

Lippia alba morphotypes cidreira and melissa exhibit significant differences in leaf characteristics and essential oil profile

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Article

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Abstract: *Lippia alba* (Mill.) N.E. Br. ex Britton & P. Wilson, Verbenaceae, is widely used in traditional Brazilian medicine for the treatment of abdominal distress. The species exhibits considerable chemical and morphological diversity, and various chemotypes have been characterized. A comparative study of *L. alba*, has been carried out of the morphoanatomical characteristics of the leaves and the profiles of the essential oils of the morphotypes cidreira and melissa grown in the Medicinal Plant Garden of the Universidade Estadual de Santa Cruz, Ilhéus, Bahia, Brazil. The mean plant height of cidreira was 1.80 m and the stems and branches were fairly erect, while melissa plants were smaller (1.60 m) and presented prostrate stems and branches. Although the leaf of the morphotypes look were similar, the mean values of length, width and area of the leaves of cidreira (respectively, 7.42 cm, 3.32 cm and 17.31 cm²) differed significantly from those of melissa (4.68 cm, 2.35 cm and 7.32 cm²). The morphotypes presented amphistomatic leaves with uniseriate epidermis on both surfaces. The mesophyll was dorsiventral, but in cidreira the palisade parenchyma was biseriate while in melissa it was uniseriate. Simple tector and capitate glandular trichomes were present on the adaxial and abaxial surfaces of the leaf blades of both morphotypes. Six distinct types of glandular trichomes could be distinguished: types I and II were present in both morphotypes, while type III was detected only in cidreira, and types IV to VI were present only in melissa. The two morphotypes also differed with respect to the composition of the essential oil, cidreira produced oil composed mainly of citral, while the oil from melissa was rich in citral, limonene and carvone.

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Introduction

Members of the family Verbenaceae (Lamiales) are distributed throughout the world, particularly in tropical and subtropical regions, and may be classified into 34 genera of trees, shrubs and herbs. The genus *Lippia*, which encompasses approximately 200 species, is native to South America, Central America and Africa (Brummitt, 1992), and is widespread in the Brazilian savannas (*cerrados*) and rocky grasslands (*campos rupestres*) (Salimena, 2010). In Brazil, *Lippia alba* (Mill.) N.E. Br. ex Britton & P. Wilson, which is popularly known as cidreira, false-melissa, sage (*sálvia-da-gripe*) or field rosemary (*alecrim-do-campo*), is commonly employed in the form of a leaf infusion or decoction in the treatment of colds, bronchitis, coughs, asthma, and stomach and intestinal disorders (Lorenzi & Matos, 2008). Furthermore, the essential oil of *L. alba*

exhibits antispasmodic and analgesic properties that have been attributed to the presence of the terpenoids citral, myrcene and limonene (Vale et al., 2002).

Although *L. alba* is considered to be a single species, some authors have grouped plants into chemotypes according to the major constituents of their essential oils (Matos 1996a,b; Zoghbi et al., 1998). More recent studies have focused primarily on the chemical characterization of *L. alba*, and have revealed considerable variability in the essential oil profiles within the species (Aguiar & Costa, 2005; Silva et al., 2006). General information relating to the morphological characterization of *L. alba* chemotypes is scarce, although Tavares et al. (2005) maintain that the phenotypic and chemical variation between citral, carvone and linalool chemotypes, originating from different areas but grown in the same location, is a function of genotypic biodiversity and does not

reflect the influence of environmental factors. Genetic diversity among *L. alba* plants has been confirmed by Pierre et al. (2011) through chromosome analysis and studies of reproductive behavior, molecular screening and DNA content.

The ethnopharmacological importance (Moreira et al., 2002; Pinto et al., 2006) and the considerable biodiversity of *L. alba*, led to a comparative study of the morphological and chemical characteristics of the two morphotypes, cidreira and melissa, most commonly employed in the relief of stomach and intestinal disorders by populations in the south of the Brazilian state of Bahia. It is reported that the essential oil of cidreira contains predominantly citral (Silva et al., 2006), but the essential oil of melissa has not been characterized chemically. The aim of the present study was, therefore, to compare the morphotypes with respect to the morphoanatomical characteristics of the leaves, most especially, of the oil secreting structures and to determine the chemical compositions of the essential oils from cidreira and melissa.

Materials and Methods

Plant material

The *Lippia alba* (Mill.) N.E. Br. ex Britton & P. Wilson, Verbenaceae, morphotypes cidreira and melissa were cultivated in the Medicinal Plant Garden of the Universidade Estadual de Santa Cruz, Ilhéus-BA, Brazil. Plants were authenticated by Fátima Regina Gonçalves Salimena, and voucher specimens deposited in the herbarium at UESC with accession numbers 14176 (cidreira) and 14178 (melissa). Fertile branches were collected from the two morphotypes during September 2009.

Morphometric, anatomical and microscopic analyses

Morphometric analyses were performed on fully expanded and mature leaves ($n=50$) that had been removed from the third, fourth and fifth nodes of five plants each of the two morphotypes. The heights of the plants and the widths and lengths of the analyzed leaves were recorded, and leaf areas were determined according to the gravimetric method described by Mielke et al. (1995).

For anatomical examinations, leaves were fixed in FAA 70 (5 mL of 37% formaldehyde, 90 mL of 70% ethanol and 5 mL of glacial acetic acid) according to Johansen (1940), subjected to serial dehydration with increasing concentrations of ethanol, embedded in paraffin, and sliced on a rotary microtome. Leaf sections were stained with 2% safranin and 1% astra blue to visualize structural layers (Bukatsch, 1972),

and subsequently viewed under an Olympus model BX 50 photomicroscope. In order to localize essential oils in secretory structures, fresh leaves were subjected to histochemical assay using Nadi reagent (David & Carde, 1964) prior to microscopic analysis.

Ultrastructural analyses under the scanning electron microscope (SEM) were carried out on leaf samples that had been fixed in modified Karnovsky solution, post-fixed with 1% osmium tetroxide, and subjected to serial dehydration with increasing concentrations of acetone. Specimens were submitted to critical point drying with liquid carbon dioxide, mounted on a double-sided adhesive tape on metallic stubs, coated with gold under vacuum and examined under a Leo Evo 40 SEM microscope (Carl Zeiss).

Extraction and chemical analysis of essential oils

Air-dried leaves (100 g dry weight) from *L. alba* morphotypes cidreira and melissa were hydrodistilled with 1.5 L of water for 1 h in a Clevenger apparatus. The collected hydrolates (aqueous and organic layers) were submitted to liquid-liquid partition with dichloromethane, the organic fractions were separated, dried over anhydrous sodium sulfate and concentrated. Hydrodistillation was carried out in triplicate for each morphotype, and the mean concentrations of essential oils were calculated as weight of oil (g) per 100 g of dry leaf biomass.

Samples (1 μ L) containing 10% essential oil in chloroform were analyzed by high resolution gas chromatography (HRGC) using a Varian Saturn 3800 gas chromatograph equipped with a flame ionization detector (FID) and a Varian VF-5ms fused silica capillary column (30 m x 0.25 mm; 0.25 μ m film thickness). Chromatographic conditions were as follows: the carrier gas was helium at a flow rate of 1.2 mL min^{-1} ; the column oven temperature started at 60 $^{\circ}\text{C}$ and was increased by 8 $^{\circ}\text{C} \text{ min}^{-1}$ to 240 $^{\circ}\text{C}$, where it was maintained for 5 min; the injector and detector temperatures were 250 and 280 $^{\circ}\text{C}$, respectively. The sample (1 μ L) was injected in the split mode (1:10). GC-mass spectrometric (GC-MS) qualitative analyses were performed using a Varian Saturn Chromopack 2000 MS/MS mass spectrometer equipped with the VF-5ms capillary column mentioned above and operated under similar conditions with an electron impact of 70 eV. The transfer line and trap temperatures were 20 and 200 $^{\circ}\text{C}$, respectively. The relative concentration (expressed as a percentage) of each constituent was determined from the normalized FID peak area. Constituents were identified by analysis and comparison of their MS fragmentation patterns with those present in the database furnished by the equipment (NIST 2.0), and by comparing the retention indices obtained from chromatograms of

samples co-injected with a homologous series of C₈-C₂₆ alkanes with those from the literature (Adams, 1980).

Statistical analysis

Mean values of the micro- and macroscopic measurements were submitted to analysis of variance (ANOVA) and the Tukey test. Differences were considered significant at the 5% probability level.

Results

Cidreira and melissa are sub-shrubby morphotypes of *L. alba*, but the size and structures of the two variants are distinct. The cidreira plants reached 1.80 m height and the stems and branches were fairly erect, while the melissa plants were smaller (1.60 m) with prostrate stems and branches. The morphotypes presented highly lignified branches, white to greenish at the base, and herbaceous and vine-like at the top. Both variants showed cross-opposite phyllotaxy with active axillary buds presenting primordial branches containing either one or two pairs of leaves or inflorescences. The leaves were elliptical in shape, pointed at the apex and attenuated to the base, with serrated borders. The discolored leaf blades were smooth on the abaxial

surface and velvety on the adaxial side. Despite the similarities between the variants, morphometric analysis revealed that the leaves of cidreira were larger than those of melissa as shown by the significant differences ($p=0.05$) observed in the mean values of length, width and leaf area (Table 1).

Table 1. Morphological characteristics of the leaves of *Lippia alba* morphotypes cidreira and melissa grown at the Medicinal Plant Garden of the Universidade Estadual de Santa Cruz, Ilhéus-BA, Brazil.

Character	Cidreira (n=50)	Melissa (n=50)
Length (cm)	7.42±0.91 ^a	4.68±0.58 ^b
Width (cm)	3.32±0.44 ^a	2.35±0.34 ^b
Ratio length/width	2.24±0.16 ^a	2.00±0.16 ^b
Leaf area (cm ²)	17.31±4.21 ^a	7.32±2.01 ^b

Values shown are means±SD: in each row, values bearing dissimilar superscript lower case letters are significantly different according to Tukey test at 5% probability.

The leaves of cidreira and melissa displayed similar anatomical patterns in that they were amphistomatic, the epidermis on both surfaces were composed of a monolayer of rectangular cells covered with a thin cuticle, and the external periclinal cell walls

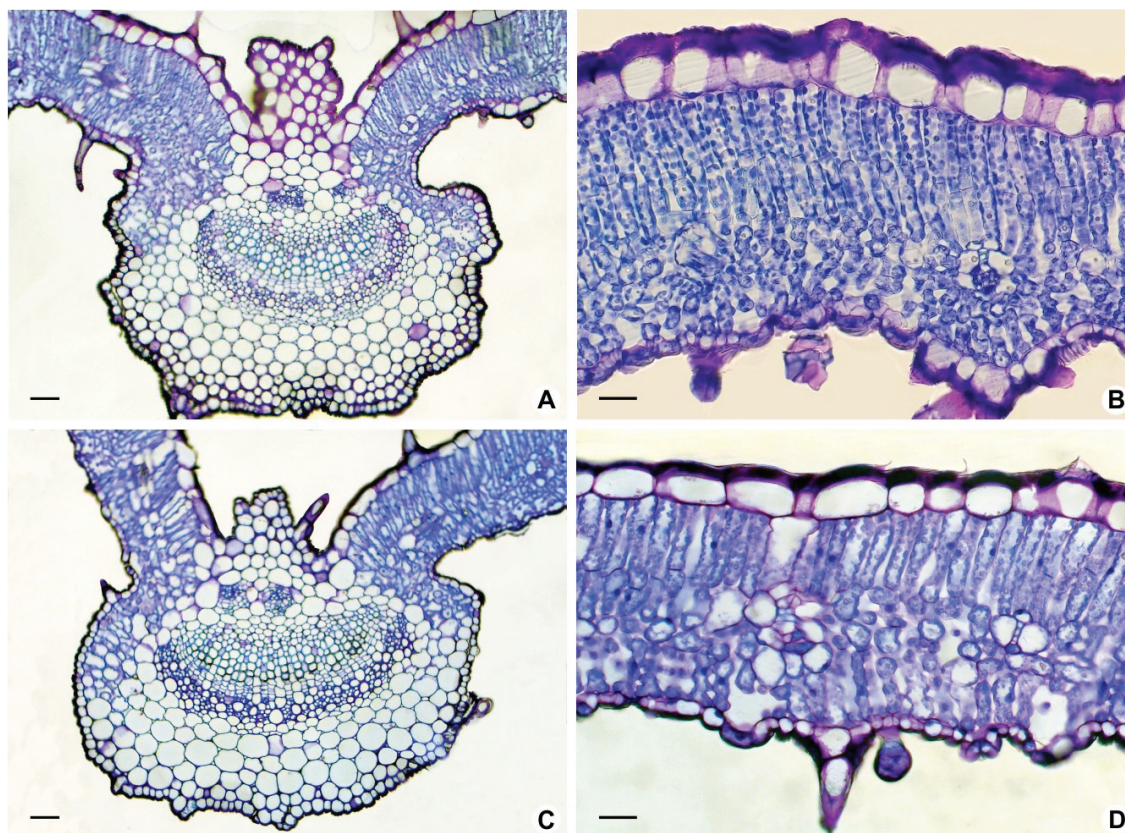


Figure 1. Photomicrographs showing cross-sections of the mid-vein (A and C: bar=30 µm) and mesophyll (B and D: bar=10 µm) of leaves from *Lippia alba* morphotypes cidreira (A and B) and melissa (Figure C and D).

Table 2. Description and occurrence of six types of glandular trichomes in *Lippia alba* morphotypes cidreira and melissa grown at the Medicinal Plant Garden of the Universidade Estadual de Santa Cruz, Ilhéus-BA, Brazil.

Type	Description	Occurrence	Figure
I	Large unicellular secretory head, collar cell present, short stalk	cidreira and melissa	2A and B, 3E and F
II	Bicellular secretory head, collar cell present, short unicellular stalk,	cidreira and melissa	2C, 2G
III	Small bicellular secretory head, collar cell present, long stalk,	cidreira	2D, 3F
IV	Bulb-shaped bicellular secretory head, short stalk	melissa	2H
V	Unicellular secretory head, collar cell present, long stalk	melissa	2I
VI	Large tricellular secretory head, collar cell present, short stalk	melissa	2J

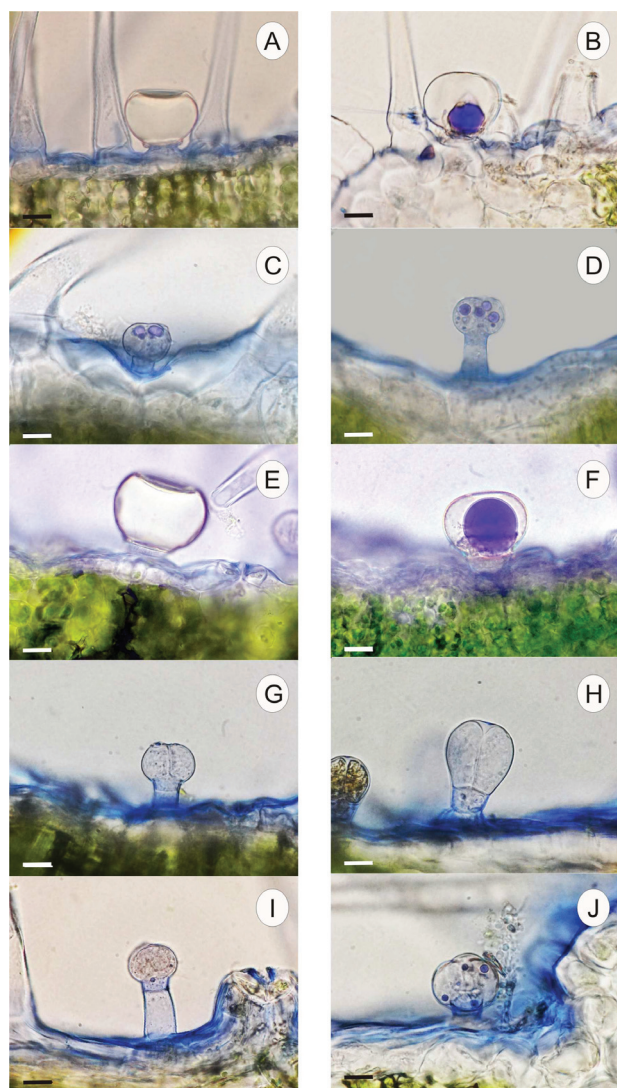


Figure 2. Secretory trichomes of *Lippia alba* morphotypes showing: A and B (type I), C (type II), and D (type III) trichomes of cidreira; and E and F (type I), G (type II), H (type IV), I (type V) and J (type VI) trichomes of melissa. The blue colored areas indicate the locations of essential oil droplets according to the NADI reagent assay (bars=10 µm).

were thicker on the adaxial compared with the abaxial surface (Figure 1). The mesophyll was dorsiventral, but in cidreira the palisade parenchyma was biseriate (Figure 1A and B) while in melissa it was uniseriate (Figure 1C and D). In cidreira, it was possible to observe the occurrence of angular collenchyma in the sub-epidermal region of the mid-vein, but this feature was not present in melissa.

Simple tector and glandular trichomes, the latter with variable morphology, were observed on the surfaces of the leaf blades of the two morphotypes (Figure 2 and 3). The abaxial surfaces of the leaves exhibited a greater density of trichomes than the adaxial sides, and the glandular trichomes were partially covered by the larger and more abundant simple tector trichomes (Figure 3E). Capitate glandular trichomes could be classified into six different morphological types as shown in Table 2 and Figure 2A-J. Types I and II were present in both morphotypes, type III occurred only in cidreira, and types IV to VI were observed only in melissa. Simple tector trichomes were of variable size, with an acute curved apex and a large basal disc comprising eight radial cells (Figure 3E).

Histochemical assays with Nadi reagent revealed the presence of essential oil droplets within the heads of some trichomes (indicated by the blue-stained areas shown in Figure 2B-D, F, J). The amount of essential oil in melissa was slightly higher than in cidreira (0.46 and 0.34% of dry weight, respectively). Fourteen oil constituents were identified and these accounted for approximately 98% of the total oil content (Table 3). Oxygenated monoterpenes predominated over all other components and comprised 87.85% of the cidreira oil and 84.44% of the melissa oil. While citral (a mixture of neral and geranial) was the major essential oil constituent in both morphotypes, distinct differences in the profiles of cidreira and melissa oils were detected. Thus, melissa oil contained 8.05% of monoterpenes while cidreira oil contained only 1.68%, and limonene and carvone were present in the oil from melissa (6.27 and 6.33%, respectively) but not in that from cidreira.

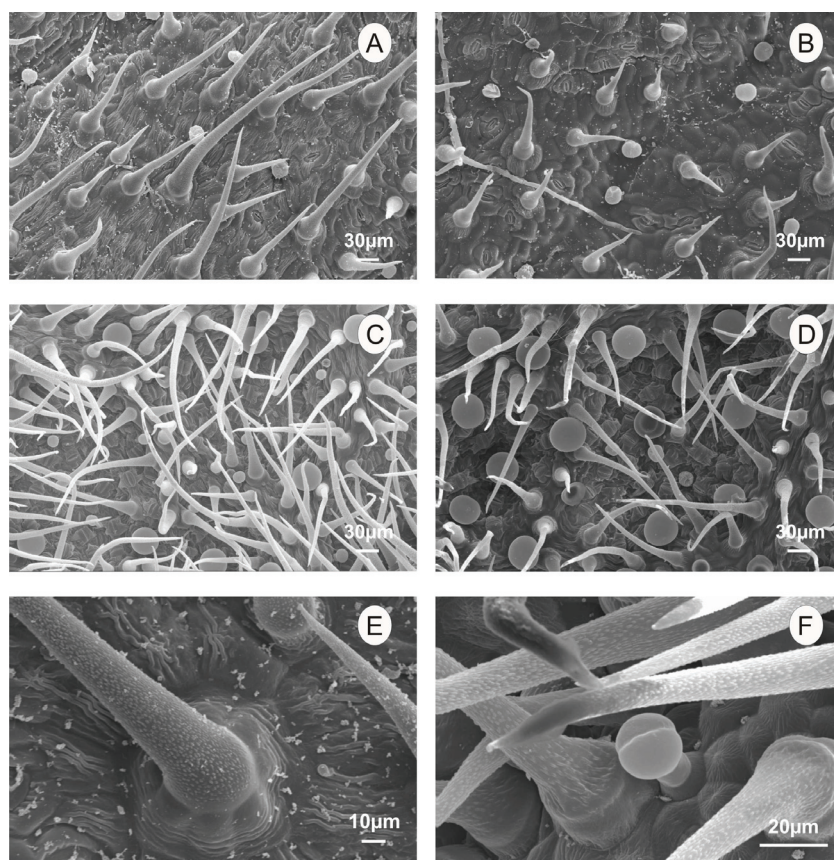


Figure 3. Scanning electron micrographs of the adaxial (A and B) and abaxial (C and D) surfaces of leaves of *Lippia alba* morphotypes cidreira (A and C) and melissa (B and D). Panel E displays the basal disc of a tector trichome comprising eight radial cells, and panel F depicts a type III capitate trichome.

Table 3. Percentage concentrations of the constituents of essential oils obtained from *Lippia alba* morphotypes cidreira and melissa grown at the Medicinal Plant Garden of the Universidade Estadual de Santa Cruz, Ilhéus-BA, Brazil.

Component	Kovats retention index	Cidreira (%)	Melissa (%)
1-octen-3-ol	981	1.99	1.04
6-methyl-5-hepten-2-one	985	1.21	1.09
β-myrcene	991	1.68	1.78
p-cymene	1029	1.61	- a
limonene	1031	-	6.27
linalool	1098	1.88	0.98
nerol	1228	1.78	2.46
neral	1240	32.56	28.81
carvone	1246	-	6.33
geraniol	1254	3.03	-
geranial	1275	48.60	45.38
isoborneol	1285	-	0.48
β-bourbonene	1375	2.86	2.49
caryophyllene oxide	1581	0.99	-
(citral = neral + geranial)		(81.16)	(74.19)
Total		98.19	97.11

^aNon-detected.

Discussion

Previous studies have shown that many species of medicinal and aromatic plants exhibit significant morphological and phytochemical variabilities. The high level of phytochemical and morphological variability among the studied populations of *Satureja khuzistanica*, an herb with interesting pharmacological and biological properties, suggests a breeding approach during the domestication, to gain new, promising, and homogenous cultivars, attractive for the industry and agriculture (Hadian et al., 2010). Jannuzzi et al. (2011) described seventeen different accessions of *L. alba* that could be classified into two distinct groups on the basis of mean values of stem length (1.48 and 1.78 m) and leaf area (10.33 and 21.98 cm²). The two *L. alba* morphotypes described in the present study also presented significant differences with respect to these variables.

The anatomical similarities observed between the leaves of cidreira and melissa corroborate the findings of Nunes et al. (2000), who noted the uniformity of the leaves among *Lippia* species and among genera of the subfamily Verbenoidae. However, despite the assertion of Santos et al. (2004) that there

were no relevant anatomical variations between *Lippia* chemotypes, the present study has revealed that the morphotypes cidreira and melissa differ with regard to the number of layers of palisade parenchyma and the types of trichomes present.

Simple, glandular, oil-producing and non-glandular trichomes, which are typically unicellular and may become silicified or calcified, are commonly found in members of the family Verbenaceae (Judd et al., 1999). The general anatomical features of the leaves of *L. alba* morphotypes cidreira and melissa described in the present study comply with earlier descriptions of the genus *Lippia* (Metcalf & Chalk, 1971), especially in respect of the occurrence of trichomes with stalks formed by one to three cells and with single or septate heads. Despite the variability in the structure of trichomes among plant groups, they can be remarkably uniform and, therefore, used for taxonomic purposes (Esau, 1977). In the present case, for example, glandular trichomes of type III were found exclusively in cidreira, while types IV, V and VI were observed only in melissa. The morphological description of the glandular trichomes presented herein increases our knowledge of the leaf anatomy of *L. alba* since only types I to III have been reported previously (Santos et al., 2004).

Based on the composition of the essential oils, the morphotypes cidreira and melissa were characterized, respectively, as citral and citral-limonene-carvone chemotypes. Interestingly, the compositions of the essential oils from cidreira and melissa differ from those of the three *L. alba* chemotypes (namely, citral-myrcene, citral-limonene and limonene-carvone chemotypes) found in the Brazilian state of Ceará (Mattos, 1996b). Such variation in the essential oils derived from plants grown in the same region (*i.e.* northeastern Brazil) confirms the existence of genotypic variability within the species *L. alba* as previously reported by Tavares et al., (2005). The chemical and morphological characterization of the species variability is the initial step of the domestication process to obtain homogenous cultivars with suitable agricultural features, with the aim of offering an attractive new crop to both industry and agriculture (Hadian et al., 2010).

Teas prepared from fresh leaves of the citral-myrcene and citral-limonene chemotypes of *L. alba* are employed widely in traditional Brazilian medicine for their analgesic, antispasmodic, sedative and anxiolytic properties (Lorenzi & Matos, 2008). However, in northeastern Brazil, the citral-limonene-carvone chemotype (melissa) is more common and is used mainly in the treatment of abdominal pain (Moreira et al., 2002; Pinto et al., 2006). It is known that limonene has analgesic activity, while carvone, which is also present in *Carum carvi* (caraway) and *Anethum graveolens* (dill), has digestive and carminative properties and

inhibits the growth of *Campylobacter jejuni*, one of the causal agents of gastroenteritis.

The comparative study described herein highlights the differences between two *L. alba* morphotypes that are important in traditional Brazilian medicine. Despite their anatomical similarities, the variants cidreira and melissa differ regarding plant size, leaf area, number of layers in the palisade parenchyma and types of trichomes. These two morphotypes also constitute different chemotypes since cidreira produces an essential oil that contains mainly citral, while the oil from melissa is rich in citral, limonene and carvone.

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Authors' contributions

CNJ, ARMFO and RSB (MSc students) contributed in collecting plant sample and identification, confection of herbarium, running the laboratory work, analysis of the data and drafted the paper. CNJ and RSB contributed to morphometric, anatomical and microscopic analyses. ARMFO contributed to essential oil quantification and CNJ contributed to chromatographic analysis. DCS, RAO and LCBC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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