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Research Article

Essential oil composition and stable isotope profile of cultivated *Lippia alba* (Verbenaceae) from Ecuador

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Abstract

Lippia alba (Mill.) N.E.Br. ex Britton & P. Wilson is a medicinal plant known for its diverse therapeutic/ethnobotanical applications. This study establishes the volatile profile (GC/MS) and stable isotope profiles of prominent volatile compounds from distinct harvest times throughout the cultivation season. Samples (*n=48*) were obtained through steam distillation from plants harvested at two distinct times: morning (9:00 hours) and afternoon (13:00 hours) over 8 weeks. Analysis by GC/MS identified the predominant compounds, including 6-methyl-5-hepten-2-one (2.4-2.7%), nerol (2.0-2.3%), neral (25.9-26.3%), geraniol (15.2-16.2%), geranial (29.8-29.9%), geranyl acetate (1.8-2.0%), β-elemene (1.3-1.5%), (E)-caryophyllene (5.7-6.1%), germacrene D (5.2-5.7%), and (E)-α-bisabolene (1.6-1.8%), which could classify this species in the Citral chemotype. While overall oil yield showed no notable differences between harvest times, variations were observed in specific compounds, including nerol, geranyl acetate, β-elemene, germacrene D, and (E)-α-bisabolene. Additionally, stable isotope values for the 3 most prominent compounds were assessed, revealing a negative linear regression for geraniol (R^2 =0.5). Overall, the findings suggest that harvest time exerts a minimal impact on the oil's composition, indicating potential flexibility in harvesting practices.

1. Introduction

Lippia alba (Mill.) N.E.Br. ex Britton & P. Wilson is a small aromatic shrub that belongs to the Verbenaceae family and is known by the common names *Incan melissa, Falsa melissa, Pronto alivio, Quita dolor*, or *Cidrela* [1]. *L. alba* grows to 0.8 m tall, and its limbs have variable forms with a "pointed apex, cuneiform, or decumbent base, and serrated or crenated border" [2, 3]. This species is native to Central and South America [4] and is used traditionally throughout Latin America [5].

In various countries, the ethnobotanical applications of *Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson span a range of ethnobotanical uses, underlining its versatility and importance in traditional medicine. In Brazil, both the leaves and roots of the plant have been employed for healing purposes. The leaves have been traditionally infused to treat conditions such as hypertension, stomach colic, nausea, and colds; they have also been applied externally for wound healing. In contrast, the roots have been predominantly used in infusions to address colds and coughs [6]. Mexico witnesses the utilization of its leaves in infusions aimed at alleviating gastrointestinal pain, vesicle discomfort, and gastritis [7, 8]. Similarly, in Guatemala, the leaves of the plant are essential; their decoctions and infusions have been used for

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conditions like coughs, skin diseases, flatulence, nausea, and headaches [9]. The versatility of *Lippia alba* is further highlighted in Colombia, where aqueous extracts of its leaves have been used as teas with properties ranging from antidiabetic and antispasmodic to diaphoretic, emmenagogue, and sedative [10]. In Peru, aqueous leaf extracts have been popularly consumed to alleviate migraine headaches [10]. Finally, in France, the plant's scope extends to its branches, which, along with the leaves, have been used in decoctions or infusions, primarily to combat conditions like insomnia and anxiety [11]. These applications accentuate the plant's widespread recognition and its pivotal role in various traditional medicinal practices. In Ecuador, the origin country of *L. alba* in this study, the leaves are cooked and used to treat cough, fever, headache, and stomachache. As part of a traditional Ecuadorian herbal mixture, *L. alba* is also used to treat diseases caused by climatic variations. The Shuar indigenous group has used this plant to relieve bone pain, stomach pain, cramps, and colic [12]. The ethnobotanical uses of this plant may begin to be elucidated by a study of the chemical constituents in the essential oil [13].

Lippia alba has been the focus of *in vitro* pharmacological studies investigating its extracts and fractions. These studies have evidenced antimicrobial activity against multidrug-resistant pathogens and antioxidant effect (DPPH assay) [3,14]. Additionally, extract from this species has demonstrated effective antimicrobial activity, particularly against Gram-positive bacteria [15]. On another note, the essential oil derived from the leaves of *L. alba* has shown significant antioxidant activity, even when compared with vitamin E, a well-known antioxidant [2].

Analyses of the essential oil composition derived from *L. alba* have revealed a diverse array of chemical profiles, indicative of seven unique chemotypes. These chemotypes are characterized by their principal compounds as follows: Chemotype I contains Citral, Linalool, and β-caryophyllene; Chemotype II is dominated by Tagetenone; Chemotype III features Limonene and Carvone; Chemotype IV is characterized by Myrcene; Chemotype V has γ-Terpinene as its major compound; Chemotype VI comprises Camphor and 1,8-Cineole; and Chemotype VII is highlighted by Estragole. Particularly, investigations focusing on chemotypes I and III have demonstrated a range of therapeutic effects, including antispasmodic, anxiolytic, and anti-inflammatory activities [4,16-19].

This study characterizes the volatile profile and distillation yield of *Lippia alba* essential oil from Ecuador. To the best of the authors' knowledge, this is the first attempt to establish its stable isotope ratios. This research will contribute to the existing body of knowledge by providing insights into the extent to which harvest time can impact the composition of essential oils, and by extension, provide insight into best harvesting practices.

2. Materials and methods

2.1 Plant material

Lippia alba was collected from February to April 2022 in Guayaquil, Ecuador (2°16'36.2"S 80°04'13.4"W). The first harvest was done in 7-month-old plants (Fig. 1), with harvest and distillations in triplicate each week, for a total of 8 weeks. All the harvests were performed in the morning (09:00 hours) and in the afternoon (13:00 hours), at a height of 10 cm above the ground. Environmental conditions (relative humidity % and temperature) were recorded at each harvest time with Vantage Vue Weather Station (Davis Instruments, USA). The voucher sample of *L. alba* was deposited in the herbarium Universidad de Guayaquil (13.552 GUAY).

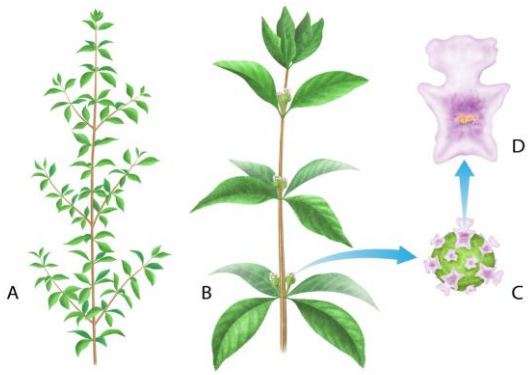

Figure 1. Botanical illustration of *Lippia alba* species used in the study. (A) Illustration of the overall plant, showing the leaf pattern and branching structure. (B) Illustration of close perspective and inflorescence. (C)/(D) Illustration of close/detailed perspective of flowering structure(s). Illustrated by Rick Simonson, Science Lab Studios, Inc. (Kearney, NE, USA).

2.2 Extraction method

The fresh plant material was chopped into small, non-uniform pieces, and the essential oil was immediately

extracted by steam distillation. Considering the three harvests per week (and per harvest time), six distillations were carried out per week, producing a total of 48 essential oil samples (n = 48).

All distillations were carried out in a 250 L distillation chamber (Albrigi Luigi S.R.L., Italy). Each batch of raw material was weighed before being placed in the chambers to get 15 kg of plant material. The steam distillations were carried out for 2 hours, and the essential oil was separated by a cooled condenser. The essential oils were collected, filtered, and stored in sealed amber vials at room temperature (25 °C) until analysis. The essential oil yield was calculated by dividing the volume of the essential oil obtained by the mass of the plant material before distillation.

2.3 Gas chromatographic analyses

The essential oil compounds were separated, identified, and quantified using GC/MS using an Agilent 7890B GC/5977B MSD (Agilent Technologies, Santa Clara, CA, USA) and Agilent J&W DB-5, 60 m × 0.25 mm, 0.25 µm film thickness, fused silica capillary column. Operating conditions: 0.1 µL of sample (20% soln. for essential oils in methylene chloride), 150:1 split ratio, initial oven temperature of 40 °C with an initial hold time of 5 min, oven ramp rate of 4.5 °C per minute to 310 °C with a hold time of 5 min, helium carrier gas. The electron ionization energy was 70 eV, scan range 35–650 amu, scan rate 2.4 scans per second, source temperature 230 °C, and quadrupole temperature 150 °C. Volatile compounds were identified using a combination of retention time data from reference compounds, (MilliporeSigma, Sigma-Aldrich, St. Louis, MO, USA) the Adams volatile oil library [20], and using Chemstation library search in conjunction with retention indices.

The stable carbon isotope ratios of *L. alba* essential oils were analyzed by GC/C/IRMS using a Thermo TRACE 1310 GC coupled to a Thermo Delta V Advantage Isotope Ratio Mass Spectrometer (ThermoFisher Scientific, Waltham, MA, USA), with an Agilent J&W DB-5, 60 m × 0.25 mm, 0.25 µm film thickness, fused silica capillary column.

Essential oil samples were prepared for GC/IRMS analysis ($^{13}C/^{12}C$) as follows: 35 mg of sample was weighed into a 2 mL clear glass vial and brought up to 1 mL with hexane. A 90 µL aliquot was added to a second vial and the final volume was brought to 1.0mL using hexane.

GC operating conditions are as follows: splitless injection of 1 µL of sample with splitless time set at 0.25 min., injection port 270 °C, initial oven temp. 40 °C with an initial hold time of 5.0 min., oven ramp rate of 6.0 °C per min. to 250 °C with a hold time of 2.0 min., then an oven ramp rate of 10.0 °C per minute to 310 °C with a hold time of 7.0 min., helium carrier gas with constant flow 1.4 mL/min. After passing through the capillary column, samples were sent through the combustion reactor for $^{13}C/^{12}C$ analysis. The combustion reactor temp. was set to 1000 °C and was conditioned with oxygen at regular intervals.

To normalize IRMS results, reference materials were purchased from Dr. Arndt Schimmelmann at Indiana University and the United States Geological Survey (USGS)–Reston Stable Isotope Laboratory. $\delta^{13}C$ isotope ratios are expressed relative to VPDB. The following three reference materials, along with their known values, were used to normalize results: hexadecane #C (USGS69), $\delta^{13}C$: −0.57‰; nonadecane #2, $\delta^{13}C$: −31.99‰; and tetradecanoic acid methyl ester #14M, $\delta^{13}C$: −29.98‰.

Samples were analyzed in quadruplicate to ensure repeatability. Isotope ratios were determined for the following prominent compounds: neral, geraniol, and geranial. $\delta^{13}C$ values are reported with a standard deviation ≤ 0.3‰.

The statistical analysis to compare the effect of harvesting time on essential oil yield and chemical profile was done with two independent sample t-tests at a confidence level of 95%. For investigating the significance of trends in stable isotope datasets, linear regressions were evaluated.

3. Results and discussion

The information presented in Table 1 contains data on environmental conditions at the time of harvest and essential oil (EO) yield for each week of collection. The relative standard deviation (RSD) is provided for the average essential oil yield per week in the 8 weeks of the study.

Table 1 shows an average essential oil yield for the morning harvest of 2.8 mL/kg and 3.1 mL/kg for the afternoon harvest. These results suggest no significant difference ($p>0.05$) in the essential oil yield of steam-distilled *Lippia alba* as a function of the harvest times selected for this study. Findings in the current study are in accordance with a prior investigation

Table 1. Averages yield data from harvest time for each week, including essential oil yield (mL), and calculated yield (mL/kg), Environmental conditions as average relative humidity percentage and average temperature.

Harvest time	Week	Avg. relative humidity [%]	Avg. temperature [°C]	Avg. EO Yield [mL/kg]
Morning	1	84.7	25.5	2.9
	2	89.3	25.5	2.6
	3	80.0	30.0	2.6
	4	69.7	32.9	3.0
	5	87.0	28.4	2.6
	6	77.7	29.6	2.5
	7	81.0	27.5	3.1
	8	78.7	30.7	3.3
	Avg	81.0	28.8	2.8
	% RSD (n=8)			10.9
Afternoon	1	67.7	31.1	3.3
	2	59.3	33.6	3.0
	3	70.3	32.6	2.8
	4	71.0	31.5	3.2
	5	68.0	32.8	2.9
	6	72.3	30.5	2.7
	7	68.0	31.8	3.6
	8	68.7	31.0	3.3
	Avg	68.2	31.9	3.1
	% RSD (n=8)			9.3

conducted on *L. alba* in Brazil, wherein no significant differences were observed in the essential oil yield at various harvest times [21]. However, a separate study focusing on the same species in Brazil, revealed contrasting outcomes, presenting differences in essential oil yield [22]. The authors of this latter study attributed their results to fluctuations in temperature and light intensity throughout the day. Furthermore, investigations encompassing distinct species have exhibited a similar tendency concerning essential oil yield, in which an increase or decrease is observed contingent upon the harvest time [23-26].

The information presented in Table 2 shows the volatile compounds of *L. alba* essential oil with the average relative area percentages from all samples in their respective harvest times.

Table 2. List of volatile compounds of *L. alba* essential oil in the morning (9:00 hours) and afternoon (13:00 hours) harvest times, GC/MS.

Compound Name	Relative Area (%)		
	KI	Morning	Afternoon
(3Z)-Hexenol	850	0.2	0.2
Camphene	946	tr	tr
1-Octen-3-ol	974	0.6	0.5
6-Methyl-5-hepten-2-one	981	2.4	2.7
Dehydro-1,8-cineole	988	0.2	0.1
3-Octanol	988	0.1	0.1
(E)-β-Ocimene	1044	0.2	0.1
Rosefuran	1093*	tr	tr
Linalool	1095	0.8	0.8
Perillen	1102	tr	tr
Exo-isocitral	1140	tr	tr
Citronellal	1148	0.1	0.2
Isoneral	1160	0.7	0.7
Borneol	1165	0.2	0.2
Rosefuran epoxide	1173	0.2	0.2
(E)-Isocitral	1177	1.3	1.2
α-Terpineol	1186	tr	tr
Nerol	1227	2.3	2.0
(Z)-Isogeraniol	1230*	0.3	0.2
Neral	1235	26.3	25.9
Geraniol	1249	16.2	15.2
Geranial	1264	29.9	29.8
Neryl acetate	1359	0.1	0.1
Geranyl acetate	1379	1.8	2.0
β-Bourbonene	1387	0.1	0.1
β-Elemene	1389	1.3	1.5
(E)-Caryophyllene	1417	5.7	6.1
β-Copaene	1430	0.2	0.2
α-Humulene	1452	tr	tr
Allo-aromadendrene	1458	0.2	0.4
γ-Muurolene	1478	tr	tr
Germacrene D	1480	5.2	5.7
α-Muurolene	1500	0.3	0.4
(E)-α-Bisabolene	1536*	1.6	1.8
Caryophyllene oxide	1582	0.1	0.2
Compound Classes			
Monoterpene hydrocarbons		0.5	0.4
Oxygenated monoterpenes		78.5	76.5
Sesquiterpene hydrocarbons		14.6	16.2
Oxygenated sesquiterpenes		0.1	0.2
Other compounds		5.2	5.6
Total		**98.9**	**98.9**

Note: Compounds detected with values less than 0.1% are denoted as trace (tr). Unidentified compounds of less than 0.5% are not included. KI is the Kovat's Index previously calculated by Robert Adams using a linear calculation on a DB-5 column [20]. *KI not previously calculated [20] and manual calculation performed using alkane standards. Data obtained from GC/MS analysis.

In an effort to identify trends, the ten major compounds present in the essential oil of *L. alba* were considered. These ten compounds are 6-methyl-5-hepten-2-one, nerol, neral, geraniol, geranial, geranyl acetate, β-elemene, (E)-caryophyllene, germacrene D, and (E)-α-bisabolene. The high content of neral (25.9-26.3%) and geranial (29.8-29.9%) in these results suggests that the *L. alba* species in this report belong to the Citral chemotype or chemotype I. Considering this chemotype, it is interesting to note the high geraniol percentage (15.2-16.2%) in the essential oil samples. Other studies on *Lippia alba* Citral chemotype have shown geraniol percentages ranging from not detected to 7.6% [27-31]. Essential oils with a high concentration of Citral, such as *Citrus limon*, *Litsea cubeba*, *Cinnamomum camphora*, and *Cymbopogon flexuosus* have garnered considerable attention in recent literature due to their array of therapeutic, culinary, and industrial applications [32-35]. For instance, the escalating demand for natural and organic products has elevated the prominence of these Citral-rich essential oils as they are frequently integrated into cosmetics, perfumes, aromatherapy, and even in the food and beverage sector [32, 34, 36]. Their economic relevance extends beyond consumer products, as they are applicable in sustainable agricultural practices, acting as potential biopesticides, thus reducing the dependence on synthetic chemicals [37, 38]. Similarly, *L. alba* from our study, which boasts high concentrations of Citral, holds significant potential in various therapeutic, culinary, and industrial applications, following the trend observed in other Citral-rich species as previously highlighted.

The essential oil obtained from *L. alba* showed quantitative significant differences ($p<0.05$) in five compounds which are presented in Table 3, comparing the morning and afternoon harvest times.

Although no literature was found comparing harvest time with the Citral chemotype of *L. alba*, the current findings diverge from those reported in the carvone chemotype *L. alba* from Brazil, where, based on harvest times, significant differences were observed in the percentages of carvone (26.7-54.9%), limonene (20.45-28.66%), sabinene (1.38-2.25%), and linalool (1.18-1.72%) [21]. The slight variability in the concentration of the five compounds (Table 3) in this study may have limited implications in terms of the biological properties of *L. alba* essential oil. The magnitude of the differences observed indicates that this influence is likely minimal in the harvest times (9:00 hours and 13:00 hours) used in this study.

Stable isotope values were determined for the 3 most prominent compounds (neral, geraniol, geranial) for each sample (n = 48). Average stable isotope values, and associated data, for both morning and afternoon samples are detailed in Table 4.

Table 3. Main volatile compounds of *L. alba* essential oil with significant differences between harvest times.

Compound	Significant differences ($p<0.05$)
Nerol	Higher in the morning (2.3%) compared to the afternoon (2.0%)
Geranyl acetate	Lower in the morning (1.8%) compared to the afternoon (2.0%)
β-elemene	Lower in the morning (1.3%) compared to the afternoon (1.5%)
Germacrene D	Lower in the morning (5.2%) compared to the afternoon (5.7%)
(E)-α-bisabolene	Lower in the morning (1.6%) compared to the afternoon (1.8%)

When considering the chemical structure of terpenes, and particularly those investigated in the current study for stable isotope analysis, plausible elements to analyze include carbon, hydrogen, and oxygen. However, carbon ($\delta^{13}C$) was selected due to inherent repeatability upon repeat injections of the same sample, where standard deviations for quadruplicate analysis range from neral (0.04 to 0.26), geraniol (0.05 to 0.22), and geranial (0.01 to 0.20). Given the confidence in carbon ($\delta^{13}C$) values, any apparent shifts or trends in the data may carry significance. When considering both morning and afternoon data points for Citral (neral + geranial), there appears to be a positive trend/slope for neral and a negative trend/slope for geranial from February to April (Fig. 2). However, the trends for both neral (R^2 = 0.1) and geranial (R^2 = 0.3) are not statistically significant, due to the broad scatter along the regression line. However, the negative linear regression for geraniol (R^2 = 0.5) is significant. Traditional taxonomy and plant identification often view chemotypes as identical, but stable isotope analysis offers a deeper understanding of the plant's molecular processes. In recent years, the significance of stable isotope analysis in authenticating the origin and quality of essential oils has gained increasing attention. Not only does this technique allow for the differentiation of products

Table 4. Stable isotope ($\delta^{13}C$) values (average, minimum, maximum, standard deviation) for neral, geraniol, and geranial for both a.m. and p.m. collection times spanning 8 weeks (n = 48). Samples were analyzed in quadruplicate and standard deviations for repeat injections are ≤ 0.3‰. $\delta^{13}C$ isotope ratios are expressed relative to VPDB.

Compounds Name	Morning/ Afternoon	Avg. Value ($\delta^{13}C$ ‰)	Min. Value ($\delta^{13}C$ ‰)	Max. Value ($\delta^{13}C$ ‰)	Std. Dev.
Neral	Morning	-26.332	-27.477	-25.062	0.691
Neral	Afternoon	-26.512	-28.262	-24.665	0.779
Geraniol	Morning	-30.685	-33.413	-25.362	1.992
Geraniol	Afternoon	-31.138	-33.900	-25.862	1.709
Geranial	Morning	-29.245	-30.663	-27.625	0.788
Geranial	Afternoon	-29.451	-30.563	-27.835	0.738

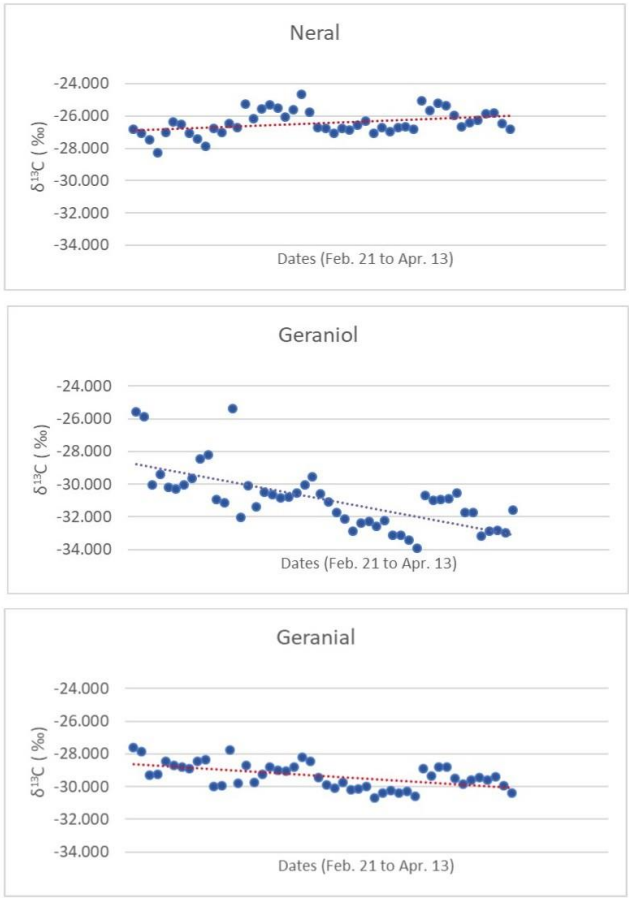

Figure 2. Scatter plot of morning and afternoon values ($\delta^{13}C$) for neral, geraniol, and geranial, for the 8 weeks.

based on geographical and temporal variables, but it also aids in distinguishing genuine essential oils from their adulterated counterparts [39-41]. A key component in the interpretation of such data is the existence of comprehensive databases, which enable the contextualization and verification of results. The present study, by contributing to these databases, amplifies the robustness of stable isotope analysis, specifically for *Lippia alba* Citral chemotype to authenticate the origin and quality of the species. Future studies with additional samples collected over a longer range of time between the hours of collection could illuminate the significance of trends in $\delta^{13}C$ values.

4. Conclusions

This study was conducted to determine how harvest time could influence the yield (volatile profile and stable isotope profile of prominent compounds) of *Lippia alba* essential oil. Results revealed L. alba from this study belongs to Citral chemotype because of the high content of neral (25.9-26.3%) and geranial (29.8-29.9%). Given the recent attention towards Citral-rich essential oils for various applications, our findings on *L. alba's* high Citral concentration add to the existing body of knowledge. Findings herein demonstrated no significant differences in essential oil yield ($p>0.05$), and significant differences ($p<0.05$) in five compounds, nerol (2.0-2.3%), geranyl acetate (1.8-2.0%), β-elemene (1.3-1.5%), germacrene D (5.2-5.7%), and (E)-α-bisabolene (1.6-1.8%). Therefore, these results suggest that harvest time might have a minor impact on the overall chemical profile of *L. alba* essential oil, providing some flexibility in harvest practices. However, it's crucial to consider that the current study was limited to examining the effect of harvest time alone, and many other biotic and abiotic factors could influence the composition and yield of the essential oil. Stable isotope values ($\delta^{13}C$) for neral, geraniol, and geranial are characteristic of other C4 carbon sequestering plants and only the linear regression for geraniol is significant in this study (R^2 = 0.5). Emphasizing the importance of stable isotope analysis, our research not only contributes to the verification of essential oil origin and quality but also adds the existing databases used for these authentications. Future lines of research could include exploring how other environmental and agronomic factors, such as

soil conditions, water availability, and temperature, can influence both the composition and stable isotope profile of *L. alba* essential oil. Moreover, a more detailed analysis of diurnal variability in essential oil composition could be undertaken, with additional sampling points throughout the day and for longer than 8 weeks.

Authors' contributions

Conceptualization, C.P.; Methodology, C.P., A.A., T.M.W., and B.J.M.; Software, C.P., A.A., and T.M.W.; Validation, C.P. and A.A; Formal Analysis (GC/MS, GC/IRMS), C.P., A.A., T.M.W., T.O., and B.J.M.; Investigation, C.P. and A.A.; Resources, C.P., E.C., O.P.; Data Curation, C.P., A.A., T.M.W, and B.J.M..; Writing – Original Draft, C.P., A.A., T.M.W., and B.J.M; Writing – Review & Editing, C.P., A.A., T.M.W., R.E.C., B.J.M, E.C., T.O., and O.P.

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Conflicts of interest

The authors declare no conflict of interest. The funding entity had no role in the design of the study, in the collection, analysis, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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