with no statistical difference ($p > 0.05$), which indicated that both populations produced EO in similar quantities. In the SPS population, specimens 5 and 8 presented statistically different ($p > 0.05$) EO content from specimen 10. In the case of the AES population, specimens 1 and 6 presented statistically different ($p > 0.05$) EO content from specimens 9 and 10, indicating an intra-population variability in both populations. This variability could be associated with genetic factors and specimen age (Gobbo-Neto and Lopes 2007).

Circadian essential oil content (SPS and AES populations)

The circadian EO content (Fig. S16) ranged from 0.6 to 1.0% (SPS and AES populations, respectively) in the different harvest times in the two months (Feb and Aug/17). However, as the EO content did not present significant differences, the circadian cycle did not affect the EO content of the leaves of the *C. sylvestris* varieties.

Physical parameters of the essential oil

We determined the refractive index and density of the EO of the SPS, AES, CB, and SA populations and found 1.500 ± 0.001 and 0.922 ± 0.001 g/mL, respectively. On the other hand, the optical rotation of the EO (Table 2) differentiated between the '*sylvestris*' and '*lingua*' varieties: in '*sylvestris*' (SPS and CB populations), the EO was dextrorotatory, whilst in '*lingua*' (AES population), the EO was levorotatory. Interestingly, in the SA population, the optical rotation of the EO was levorotatory in December and dextrorotatory in July, and the specimens of this population had some morphological characteristics that could lead to their classification as intermediate varieties between '*sylvestris*' and '*lingua*'. These physical parameters may be employed together to establish control quality specifications for the EO of *C. sylvestris* to check its authenticity and purity, highlighting the optical rotation as a potential tool for the differentiation of the varieties.

Infrared spectroscopy of the essential oil

The infrared spectra of the *C. sylvestris* EO of the SPS, AES, CB, and SA populations presented absorption bands with similar intensities (Table S5 and Fig. S17). Noteworthy, absorption bands characteristic of aromatic compounds (e.g., phenylpropanoids) or carbonyl compounds were not observed. The observed bands were coherent with the compounds identified in the EO by GC–MS.

Soil analysis

According to the Brazilian Soil Classification System, the soils of the AES, CB, and SA populations were classified as sandy loam, whilst the soil of the SPS population corresponded to clayey sandy loam (Embrapa 2013). Table S6 lists the chemical and granulometric soil composition of the *C. sylvestris* populations.

On the basis of the criteria reported by Viecelli (2017), the P content (2.0 mg/dm^3) of the soil of the AES population was very low (≤ 6.0 mm/dm^3), which influenced the delayed flowering and fructification (July to October) as compared to the SPS population (May to September), whose soil had P content of 10.0 mg/dm^3. The K content (0.9 mmol$_c$/dm^3) in the soil of the AES population was low (1.5 mmol$_c$/dm^3), and such K deficiency could cause adaxial curvature observed in the leaves of the '*lingua*', making the stems dry and brittle (Cecílio et al. 2016; Viecelli 2017). These characteristics were predominant in the AES population (Fig. S18), but we did not observe them in the other populations. According to Schymanski et al (2013), longer sunlight exposure favors water loss and overheating in the leaves, as in the case of the AES population, which contributed to adaxial curvature in the '*lingua*' variety.

Table 2 Optical rotation of the EO of the SPS, AES, CB, and SA populations of *C. sylvestris* in July and December 2016

Optical Rotation	SPS (°)	AES (°)	CB (°)	AS (°)
July	+ 82.3 ± 0.6	− 99.5 ± 0.3[a]	+ 117.1 ± 0.5	+ 2.3 ± 0.3
December	+ 180.1 ± 0.4	− 98.7 ± 0.6[1]	+ 139.7 ± 0.4	− 39.7 ± 0.5

[a]Values without a significant statistical difference, $p > 0.05$

Conclusions

Morphological differences that are used to distinguish *C. sylvestris* '*sylvestris*' and '*lingua*' varieties, like palisade index, epidermal cell wall, stomata distribution, trunks with more significant fissures or leaves with adaxial curvature can be associated to different soil composition, and relative humidities. This study has shown that these factors impact the EO content in population, intrapopulation, seasonal, and circadian of these two varieties. Differences between their EO composition were reported in this study for the first time with the aid of clustered analysis of four different *C. sylvestris* populations. Higher contents of germacrene D, α-muurolol, and α-cadinol in the '*lingua*' variety were verified. However, the (*E*)-caryophyllene, spathulenol, β-elemene, and bicyclogermacrene in the '*sylvestris*' variety were the main components. These differences, added to different optical rotation for the two varieties, can be useful to distinguish *C. sylvestris* '*sylvestris*' and '*lingua*'. In summary, this study demonstrates the role that the EO can also play as a complementary tool for distinguishing these two varieties of such an important plant species in the folk medicine, in combination glycosylated flavonoids (in *C. sylvestris* '*lingua*') and casearin-like diterpenes (in *C. sylvestris* '*syvestris*') and morphological aspects.

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Author contributions FAC performed plant collection, leaf drying, morphological analyses, EO extraction, EO physical–chemical characterization, GC data analysis, and chemometric data analysis. FBO participated in plant collection and morphological analyses. LVSS performed botanical analysis. AEMC and EJC carried out GC analyses and data analysis. FMVB performed chemometric analyses. AGS

participated in design and coordination. All the authors contributed to the critical reading of the final manuscript and approved its submission.

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Data availability Not applicable.

Materials and code availability Not applicable.

Declarations

Conflict of interest There is no conflict of interest among the authors.

Ethical approval This article does not contain any studies with human or animal subjects.

Consent for publication Not applicable.

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