International Journal of
Agriculture, Environment and Food Sciences
e-ISSN: 2618-5946 https://dergipark.org.tr/jaefs

DOI: https://doi.org/10.31015/jaefs.2024.4.28 Int. J. Agric. Environ. Food Sci. 2024; 8(4): 986-993

Effects of harvest time and plant part on essential oils, phenolics, and antioxidant activity in *Lippia citriodora*

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Article History
Received: November 04, 2024
Revised: December 23, 2024
Accepted: December 27, 2024
Published Online: December 27, 2024

Article Info
Article Type: Research Article
Article Subject: Enzyme and Microbial Biotechnology in Agriculture

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Available at
https://dergipark.org.tr/jaefs/issue/87864/1579193

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Abstract
This study investigates the effects of harvest time and plant parts on the concentrations of essential oils, phenolic content, flavonoids, and antioxidant activities (ABTS and DPPH) in the *Lippia citriodora* plant to determine the best outcome. The experiment consists of four different harvest times during flowering period and two plant parts (upper and lower). Harvest times were scheduled at weekly intervals.Significant variations were observed during experiment, Essential oil (EO) content reached peak value at the first harvest in upper parts of plants (L1U: 1.18%) and lowest value in lower parts by the fourth harvest (L4L: 0.25%). The highest phenolic content determined at the first harvest (L1: 44.04 mg GAE/g DW), while flavonoid levels reached peak value at the fourth harvest (L4: 314.07 mg rutin/g DW). Antioxidant activities, measured by ABTS and DPPH assays, were significantly greater in lower plant parts. Partial Least Squares Discriminant Analysis (PLS-DA) and Principal Component Analysis Discriminant Analysis (PCA-DA) confirmed clear distinctions between the upper and lower parts of the plant regarding bioactive compounds concentrations. The findings shows the importance of targeted harvest timing and plant parts in optimizing bioactive compound in *Lippia citriodora*, with implications for getting better benefits from plant.

Keywords: Plant parts, Harvest times, Bioactive compounds, Lippia

Cite this article as: Tan, U., Goren, H.K. (2024). Effects of harvest time and plant part on essential oils, phenolics, and antioxidant activity in *Lippia citriodora*. International Journal of Agriculture, Environment and Food Sciences, 8(4), 986-993. https://doi.org/10.31015/jaefs.2024.4.28

INTRODUCTION

Lippia citriodora, or Lemon verbena, is a species of the Verbenaceae family, primarily cultivated because of its aromatic lemon-scented leaves, which are widely used in herbal teas. This herbal teas are believed to have antispasmodic, fever-reducing, sedative, and digestive benefits (Carrera-Quintanar et al., 2010; Lenoir et al., 2011). *L. citriodora* serves both culinary and medicinal purposes, being abundant in flavonoids and phenolic acids, which enhance its therapeutic properties. Moreover, its essential oil is extensively utilized in pharmaceuticals, perfumes, and cosmetics (Kaskoos, 2019). *L. citriodora*, originally from South America, especially Argentina, Brazil, and Paraguay, has been extensively cultivated in southern Europe and North Africa for its medicinal and aromatic properties (Aghdam et al.,2019; Farahmandfar et al., 2018). The plant grows in warm climates and is commonly cultivated in gardens for its aromatic leaves (Argyropoulou et al., 2010; Ebadi et al., 2016; Kara et al., 2018).

Lippia plants also serve as important sources of essential oils, which have been researched for their potential applications in the food industry (Mustafa, 2015; Kamal et al., 2011). The essential oils and bioactive substances found in medicinal and aromatic plants exhibit considerable variation influenced by internal and external factors, such as the specific plant part, growth stage, and harvesting time (Figueiredo et al., 2008; Telci et al., 2009).

The correlation among harvesting time, plant parts, and the chemical composition of medicinal plants is crucial for optimizing their therapeutic efficacy. This is particularly important for tall plants such as *Lippia citriodora*, which can attain heights of 3 to 5 meters. The upper regions of the plant are exposed to much more sunlight than the lower areas, which may cause variations in the concentration of bioactive compounds. Understanding these variations are important for enhancing the plant's medicinal and culinary efficacy. This study aims to assess the

variations in bioactive content across different harvest times and plant parts to maximize the potential benefits derived from the *Lippia citriodora* plant.

MATERIALS AND METHODS

Plant Material

The plant material used for the current study was *Lippia citriodora*, cultivated at the Faculty of Agriculture, Aydın Adnan Menderes University. Plant material were six years old, fully grown and mature. The harvests were done during the fall, blooming period (23 October-13 November), ensuring the plants were at a suitable stage of development for the extraction of bioactive chemicals. Harvest proceeded in several phases, with a weekly gap between every harvest. This approach allowed us to observe variations in chemical composition that could occur throughout the period of flowering.

Experimental Design

The experiment consists of four different harvest times (L1, L2, L3, and L4) and two distinct plant parts.Harvest times were spaced weekly. The plant parts were divided into two sections: the upper and lower portions of the plant. To avoid the effects of diurnal variability, all harvests were conducted between 10:00 AM and 12:00 PM. After harvesting, the plant samples were shade-dried to minimize the loss of bioactive substances.The samples were taken in four replicates, and the experiment was designed according to a completely randomized factorial design. The plants left to dry in the shade were weighed daily until their weight stabilized, indicating that they were fully dried. Once dried, the stems and the drog (dried medicinal plant parts used for medicinal or culinary purposes) parts were separated, and the following analyses were performed on drog parts of plant.

Determination of essential oil content

The essential oil content was assessed using the hydro-distillation method. Ten grams of dried plant material were added to 100 mL of distilled water and hydrodistilled using a Clevenger-type equipment. The distillation process took one hour at a temperature of 180°C. After the distillation process was finished, the mixture was allowed to cool for 5 minutes so that any condensed vapor may settle. The essential oil was then accurately quantified and collected. The oil content (%) was calculated using the following formula and expressed as a percentage.

Oil Content (%) = (Dry Sample Weight (g) / Extracted Oil Volume (ml)) × 100

Extraction of samples

Extraction was done on dried plant samples following the method of Skerget et al., (2005), with slight modifications. The samples were ground and subsequently sieved to ensure uniformity. A 500 mg portion of the dried powdered sample was mixed with 50 ml of 80% methanol and extracted in a shaking incubator at 40°C for 2 hours (150 rpm). Then, the extract was filtered and immediately used for the analyses described at below.

DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay

The evaluation of antioxidant capacity was carried out via the DPPH assay, following to the methodologies defined by (Gadow et al., 1997; Maisuthisakul et al., 2007). A portion of 100 µL of the extract was diluted to create four different concentrations, afterward added to 3.9 mL of a freshly prepared 0.1 mM DPPH solution (2,2-diphenyl-1-picrylhydrazyl radical). The mixture was gently shaken and then allowed to incubate in the dark at room temperature (23-25°C) for a duration of 30 minutes. Following the incubation period, the absorbance of the final solution was assessed spectrophotometrically at 516 nm with the aid of a microplate reader. The antioxidant activity of the samples was represented as Trolox equivalent antioxidant capacity (mg TEAC/g DW).

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) Assay

The ABTS assay was performed according to the method described by Re et al., 1999. To generate the ABTS radical cation (ABTS•+), a 7 mM ABTS solution was mixed with 2.45 mM potassium persulfate (1:1 ratio) and allowed to react in the dark for 16 hours. The resulting ABTS•+ solution was then diluted with methanol to achieve an absorbance of 0.700 at 734 nm. A 5 µL aliquot of plant extract was added to 3.995 mL of the diluted ABTS•+ solution and incubated for 30 minutes in dark. After that the absorbance of sample was measured, and the antioxidant capacity was expressed as Trolox equivalent antioxidant capacity (mg TEAC/ g DW).

Determination of total flavonoid content

To determine the flavonoid content, 0.5 mL of the sample extract was combined with 2.5 mL of distilled water and 150 µL of 5% sodium nitrite (NaNO2), followed by gentle shaking. The mixture was left to stand for 5 minutes, after 300 µL of 10% aluminum chloride ($AlCl_3$) was added and allowed to react for another 5 minutes. Subsequently, 1 mL of 1 M sodium hydroxide (NaOH) was added, and the total volume was adjusted to 5 mL with distilled water. The solution was then incubated for 30 minutes, and the absorbance was measured at 510 nm using a microplate reader. The flavonoid content was expressed as rutin trihydrate equivalents (MW: 664.56), following the method described by Chang et al., 2006.

Determination of total phenolic content

The phenolic content was determined using the procedure described by Skerget et al., 2005. To perform this procedure, mix 0.5 mL of sample extract, 2.5 mL of 0.1 N Folin-Ciocalteu reagent, and 2 mL of sodium carbonate solution (75 g/L Na_2CO_3). The reaction mixture was incubated for 5 minutes at 50°C to promote the formation of

the blue color, which shows the presence of phenolic chemicals. Following incubation, the mixture was shoked with ice and cooled to room temperature. The absorbance of the solution was then measured at 760 nm with a microplate reader. The phenolic content was represented in gallic acid equivalents (mg GAE per gram dry weight).

Statistical Analyses

The data were subjected to a one-way analysis of variance (ANOVA) to evaluate differences among treatment groups. Following ANOVA, multiple comparisons between treatment levels and controls were performed using Tukey's HSD, post hoc test to control for multiple comparisons. Additionally, Principal Component Analysis (PCA), incorporating discriminant analysis to enhance group separation, and Partial Least Squares Discriminant Analysis (PLS-DA) were conducted to further explore patterns and relationships within the data. All statistical analyses were conducted using JMP Pro 16 software (SAS Institute, Cary, NC, USA), while data visualization, including the PCA-DA and PLS-DA plots, was carried out using Python's Matplotlib library.

RESULTS AND DISCUSSION

ANOVA results

Table 1 shows the ANOVA results for essential oil content (EO), phenolic content (Phn), ABTS and DPPH radical scavenging capabilities, and flavonoid content (Flv). Harvest time (HT), plant part (PP), and their interaction (HT x PP) had a significant impact on all evaluated parameters, as indicated by the P values. Harvest time showed highly significant effects ($P<0.01$) across all parameters. Plant part also had significant effects, with phenolic content significant at $P<0.05$ and other parameters at $P<0.01$. The interaction between harvest time and plant part (HT x PP) was significant for all parameters at $P<0.01$.

Table 1. Effects of Harvest Time (HT) and Plant Part (PP) on Essential Oil (EO), Phenolic Content (Phn), ABTS Radical Scavenging Activity (ABTS), DPPH Radical Scavenging Activity (DPPH), and Flavonoid Content (Flv) in *Lippia citriodora* Plants.

Factors		**EO**	**Phn**	**ABTS**	**DPPH**	**Flv**
				HT (harvest time)		
L1		0.85 **ab**	44.04 **a**	101.14 **a**	104.74 **a**	239.95 **c**
L2		0.95 **a**	33.59 **c**	70.87 **c**	78.02 **d**	262.89 **b**
L3		0.73 **bc**	35.81 **c**	85.67 **b**	83.37 **c**	226.36 **d**
L4		0.63 **c**	40.67 **b**	85.58 **b**	92.63 **b**	314.07 **a**
				PP (plant part)		
L		0.62 **b**	37.68 **b**	70.26 **b**	86.78 **b**	246.33 **b**
U		0.95 **a**	39.37 a	101.37 **a**	92.60 **a**	275.30 **a**
				HTxPP		
L1	L	0.52 **d**	40.74 **b**	69.91 **e**	104.19 **a**	229.00 **f**
L2	L	1.02 **ab**	33.88 **d**	63.31 **e**	70.83 **d**	284.40 **b**
L3	L	0.70 **cd**	36.69 **bcd**	100.78 **c**	84.35 **c**	190.71 **f**
L4	L	0.25 **e**	39.42 **bc**	47.03 **f**	87.75 **c**	281.22 **b**
L1	U	1.18 **a**	47.35 **a**	132.37 **a**	105.28 **a**	250.91 **cd**
L2	U	0.88 **bc**	33.30 **d**	78.43 **d**	85.21 **c**	241.37 **de**
L3	U	0.75 **cd**	34.93 **cd**	70.56 **e**	82.40 **c**	262.00 **c**
L4	U	1.00 **ab**	41.91 **b**	124.13 **b**	97.52 **b**	346.93 **a**
Anova	df			***Mean Square***		
HT	3	0.124**	133.532**	916.579**	822.392**	8927.054**
PP	1	0.657**	17.135*	5808.947**	203.558**	5035.829**
HT*PP	3	0.293**	20.951**	3557.762**	85.801**	4186.839**

*EO: Essential Oil, Phn: Phenolic Content, ABTS: ABTS Radical Scavenging Activity ,DPPH: DPPH Radical Scavenging Activity, Flv: Flavonoid Content, HT: Harvest Time ,PP: Plant Part (HTxPP: Interaction between Harvest Time and Plant Part df: Degrees of Freedom and four harvest times (L1, L2, L3, L4)

A significant reduction in essential oil content (EO) was observed in the lower parts of the plant during all harvests, particularly in the fourth harvest (L4L: 0.25%). The harvest time significantly influenced the essential oil content showing a consistent pattern of increased in the early harvests (L1 and L2) and a decline in following harvests (L3 and L4), particularly in the lower parts of the plants. The upper parts (U) consistently exhibited a greater essential oil content than the bottom parts (L) during all harvests. The essential oil amount in the upper parts remained comparatively elevated in successive harvests (L4U: 1.00%), whereas in the lower parts, the essential oil content significantly decreased over time (Figure 1).

The results showed a significant variation in EO content at various harvest times. The second harvest recorded the highest concentration (L2: 0.95%), whereas the fourth harvest showed the lowest concentration (L4: 0.63%). The observed variations could result from physiological shifts occurring during the plant's growth cycle, which may impact the synthesis of secondary metabolites (Verma et al., 2015). The upper parts of the plants consistently demonstrated a higher essential oil content than the lower parts (U: 0.95%, L: 0.62%). It is known that environmental factors, including temperature, humidity, and soil nutrients, significantly influence essential oil production. The consistent trend observed in the upper parts of plants indicates that light availability can be a primary factor in here. Research by Zhao et al. (2022) shows that the synthesis of essential oils is closely associated with the metabolic pathways activated during photosynthesis. The upper leaves, due to their greater contact with light, show higher chlorophyll content, enhancing photosynthetic efficiency and resulting in increased essential oil production. Research conducted by Malayeri et al. (2010) shows that changes in light intensity can significantly affect both the composition and the quantity of essential oils produced. The relationship between harvest time and plant part suggests that harvest timing matters for optimizing essential oil production, as the highest essential oil content was recorded in the upper part of the plant during the early harvests during flowering period. The studies by Karık et al. (2019) and Toncer et al. (2022) on *Lippia citriodora* shows slightly different results, likely due to variations in methodology. Both studies focus on a single harvest, which may explain the discrepancies. For instance, Toncer et al. (2022) reported significantly lower essential oil content (0.32%-0.37%) with lower parts have higher values than upper parts of plants, while Karık et al. (2019) found higher essential oil content in the upper parts of the plant in (10 a.m.) morning harvest conditions but they obtained the opposite results under (4 p.m.) afternoon conditions. These differences in findings lead to varying conclusions and interpretations. In our study, the highest average essential oil content across all harvest times was obtained from the upper parts of plant. However, when examining individual harvests, it was observed in L2 harvest that lower parts of plant exhibit higher essential oil content compared to the upper parts of plant which similiar with Karık et al. (2019) and Toncer et al. (2022). This indicates that, although the essential oil content may vary between different plant parts depending on the harvest period, on average, the upper parts of plants contain higher levels of essential oil than lower parts of plants.

Phenolic content varied significantly across different harvest times, the highest level of phenolic content recorded during the first harvest (L1: 44.04 mg GAE/DW) and the minimum levels observed during the second and third harvests (L2: 33.59 mg GAE/DW, L3: 35.81 mg GAE/DW). The phenolic content (Phn) exhibited a small rise when it comes to the fourth harvest (L4L: 39.42 mg GAE/DW, L4U: 41.91 mg GAE/DW); however, these values remained lower compared the first harvest. The upper plant parts showed a consistently increased phenolic content compared to the lower parts across all harvests, significant difference between the lower and upper parts was observed during early harvesting times, with the upper parts showed higher phenolic content. The interaction between harvest time and plant part demonstrates that accurate timing of harvest is important for improving both essential oil and phenolic production, especially as the upper part exhibited the highest phenolic concentration during the first harvest (L1U: 47.35 g GAE/mg DW). When evaluating the ABTS value, the second harvest exhibited the lowest overall values, particularly in the lower plant parts (L2L: 63.31 mg/g DW), indicating a reduction in ABTS activity during this period. Similar to phenolic content, the upper plant parts consistently demonstrated higher ABTS activity compared to the lower parts across all harvests. This difference can be seen especially in both the first and final harvests. ABTS activity in the lower plant parts was generally below the overall mean, except during the third harvest (L3L: 100.78 mg/g DW). The DPPH activity was lowest in the second harvest (L2), in the lower portion of the plant (L2L: 70.83 mg/g DW). The fourth harvest showed an elevation in DPPH activity in the upper part of the plant (L4U: 97.52 mg/g DW). In the early harvest, DPPH activity in both the upper and lower sections was comparable, with both values above the overall mean, shows that the time of the first harvest produces the most balanced and strongest antioxidant activity throughout plant parts. In following harvests (L2, L3, L4), the upper sections continuously exhibited elevated DPPH activity relative to the lower sections, especially during the second and fourth harvests. DPPH activity maximized at the first harvest for both the upper and lower sections of the plant, but showed a considerable decline in the subsequent second and third harvests. The fourth harvest demonstrated a rise in DPPH activity, especially in the upper parts. The ABTS and DPPH radical scavenging activities reached its peak at the beginning of harvest, showing significantly higher activity in the upper parts of the plant. The relationship between antioxidant capacity and phenolic content suggests that phenolics are key contributors to antioxidant activity in *Lippia* (Feduraev et al., 2019). This finding shows the

upper parts of plants as an important source of natural antioxidants, with harvest timing also important. Gathering of samples during the beginning of harvest improves antioxidant activity.

The lowest flavonoid content was determined in the lower part of the plant during the third harvest (L3L: 190.71 mg rutin/g DW), but the higher part of the plant in the same harvest (L3U: 262.00 mg rutin/g DW) showed higher levels. The fourth harvest (L4) yielded the highest flavonoid content, while the third harvest showed the lowest values. The upper parts of the plant exhibited elevated flavonoid levels compared to the lower parts across all harvests, with the most significant differences observed during the third and fourth harvests. The maximum flavonoid concentration was observed in the upper part of the fourth harvest (L4U: 346.93 mg rutin/g DW), while the minimum content was found in the lower section of the third harvest (L3L: 190.71 mg rutin/g DW). The flavonoid concentration generally increased over time, reaching its peak around the fourth harvest. The upper parts consistently exhibited higher flavonoid concentrations compared to the lower parts in every harvest time. The flavonoid content was highest at the fourth harvest, with upper parts showing generally higher flavonoid levels than lower parts (L4: 314.07 mg rutin/g DW; U: 275.30 mg rutin/g DW vs. L: 246.33 mg rutin/g DW). The delayed maximum in flavonoid accumulation shows that flavonoids may be synthesized later in the flowering cycle. The raised flavonoid content in the upper plant parts at the fourth harvest (L4U: 346.93 mg rutin/g DW) likely shows improved synthesis due to extended sunlight exposure, which is recognized to affect flavonoid biosynthesis via light-responsive pathways (Wang et al., 2022). Zoratti et. al., (2014) and Shi et. al., (2024) also suggest that sunlight is a significant environmental factor inducing flavonoid biosynthesis and light exposure enhances flavonoid accumulation.

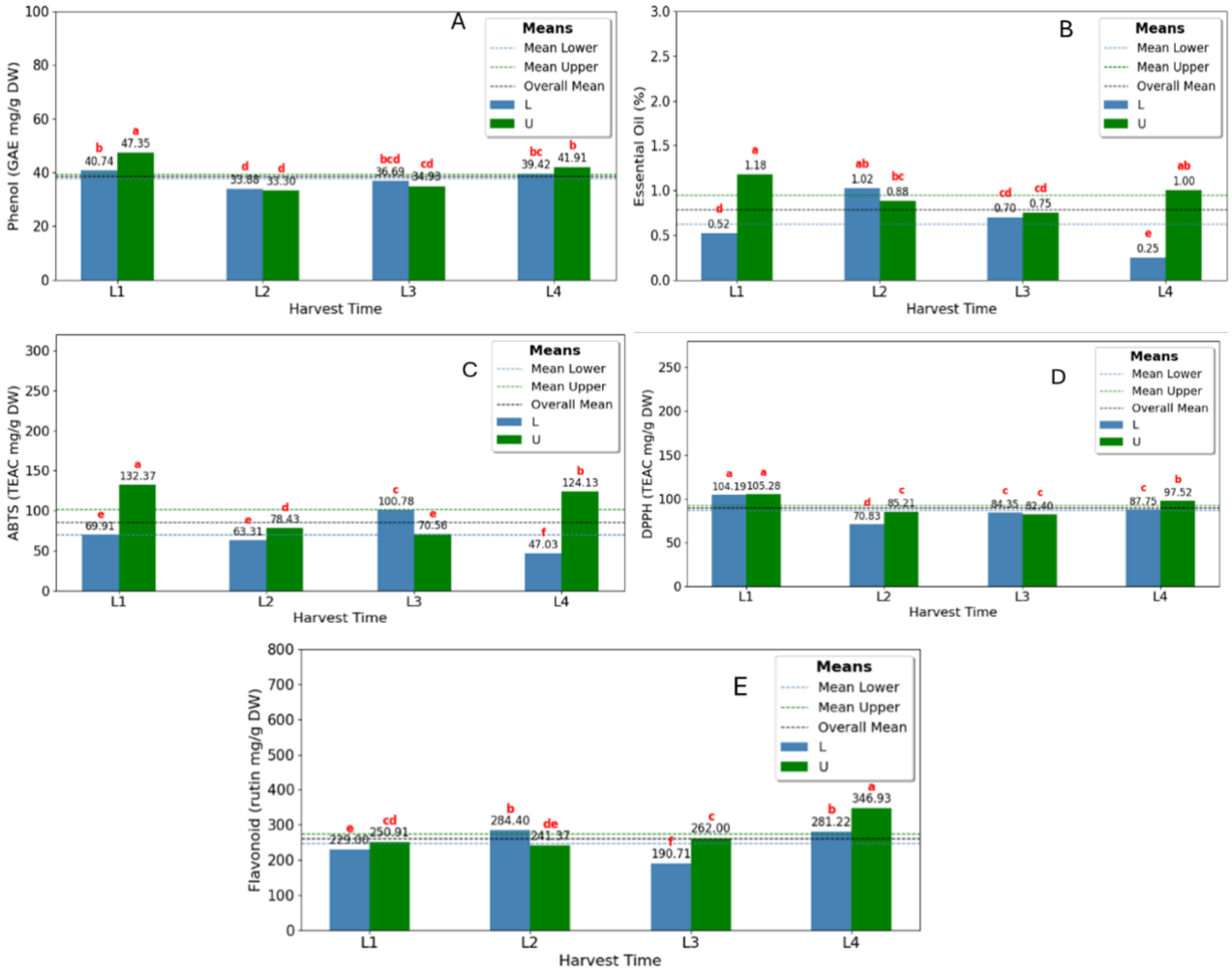

Figure 1. Variation in Phenol (A), Essential Oil Content (B), ABTS (C), DPPH (D) and Flavanoid (E) Between Lower (L) and Upper (U) Parts of the Plant Across Four Harvest Times (L1, L2, L3, L4), Highlighting the Interaction Between Harvest Time and Plant Part. (P<0.01).

PCA-DA and PLS-DA Analysis results

Partial Least Squares-Discriminant Analysis (PLS-DA) and Principal Component Analysis-Discriminant Analysis (PCA-DA) was used to further examine the results and determine variation in

bioactive parameters including flavonoids, essential oil (EO), ABTS, phenols, and DPPH, in different parts (upper and lower) of the *Lippia* plant across four separate harvest times.

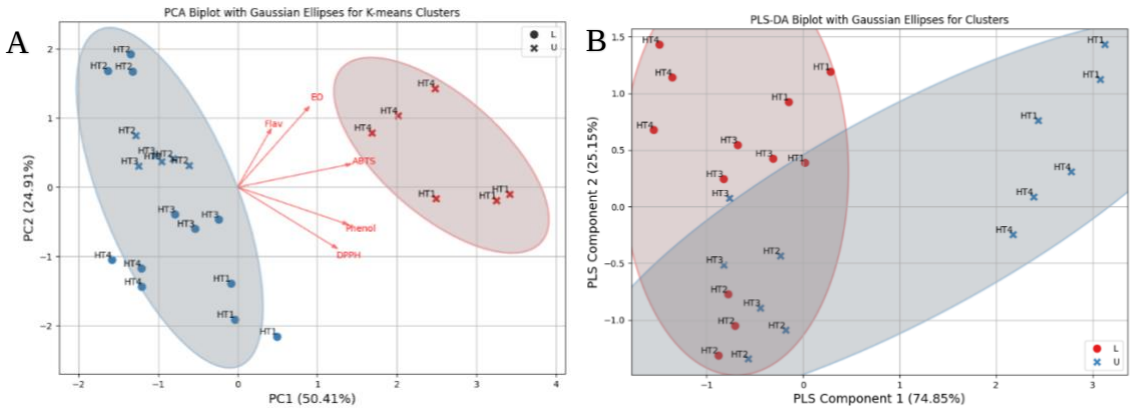

Figure 2. PCA-DA (A) and PLS-DA (B) biplots show the distinction of lower and upper plant parts according to specified parameters.

In the PCA-DA graph, the x-axis (PC1) accounts for 50.41% of the variance, while the y-axis (PC2) explains for 24.91%. Together, these two components reflect 75.32% of the overall variance in the data, the first two principal components capture an important portion of the variation (Figure 2A). The PCA graph illustrates two separate clusters represented by Gaussian ellipses: the blue ellipse on the left reflects samples assigned "HT1," "HT2," "HT3," and "HT4" within the "L" group (marked by filled blue circles), while the red ellipse refers to "HT1," "HT4," etc., classified as "U" (identified by red crosses). The two clusters clearly distinguish the two groups, linked to plant parts (L = lower, U = upper). The red arrows show the measured parameters. The position and size of the arrows indicate the effect of these parameters on the main components. Essential oil (EO), flavonoids (Flv), ABTS, phenol, and DPPH are factors affecting the differentiation of the clusters. Samples in the upper part (U) class show more impact from higher values of EO, Flav, ABTS, phenol, and DPPH compared to lower part (L) class. The L class (blue cluster) is positioned further from these variables, showing decreased values of these components relative to the U group.

PLS-DA is a supervised multivariate statistical approach that distinguishing the examimined parameters of the upper (U) and lower (L) parts of the plant and harvest times. The x-axis of the PLS-DA model (PLS Component 1) elucidates 74.85% of the variance, while the y-axis (PLS Component 2) reflects 25.15%. Together, both of these variables explain approximately 100% of the variance in the data, signifying that the PLS-DA model effectively captures almost all the information required for group distinction (Figure 2B).

The distinct separation within the two groups, shown with the red and blue ellipses, shows the efficacy of PLS-DA based on the defined parameters. The U category (upper parts of plants) is mainly located on the right side of the plot, while the L group (lower plant parts) positioned on the left. The observed separation proves that the variables used in this model are competent in distinguishing between the two groups. In contrast to the PCA-DA which shows greater overlap, the separation observed in PLS-DA is stronger, showing the supervised characteristic of PLS-DA.

CONCLUSION

This study shows the significant variation in the bioactive content of *Lippia citriodora*, influenced by both harvest timing and plant parts. Essential oil (EO) content peaked during the second harvest, with consistently higher levels observed in the upper plant parts across all harvests. This results may result from greater sunlight exposure in the upper regions compared to the lower parts. Similar patterns were observed for other parameters. Phenolic content reached its maximum during the early harvests, and antioxidant activities (ABTS and DPPH assays) followed a same pattern, exhibiting higher activity in the upper plant parts. Flavonoid content, however, peaked during the fourth harvest, dominantly in the upper sections of the plants. These findings underscore the importance of optimizing harvest timing during the flowering period and considering specific plant regions to maximize the yields of essential oils, phenolics, flavonoids, and antioxidants in *Lippia citriodora*. This can be used as practical implications for medicinal and culinary purposes of *Lippia citriodora* plant.

Compliance with Ethical Standards

Peer-review

Externally peer-reviewed.

Conflict of interest

The authors declare that they have no competing, actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

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