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CHARACTERIZATION AND COMPARISON OF THE BIOCHEMICAL PROFILE AND ANTIOXIDANT CAPACITY OF TWO LETTUCE VARIETIES (COSTINA AND ESCAROLE)

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ABSTRACT

Leaves are a potential source of biologically active lipids; however, effective extraction requires cell disruption. The objective of this study was to characterize and compare the lipid content, total phenolic compounds, vitamin C, nitrogen, and total chlorophyll levels in two lettuce varieties (costina and escarole), in order to evaluate their biochemical profiles and antioxidant capacities. Nitrogen and total chlorophyll levels in leaves were measured. Lipid extraction was carried out using vortex-agitation with hexane and distilled water as solvents. Phenolic content was determined using the Folin-Ciocalteau method, and vitamin C was measured using spectrophotometry in the extracted lipid fractions. Escarole lettuce exhibited lower nitrogen (3.15 ± 1.74 mg kg^{-1}) and total chlorophyll levels (10 ± 5.38 SPAD). Conversely, it showed a higher concentration of polar lipids (5.28 ± 1.28 g g^{-1} dry weight), while non-polar lipids were not detected in either variety. The phenolic content was higher in costina lettuce (60.34 ± 17.08 mg tannic acid g^{-1} dry polar lipid) compared to escarole lettuce (21.24 ± 12.76 mg tannic acid g^{-1} dry polar lipid). Similarly, vitamin C content was greater in costina with respect to escarole lettuce, with values of 0.2167 ± 0.0347 and 0.1038 ± 0.0166 mg ascorbic acid g^{-1} dry polar lipid, respectively. The results suggest that lettuce leaves are a valuable source of lipids, phenolic compounds, and vitamin C. The concentration of these bioactive compounds appeared to be influenced by nitrogen and total chlorophyll levels in the leaves, as well as by lettuce variety.

Keywords: Green leaves, lipid extraction, hexane, antioxidant activity.

INTRODUCTION

Plants are exposed to intense environmental conditions such as drought and salinity, which tend to limit their growth and development, reducing their productivity and yield (Seth et al., 2024).

Chloroplasts are organelles of the plant cell that contain a network of internal membranes known as thylakoids, which host photosynthetic complexes and support numerous essential biochemical pathways, including the synthesis of chlorophyll, proteins, and lipids (Chegeni et al., 2016). Under stress conditions, nitrogen deficiency

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in plants can lead to alterations in chlorophyll content and shifts in lipid accumulation. As nitrogen is a crucial component of chlorophyll, its shortage can reduce chlorophyll levels, thereby diminishing photosynthetic capacity. Therefore, nitrogen limitation may trigger lipid accumulation, potentially as a mechanism for storing excess carbon or as a physiological response to stress due to a lack of nitrogen (Li et al., 2020).

The lipid content of plants plays a major role in energy storage and cell signaling and include fatty acids, phospholipids, sphingolipids, galactolipids, carotenoids, tocopherols, sterols, fat-soluble vitamins, waxes, and other related compounds; however, their extraction requires cell disruption (Mumtaz et al., 2020; Reszczyńska and Hanaka, 2020). Total lipids are generally classified into polar and non-polar fractions; the former are composed of polyunsaturated fatty acids, while the latter consist of monounsaturated and saturated fatty acids (Romsdahl et al., 2022).

For lipid extraction, the vortex-agitation is a low-cost mechanical stirring method widely used for cell wall disruption and for dispersing extracting solvents in lipid samples (Zimila et al., 2021). The formation of fine droplets by vortex agitation can significantly increase the interfacial area available for mass transfer, thereby reducing diffusion distances and improving extraction efficiency within minutes (Psillakis, 2019).

Approximately 80% of all extractable lipids in green leaves are found in chloroplasts (Hölzl and Dormann, 2019). A low chloroplast lipid content is often associated with reduced chlorophyll levels in leaf tissues (Matsuzaki et al., 1984). Under environmental stress, chloroplast lipids are protected by the antioxidant activity of plants, which relies on non-nutritional compounds with biological activity, such as polyphenols (e.g., flavonoids, isoflavones, flavonoids, quercetin, gallic acid, and resorcinol) (Hano and Tungmunnithum, 2020), as well as vitamin C (Leiva-Vega et al., 2024). These compounds play a key role in mitigating oxidative damage and maintaining cellular function.

Therefore, this study aimed to characterize and compare the lipid content, total phenolic compounds, vitamin C, nitrogen and total chlorophyll levels in two lettuce varieties (costina and escarole), in order to evaluate their biochemical profiles and antioxidant capacities.

MATERIALS AND METHODS

Chemicals

Chemicals can be classified based on their chemical properties and typical behavior when exposed to other substances or environmental conditions. Table 1 summarized the types of reactive chemicals used in the present study.

Biological material

Lettuce leaves (*Lactuca sativa* L.), belonging to the Asteraceae family, and identified as costina and escarole varieties were used for the analyses. The samples were purchased from the local market in Temuco, Chile, during August 2024.

Nitrogen and total chlorophyll levels in lettuce leaves

Nitrogen (mg kg^{-1}) and total chlorophyll (SPAD) were measured using a ZYS-4N Portable Plant Nutrition Test Analyzer Machine (WANT Balance Instrument Co., Changzhou, CN). Nitrogen levels were determined based on the strong linear correlation between chlorophyll and nitrogen content, with a reported accuracy of ±5% according to equipment manufacturer (Leiva-Vega et al., 2024). The chlorophyll measurement principle was based on the quantitative evaluation of the intensity of the green color in leaves within the 650 to 940 nm wavelength range, with an accuracy of ±1 SPAD unit (Cunha et al., 2015). Samples were analyzed in triplicate, and each analysis was conducted in duplicate.

Lettuce extracts

Lettuce extracts were prepared from costina and escarole lettuce leaves following the methodology

Table 1. Reactive nature of chemicals used in the study.

Chemical names	Chemical formulas	CAS numbers	Hazards
2,6-dichloroindophenol	$C_{12}H_7NCl_2O_2$	620-45-1	Skin and eye irritation
Ascorbic acid	$C_6H_8O_6$	50-81-7	-
Folin-Ciocalteau reagent	-	12111-13-6	Corrosive
Hexane	C_6H_{14}	110-54-3	Skin and eye irritation
Oxalic acid	$C_2H_2O_4$	144-62-7	Corrosive
Sodium carbonate	Na_2CO_3	497-19-8	Corrosive
Tannic acid	$C_{76}H_{52}O_{46}$	1401-55-4	-

described by Altunkaya et al. (2009), with some modifications. Briefly, randomly selected lettuce leaves were washed with clean water, and 50 g of ground lettuce were homogenized in 100 mL hexane using a mini vegetable chopper for 1 min at room temperature (22 °C). The soluble solid concentration (30 g soluble solids 100 g^{-1} of lettuce extract) in both extracts was measured. The resulting extracts were placed in amber containers with airtight lids and stored in the dark at 22 °C until further analysis.

Lipid extraction

Lipid extraction was performed from hexane-wet lettuce extracts. First, 0.120 g aliquot of the hexane-wet extract was mixed with 0.25 mL of hexane and 0.25 mL of distilled water, followed by vortex agitation at 10,000 rpm for 7 min (VM–300 Model, Shenzhen Junmiao Technology Co. Ltd., China). The mixture was then centrifuged at 4,000 rpm for 3 min (MC-4K Model, JOANLAB, China), resulting in three distinct phases: (i) an upper or hexane phase, rich in non-polar lipids; (ii) an intermediate phase, consisting of depleted residue; and (iii) a lower phase or aqueous phase, rich polar lipids. Following centrifugation, the phases were carefully separated using Pasteur pipettes, transferred to unsealed Eppendorf tubes, and stored in the dark at room temperature (22 °C) for 14 h to allow solvent evaporation. Lipid content was quantified gravimetrically, and all measurements were conducted in triplicate.

Total phenolic content

Total phenolic content was determined using the Folin-Ciocalteau method, according to the procedure described by Makkar et al. (1993), with some modifications. Briefly, 0.1 mL of lipid sample was transferred to a 10 mL volumetric flask containing 6 to 7 mL of deionized water. Subsequently, 0.5 mL of Folin-Ciocalteau reagent (0.2 N) was added, followed by 1.5 mL of sodium carbonate solution (20 g per 100 mL^{-1}). The mixture was stirred and brought to volume with deionized water. After standing in the dark at room temperature (around 22°C) for 2 h, absorbance was measured at 760 nm in triplicate against a blank. A calibration curve was prepared using a tannic acid standard solution (0.18 mg mL^{-1}) as the reference substance, and the mean absorbance values from three measurements were used to construct the curve (Equation 1). Total phenolic concent was expressed as milligrams of tannic acid equivalents per gram of dry polar lipid.

$$y = 4.2738x - 0.0135, R^2 = 0.9973 \quad \text{Equation (1)}$$

Where: y = absorbance values, x = tannic acid concentrations in mg mL^{-1}, R^2 = determination coefficient.

Vitamin C content

Vitamin C content was measured spectrophotometrically using the indicator dye 2,6-dichloroindophenol (0.0012 g per 100 mL^{-1}), following the method described by Laguna and Arroy (2015), with some modifications. Specifically, 2 g of lettuce were crushed in a mortar with 20 mL of oxalic acid solution (0.4 g 100 mL^{-1}). The homogenate was filtered using Whatman grade 41 filter paper. Subsequently, 1 mL of the filtrate was diluted with 9 mL of distilled water to serve as the blank. For the assay, 1 mL of the filtered sample was mixed with 9 mL of 2,6-dichloroindophenol solution (0.0012 g per 100 mL^{-1}), followed by the addition of 1 mL of oxalic acid solution (0.4 g per 100 mL^{-1}). Absorbance readings were obtained at 520 nm in triplicate against the blank. The average room temperature during the analysis was around 22 °C.

A calibration curve was constructed using a standard ascorbic acid solution (0.06 mg mL^{-1}), with mean absorbance values from three replicate measurements used to generate the curve (Equation 2). Ascorbic acid concentration was expressed as milligrams of ascorbic acid per gram dry polar lipid.

$$y = 0.0468x - 0.0025, R^2 = 0.9998 \quad \text{Equation (2)}$$

Where: y = absorbance values, x = ascorbic acid concentrations in mg mL^{-1}, R^2 = determination coefficient.

Statistical analysis

Data were analyzed using Statgraphics Centurion XVI statistical software (Statistical Graphics Corp., Herdon, VA, USA). Tukey´s test was applied to determine significant differences between means at a p-value ≤ 0.05. Results are reported as mean ± standard deviation.

RESULTS AND DISCUSSION

Nitrogen and total chlorophyll levels in lettuce leaves

Nitrogen and total chlorophyll levels measured on the surface of costina and escarole lettuce leaves are presented in Table 2. Costina lettuce showed a significantly higher nitrogen content compared to escarole lettuce, with values of 11.27 ± 3.63 and 3.15 ± 1.74 mg N per kg, respectively. A similar trend was observed for total chlorophyll, with costina lettuce exhibiting a higher level than

Table 2. Nitrogen and total chlorophyll levels measured in lettuce leaves.

Lettuce types	Nitrogen ($mg\ kg^{-1}$)	Total chlorophyll (SPAD)
Costina	11.27 ± 3.63 a	37.42 ± 9.50 a
Escarole	3.15 ± 1.74 b	10.00 ± 5.38 b

Different superscript letters in the same column indicate significant differences ($p < 0.05$).

escarole lettuce, recording 37.42 ± 9.50 and 10 ± 5.38 SPAD units, respectively.

Chlorophyll synthesis is known to be closely dependent on nitrogen, which is essential for the formation of chloroplast-associated compounds within green leaf structures. Specifically, plants need nitrogen to produce chlorophyll, and higher nitrogen contents generally lead to higher chlorophyll concentrations, resulting in a darker green leaf color (Fathi, 2022; Wang et al., 2014). Therefore, the elevated nitrogen level in costina lettuce suggests a more stable chlorophyll synthesis process compared to escarole lettuce.

Total lipid content

Total lipid content in lettuce leaves, which was extracted using vortex-agitation with a 1:1 mixture of hexane:distilled water, consisted primarily of polar lipids, while non-polar lipids were not detectable in either lettuce variety.

Polar lipids, key components of the cell membrane, were quantified as 5.28 ± 1.28 g and 2.50 ± 0.36 g polar lipids per g dry weight for escarole and costina lettuce, respectively (Fig. 1). These results are in agreement with previous findings reported in the literature. In general, total lipid distribution in plants varies depending on the part of the plant, with seeds typically exhibiting the highest lipid content, followed by the pericarp of fruits, and then the mesophyll of green leaves (Mumtaz et al., 2020). Within green leaves, thylakoid membrane lipids are particularly rich in polar lipids, representing approximately 5.33 g per g dry weight (Köber et al., 2022). Sularz et al. (2020) reported a lipid content of 3.2 g per 100 g dry weight in lettuce (*L. sativa* L.) leaves.

Non-polar lipids, such as oils and waxes, are completely non-polar molecules that play a key role in plant protection and survival. Oils, typically found in young leaves, help protect plants against insect damage, while waxes, located on the leaf surface, form a protective barrier that repels water and reduce leaf dehydration (Fei and Wang, 2017). In the present study, non-polar lipids were not detected in either costina or escarole lettuce samples, which may be attributed to the nature of the plant material used since commercially available lettuce typically present older leaves and are subjected to industrial washing processes. These factors could have contributed to the depletion of oil molecules (relative to younger leaves), and the removal of wax molecules during washing processes, resulting in undetectable levels of non-polar lipids in the analyzed samples.

In summary, escarole lettuce exhibited lower nitrogen (3.15 ± 1.74 mg N kg^{-1} of lettuce) and total chlorophyll (10 ± 5.38 SPAD) levels, but a higher lipid content (5.28 ± 1.28 g polar lipids per g dry weight) (Table 2, Fig. 1). These results may reflect a plant response, in which the plant increases lipid production as a means of storing carbon sources and mitigates stress related to nitrogen deficiency. Furthermore, the elevated lipid content could also serve a protective function for chlorophyll molecules by favoring competition between lipid and chlorophyll molecules for singlet oxygen (Lee et al., 2014).

In costina lettuce, higher nitrogen content (11.27 ± 3.63 mg N per kg of lettuce) and total chlorophyll levels (37.42 ± 9.50 SPAD), combined with a lower lipid content (2.50 ± 0.36 g polar lipids per g dry weight) were observed (Table 2, Fig. 1). Elevated nitrogen and chlorophyll levels in leaves are associated with increased chloroplast activity and, consequently, enhanced photosynthetic productivity (Fathi, 2022). These results suggest that costina lettuce may exhibit a more stable chlorophyll synthesis process than escarole lettuce due to its higher nitrogen level, reducing the need for a higher number of lipid molecules to protect chlorophyll molecules.

Therefore, the observed relationships between nitrogen, total chlorophyll, lipid content in lettuce leaves could serve as a predictive framework for selecting green leafy vegetables with significant lipid accumulation.

Total phenolic content

Total phenolic content was measured in the polar lipid fraction extracted from lettuce leaves.

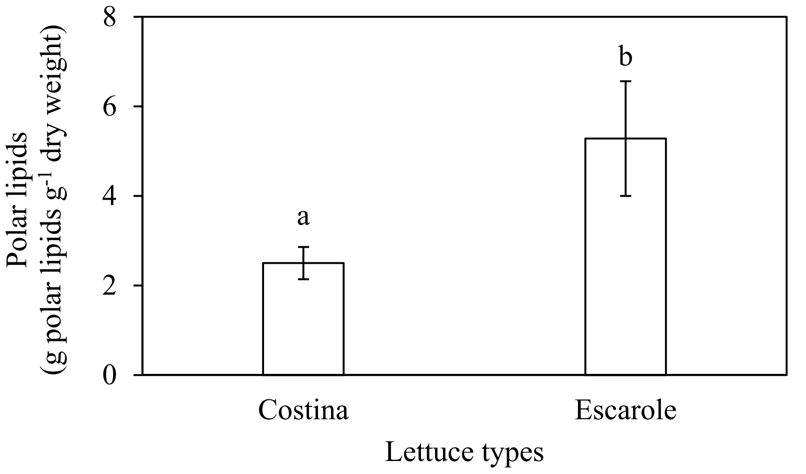

Fig. 1. Polar lipids measured in lettuce leaves.

A significantly higher phenolic content was observed in costina lettuce compared to escarole lettuce, with values of 60.34 ± 17.08 and 21.24 ± 12.76 mg tannic acid per g of dry polar lipid, respectively (Fig. 2). These results are consistent with those of Brazaitytė et al. (2022), who reported a phenolic content of 14 mg dry weight in lettuce (*L. sativa* L.) leaves. In the present study, the levels observed in costina lettuce indicate that phenol molecules may provide antioxidant protection to lipid molecules, aligning with evidence that phenolic compounds can effectively scavenge free radicals and reduce their harmful effects (Oluwagunwa et al., 2024). In contrast, the lower phenolic content in escarole lettuce may suggest reduced antioxidant capacity in its lipid fraction, i.e., a high competition of its lipids for singlet oxygen (Lee et a., 2014).

Vitamin C

Vitamin C content was also analyzed in the polar lipid fraction of lettuce leaves. A higher concentration was detected in the polar lipid fraction extracted from escarole compared to costina lettuce, with values of 0.2167 ± 0.0347 and 0.1038 ± 0.0166 mg ascorbic acid per g dry polar lipid, respectively (Fig. 3).

Comparable vitamin C levels in lettuce (*L. sativa* L.) were reported by Medina-Lozano et al. (2021), who established a range between 0.08 and 0.21 mg ascorbic acid per g dry weight. These findings may suggest an antioxidant role of vitamin C that can help protect against oxidative stress in both lettuce types, primarily through direct scavenging of reactive oxygen species (Stasiuk and Kozubek, 2010). However, the effect may be more pronounced in escarole lettuce due to its higher vitamin C content.

In summary, lipids found in lettuce leaves may readily compete for singlet oxygen, suggesting a high susceptibility to oxidative degradation. This high reactivity is attribute to the fact that many reactants of lipid molecules are in the singlet basal state, making singlet-singlet reactions more probable than triplet-singlet reactions, which are required for reactions with oxygen in its basal state (Andrés et al., 2024). In that context, total lipids in escarole and costina lettuce could exhibit two mechanisms of protection against lipid oxidation: phenolic compounds and vitamin C. The antioxidant effect of these compounds may become more evident when lipid concentrations in lettuce leaves are at low (phenolic compounds) or high (vitamin C) concentrations.

Practical applications

Polar lipids (e.g., phospholipids) have demonstrated health-promoting properties, including antioxidant and anticancer activities (Mendes et al., 2024). Phenolic compounds have been used for skin disinfection and to relieve itching (Downs and Wills, 2023). Vitamin C acts as a strong antioxidant with anti-inflammatory and anti-aging properties, contributing to skin health (Mumtaz et al., 2021). Chlorophyll, the green pigment found in plants, has shown to exert antioxidant, anticancer and antimicrobial effects (Vaňková et al., 2018). Therefore, the polar lipids present in lettuce leaves and their content of phenolic compounds and vitamin C,

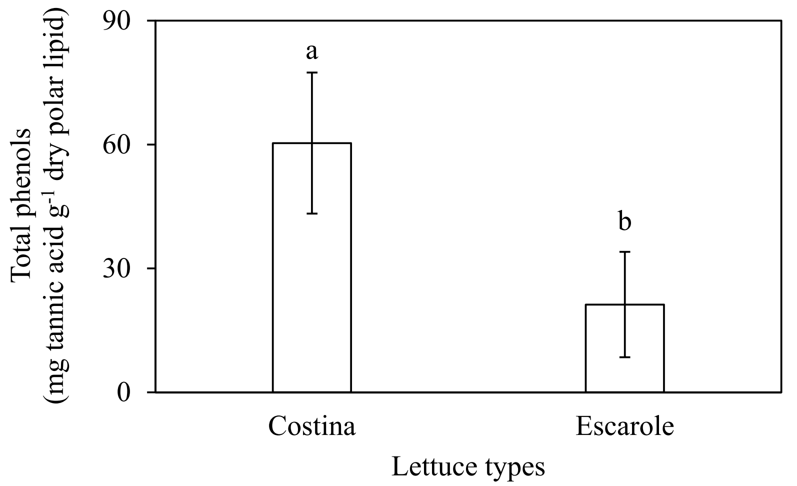

Fig. 2. Total phenolic compounds measured in the polar lipid fraction of lettuce leaves.

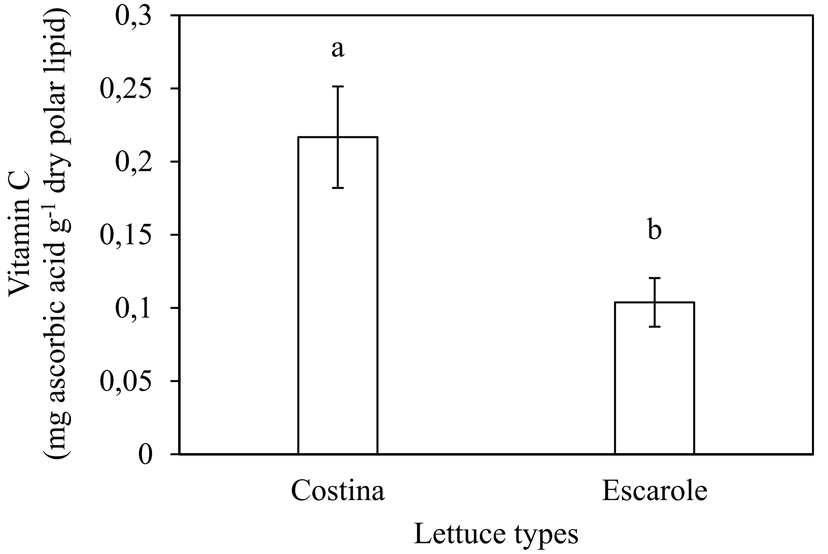

Fig. 3. Vitamin C measured in the polar lipid fraction of lettuce leaves.

in combination with the presence of nitrogen and chlorophyll molecules in the leaves, may have potential applications in cosmetic products for skin care.

CONCLUSIONS

Lipid extraction from lettuce leaves by vortex-agitation, along with the characterization of their phenolic and vitamin C contents, and the relationship of these components with nitrogen and total chlorophyll levels in green leaves, was investigated.

- Estimating nitrogen and total chlorophyll in lettuce leaves may serve as a predictive strategy for assessing lipid content.
- Lipids content varies depending on the presentation status as commercial product (or harvested) of lettuce leaves.

- Polar lipids constituted the major fraction of total lipids in the lettuce leaves, with higher levels observed in escarole lettuce.
- Characterization of the lipid fraction revealed an inverse relationship between phenolic compounds and vitamin C.

For a future study, it is recommended to investigate the oxidative stability of polar lipids and the antioxidant effects of their associated phenolic and vitamin C contents.

Author contributions

Bibliographic review: Javier Leiva-Vega, Lucía De La Fuente-Jiménez and Luis Ríos-Soto; methodology: Javier Leiva-Vega and Luis Ríos-Soto; discussion: Javier Leiva-Vega and Luis Ríos-Soto; writing, review and editing: Javier Leiva-Vega, Lucía De La Fuente-Jiménez and Luis Ríos-Soto. All authors have read and agreed to the published version of the manuscript.

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Declaration of conflicts of interest

The authors declare no conflict of interest.

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