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Research Article

Valorization of Cultivated and Wild Spinach Varieties: Comparative Phytochemical Screening and Evaluation of Antioxidant and Anti-Inflammatory Activities

Hayet Cherrad[1]*, Abdesselem Si Mohammed[2], Fatima Zohra Alachaher[3, 4], Djamil Krouf[5]

[1]Laboratory Of Biochemistry, Molecular Biology and Environmental Toxicology, Medicine Department, Faculty of Medicine, Abdelhamid Ben Badis University, Mostaganem, Algeria.
* **Corresponding Author Email:** hayet.cherrad@univ-mosta.dz- **ORCID:** 0009-0006-0630-5106

[3]Laboratory of Environment and Sustainable Development, Biological Sciences Department, Faculty of Natural Sciences and Life, Ahmed Zabana University, Relizane, Algeria.
Email: abdesselem.simohammed@univ-relizane.dz - **ORCID:** 0009-0000-6860-7165

[3]Laboratory of Beneficial Microorganisms, Functional Foods and Health (LMBAFS), Food Sciences Department, Faculty of Life and Natural Science Abdelhamid Ben Badis University, Mostaganem, Algeria.
[4]Laboratory of Clinical and Metabolic Nutrition, Food Sciences Department, Faculty of Life and Natural Science, Ahmed Ben Bella University, Oran, Algeria.
Email: fatima.alachaher@univ-mosta.dz- **ORCID:** 0009-0003-2102-2938

[5]Laboratory of Clinical and Metabolic Nutrition, Food Sciences Department, Faculty of Life and Natural Science, Ahmed Ben Bella University, Oran, Algeria.
Email: lncmkrouf@gmail.com- **ORCID:** 0000-0003-2159-9422

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Abstract:

This study aims to compare the cultivated spinach Spinacia oleracea and wild spinach Chenopodium bonus-henricus through detailed phytochemical screening and evaluation of their biological activities. Quantitative analysis shows that C. bonus-henricus leaves have the highest polyphenol (102.9 ± 6.8 mg GAE/g) and flavonoid (74.3 ± 4.7 mg QE/g) contents. Screening by thin-layer chromatography (TLC) reveals greater molecular diversity in leaf tissues, with the identification of flavonoids, coumarins, and the exclusive presence of terpenoids and quinones. On the biological level, the wild species consistently outperforms the cultivated variety, displaying an antioxidant capacity of 486.5 ± 14.1 µmol TE/g (FRAP assay) and a stronger radical scavenging activity with a lower DPPH IC_{50} value (37 µg/mL) compared to the cultivated spinach (43.65 µg/mL). Similarly, anti-inflammatory activity assessment via bovine serum albumin (BSA) denaturation inhibition revealed a lower IC_{50} for C. bonus-henricus leaves (216.1 µg/mL) relative to S. oleracea (416.1 µg/mL), reaching 81.2% inhibition at 800 µg/mL. These findings confirm that tissue compartmentalization and wild origin promote the accumulation of protective secondary metabolites, positioning C. bonus-henricus as a promising bioactive resource for nutritional and pharmaceutical applications.

1. Introduction

Oxidative stress results from an imbalance between excessive production of reactive oxygen species (ROS) and endogenous antioxidant defense systems, leading to oxidative alterations in lipids, proteins, and DNA (Jomova *et al*., 2023; Reddy, 2023). This phenomenon is now recognized as a central mechanism involved in the pathophysiology of many chronic diseases, including diabetes, cardiovascular disease, neurodegenerative disorders, and persistent inflammatory conditions (Alruways *et al*., 2024; Chong and Souayah, 2025). The increased incidence of these diseases has stimulated scientific interest in identifying bioactive compounds able to restore cellular redox homeostasis (Tretter *et al*., 2022). Natural antioxidants, especially polyphenols from plants, are getting more attention because they can neutralize free radicals, chelate transition metals,

and modulate signaling pathways that are sensitive to oxidative stress (Jalouli *et al*., 2025). Polyphenols, including flavonoids, tannins, and some terpenoids, are widely recognized for their antioxidant and anti-inflammatory properties (Rudrapal *et al*., 2022). Their dual biological activity makes them promising candidates for nutritional and pharmaceutical applications (Nani *et al*., 2021; Bolat *et al*., 2025). *Spinacia oleracea* L., a cultivated plant belonging to the Amaranthaceae family, is widely consumed and appreciated for its high nutritional value and rich content of bioactive compounds such as phenolic compounds, vitamins, and carotenoids (Roberts and Moreau, 2016). In contrast, *Chenopodium bonus-henricus* L., a local wild variety, remains relatively understudied. This species is described as rich in biologically active compounds, including mucilage, vitamins, and minerals, and has antioxidant and anti-inflammatory potential that has not yet been sufficiently explored (Kokanova-Nedialkova *et al*., 2017; Kondeva *et al*., 2025), particularly in the context of Algerian flora. The assessment of the antioxidant activity of plant extracts is based on complementary analytical approaches targeting different mechanisms of action. The most commonly used methods include the determination of total polyphenols using the Folin-Ciocalteu method, the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, and the measurement of reducing power using the FRAP assay (Gülçin, 2020; Munteanu and Apetrei, 2021). In addition, phytochimecal screening by thin-layer chromatography (TLC) is a relevant qualitative tool for identifying the main classes of secondary metabolites present in plant extracts (Kazi *et al*., 2025; Pandey *et al*., 2025). The integration of these approaches allows for a global characterization of the bioactive potential of the plants studied. In Algeria, comparative studies focusing on the antioxidant and anti-inflammatory potential of cultivated and wild spinach varieties remain limited. Therefore, this study aims to evaluate and compare the antioxidant and anti-inflammatory activity of methanolic extracts from the leaves and stems of *S. oleracea* and *C. bonus-henricus*. This analysis is supplemented by detailed phytotherapeutic screening to characterize their main bioactive compounds. The originality of this work lies in the comparative approach between a widely documented cultivated species and a local wild variety that has been poorly studied, with the aim of contributing to the scientific and potentially pharmaceutical and nutritional value of these plant resources.

2. Materials and methods

2.1. Preparation of plant material

More than twenty varieties of spinach have been documented worldwide, showing morphological and biochemical variations influenced by climatic conditions, growing seasons, and regional specificities. This study focused on the two most commonly consumed varieties in Algeria: cultivated spinach *S. oleracea* and wild spinach *C. bonus-henricus*. Specimens of *S. oleracea* were collected in the municipality of Misserghin (Oran, Algeria), while *C. bonus-henricus* was collected in the municipality of Es-Senia (Oran, Algeria). The botanical identification of the two species was carried out and validated by a taxonomist affiliated with the plant biology laboratory of the University of Oran 1. After harvesting, the aerial parts (leaves and stems) were carefully washed with distilled water to remove any exogenous contamination (dust, soil residues). The samples were then dried in a ventilated oven at 40°C for 48 hours until the mass stabilized in order to preserve the integrity of the thermosensitive compounds. The dried plant material was finely ground using a mechanical grinder to obtain a homogeneous powder with a uniform particle size. The powders obtained were packaged in airtight containers and stored away from moisture, light, and heat until their use in the preparation of methanolic extracts and subsequent phytochemical analysis.

2.2. Preparation of methanolic extracts

The methanol extracts were obtained by cold maceration, a method suitable for extracting phenolic compounds and other bioactive polar metabolites. 20 g of dry plant powder was subjected to extraction in 200 mL of analytical grade methanol (99%, v/v), using a solid/solvent ratio of 1:10 (m/v). The extraction was conducted at room temperature (25 ± 2 °C) under continuous magnetic agitation for 24 hours to optimize mass transfer and promote the diffusion of bioactive compounds into the solvent. After maceration, the mixtures were filtered through Whatman No. 1 filter paper to remove insoluble residues. The filtrates were then concentrated under reduced pressure at 40 °C using a rotary evaporator, allowing the solvent to be removed while preserving the integrity of the thermosensitive compounds. The extraction yield was calculated using the following formula (Sasidharan *et al*., 2011):

$$\textbf{Yield}\ (\%) = \frac{\text{Weight of dried extract (g)}}{\text{Initial weight of dry plant material (g)}} \text{X } 100$$

Where **weight of dried extract** is the mass of the extract obtained after solvent removal, and **initial weight of dry plant material** is the mass of powdered plant material used for extraction.

The dry extracts were then stored in sealed amber glass bottles and kept at -20°C until used for phytochemical analysis and biological activity assessment.

2.3. Phytochemical screening

Qualitative phytochemical screening of methanol extracts was performed in accordance with standard protocols described by Terease and Evans (1989), Harborne (1998), and Bruneton (1999). These methods are based on specific colorimetric reactions that enable the preliminary detection of the main classes of secondary metabolites. They were used to qualitatively identify the presence or absence of flavonoids, gallic tannins, catechin tannins, coumarins, anthocyanins, alkaloids, carotenoids, and saponosides. The interpretation of the results was based on characteristic color changes or the formation of precipitates specific to each class of metabolites (Table 1), which provided essential information on the phytochemical profile of the extracts and their potential contribution to biological activities.

2.4. Thin layer chromatography (TLC)

TLC is a rapid and simple analytical technique based on the interaction between a mobile phase (solvent or mixture of solvents) and a stationary phase fixed on a support. The compounds present in the sample migrate along the plate at different speeds depending on their chemical nature and the type of solvent (Akash *et al*., 2020; Hameed *et al*., 2023).

- Stationary phase: 0.25 mm thick, G60 F254 silica gel plates (Merck), fixed on an aluminum support, and were activated in an oven at 80°C for 20 min before use.
- Sample: a few microliters of methanolic extract (spinach leaves and stems) were deposited at a reference point 1 cm above the base of the plate. Once dry, the plate was then immersed in the appropriate migration system.
- Eluent: a pure solvent or a mixture of solvents was chosen according to the type of compounds to be separated, allowing the sample components to migrate along the plate.

Table 2 shows the different eluent systems used for the separation of the main phytoconstituents.

Migration is stopped when the solvent front reaches 17 cm from the top edge. The plate is then dried, examined under UV light (Bioblock Scientific Vilber Lourmat CN-15LC) at 254 and 365 nm, and then developed using a specific detection reagent (Table 02). The retention factor (*Rf*) is determined for each constituent as follows:

$$\mathbf{Rf} = \frac{\mathrm{d}}{\mathrm{D}}$$

Where **d** is the distance traveled by the sample and **D** is the distance traveled by the solvent front.

The retention factor is characteristic of a given compound for a specific eluent and stationary phase, and is independent of the compound's concentration in the mixture (Litvinova, 2008).

2.5. Quantification of bioactive compounds

2.5.1. Total polyphenol content

The total polyphenol content of leaf and stem extracts was determined using the Folin-Ciocalteu colorimetric method, according to the original protocol by Singleton and Rossi (1965) with minor modifications. This method is based on the reduction of the phosphotungstic and phosphomolybdic complexes of the Folin-Ciocalteu reagent by phenolic compounds in an alkaline medium, resulting in the formation of a blue color that can be measured spectrophotometrically. The Folin-Ciocalteu reagent was diluted 1:10 (v/v) with distilled water immediately before use. The sodium carbonate (Na_2CO_3) solution was prepared at 7.5 g/L in distilled water. In test tubes, 200 µL of the methanolic extract (or gallic acid standard solution) was mixed with 1000 µL of the diluted Folin reagent and incubated for 5 minutes at room temperature. Next, 800 µL of the Na_2CO_3 solution was added to create the alkaline environment necessary for the reaction. The tubes were vortexed and then incubated in the dark at room temperature for 2 hours to allow the color to develop completely. The absorbance (A) was measured at 725 nm against a blank containing only the extraction solvent (methanol). Each measurement was performed in triplicate (n = 3). A calibration curve was established simultaneously with gallic acid (20-200 µg/mL), and the results were expressed in milligrams of gallic acid equivalent per gram/dry extract (mg GAE/g DE).

2.5.2. Total flavonoid content

The total flavonoid content was determined using the colorimetric method with aluminum trichloride ($AlCl_3$), based on the formation of stable complexes between aluminum and the hydroxyl or ketone groups of flavonoids, as described by Zhishen et al.

(1999) with minor adaptations. The following reagents were prepared: $AlCl_3$: 2 g/L in methanol; $NaNO_2$: 5 g/L in distilled water; NaOH: 1 M in distilled water. In test tubes, 500 µL of diluted extract (or quercetin standard solution) was mixed with 500 µL of the $NaNO_2$ solution. After 5 minutes of incubation at room temperature, 500 µL of the $AlCl_3$ solution was added and the mixture was left to incubate for 6 minutes. Next, 2 mL of 1 M NaOH was added to stop the reaction and stabilize the color, bringing the final volume to 3.5 mL. Each measurement was performed in triplicate (n = 3). Quercetin was used as a calibration standard with concentrations ranging from 25 to 200 µg/mL. A calibration curve was established by plotting the absorbance against the quercetin concentrations. The absorbance (A) was measured at 510 nm against a blank with the same reagents, replacing the extract with the extraction solvent (methanol). Results were expressed in milligrams of quercetin equivalent per gram/dry extract (mg EQ/g DE).

2.6. Evaluation of antioxidant activity

2.6.1. DPPH radical scavenging assay

The antioxidant activity of the extracts was evaluated using the stable free radical scavenging method 2,2-diphenyl-1-picrylhydrazyl (DPPH), according to the protocol of Brand-Williams et al. (1995) with minor modifications. This method is based on the reduction of the purple DPPH radical in the presence of antioxidants, resulting in a spectrophotometrically measurable discoloration. A methanolic solution of DPPH (0.1 mM) was prepared and stored in the dark at 4°C prior to use. Different dilutions of the extracts were prepared in methanol, covering a concentration range of 25 to 200 µg/mL. Ascorbic acid (vitamin C) was used as a positive control, with concentrations ranging from 5 to 100 µg/mL. For the test, 100 µL of each extract or positive control dilution was mixed with 3.9 mL of the DPPH solution (0.1 mM) in test tubes. The mixtures were homogenized and incubated in the dark for 30 minutes at room temperature to prevent photodegradation of the radical. The absorbance (A) was measured at 517 nm against a blank containing 100 µL of methanol and 3.9 mL of DPPH. The percentage inhibition (I %) of the DPPH radical was calculated using the following formula:

$$\textbf{Inhibition}\ (\%) = \frac{\text{A control} - \text{A sample}}{\text{A control}} \text{x}\ 100$$

Where ***A control*** *is the absorbance of the DPPH solution without extract, and* ***A sample*** *is the absorbance in the presence of the extract.*

The 50% inhibitory concentration (IC_{50}), corresponding to the concentration required to inhibit 50% of DPPH radicals, was determined by graphical interpolation or non-linear regression. The results were expressed in µg/mL.

2.6.2. Ferric reducing antioxidant power (FRAP) assay

The antioxidant reducing power of the extracts was evaluated using the adjusted method of Benzie and Strain (1996), which measures the ability of compounds to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), forming a colored complex with a maximum absorbance at 593 nm. The FRAP reagent was freshly prepared by mixing three solutions in a volume ratio of 10:1:1 (v/v/v):

- Sodium acetate buffer (300 mM, pH 3.6)
- TPTZ (2,4,6-tripyridyl-s-triazine) 10 mM in 40 mM HCl
- Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) 20 mM in distilled water

In test tubes, 200 µL of the diluted extract (or standard solution) was mixed with 1.8 mL of FRAP reagent. The tubes were sealed and incubated for 30 min at 37°C in a water bath. The absorbance was then measured at 593 nm against a blank prepared in the same way, but with the extraction solvent instead of the extract. All analyses were performed in triplicate (n = 3). A standard curve was established using standard solutions of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at concentrations ranging from 50 to 500 µM. The results were expressed in micromoles of Trolox equivalent per gram/dry extract (µmol TE/g DE).

2.7. Evaluation of anti-inflammatory activity

In vitro anti-inflammatory activity was evaluated using the bovine serum albumin (BSA) thermodenaturation inhibition assay, according to Mizushima and Kobayashi (1968) with minor modifications. This assay is based on the ability of extracts to prevent heat-induced denaturation of BSA, a phenomenon involved in inflammatory processes. BSA was prepared at 0.2% in Tris-HCl buffer (0.05 M, pH 6.6). Different concentrations of the extracts (50-800 µg/mL) were prepared. Diclofenac was used as a positive control and treated in the same way as the extracts. For the test, 1 mL of each extract dilution was mixed with 1 mL of BSA solution. The mixtures were incubated at 37°C for 15 minutes, then at 72°C for 5 minutes. After incubation, the tubes were vortexed and

rapidly cooled. The turbidity resulting from protein denaturation and aggregation was measured at 660 nm. Two blanks were used to correct the measurements:

- Sample blank: 1 mL of each dilution + 1 mL of Tris-HCl buffer.
- BSA blank: 1 mL of BSA + 1 mL of the solvent used for the extracts, representing maximum denaturation in the absence of inhibitor.

The percentage of denaturation inhibition was calculated using the following formula:

$$\textbf{Inhibition}\ (\%) = \frac{\text{A blank} - \text{A sample}}{\text{A blank}}$$

Where **A blank** is the absorbance of the BSA blank and **A sample** is the absorbance in the presence of the extract.

2.8. Statistical analyses

All tests were performed in triplicate (n = 3) and expressed as mean ± SD. The data were analysed by two-factor ANOVA (Statistica 13.0, TIBCO Software Inc.) to evaluate the effects of species (*S. oleracea* vs. *C. bonus-henricus*) and plant part (leaf vs. stem), followed by Tukey's HSD test in case of significance ($p < 0.05$).

3. Results and discussion

3.1 Extraction yields

Methanolic extraction yields vary depending on the species and type of plant tissue (Table 3). Indeed, the leaves of both species studied show higher yields (*S. oleracea*: 8.3%; *C. bonus-henricus*: 8.6%) than the stems (*S. oleracea*: 4.1%; *C. bonus-henricus*: 4.7%). This trend suggests that leaves concentrate more methanol-soluble compounds, such as polyphenols and other secondary metabolites, while stems, which are richer in fiber and lignin, limit extraction. These differences are consistent with observations reported in the literature, where leaf tissues of spinach and related plants are known to preferentially accumulate bioactive compounds (Kabir *et al*., 2017; Chatepa *et al*., 2025).

3.2. Phytochemical profile

Qualitative phytochemical screening reveals marked differences between the leaves and stems of the two species (Table 4). The leaves are distinguished by a high presence (+++) of flavonoids, gallic tannins, and carotenoids, and a moderate presence (++) of saponins and coumarins, while the stems show low to moderate levels (+ to ++) of these compounds. Alkaloids are limited to a low presence (+) in the leaves only, and catechin tannins are absent (-) in all tissues, with anthocyanins ranging from trace amounts (±) to low presence (+).

The differences observed between leaves and stems mainly concern bioactive compounds. Leaves accumulate high levels of flavonoids, gallic tannins, and carotenoids, as well as saponins and coumarins to counteract photosynthetic oxidative stress (Agati *et al*., 2020). Stems have reduced levels, with no catechin tannins or alkaloids, and traces of coumarins or anthocyanins, due to their high cellulose and lignin content, which limits soluble metabolites (Zhong *et al*., 2018). Flavonoids and leaf carotenoids dominate as photoprotective antioxidants, dissipating excess energy and preventing membrane oxidation (Lingwan *et al*., 2023). This distribution is genetically regulated, favoring leaf bioactives and explaining higher yields (Badmus, 2022).

3.3. Thin layer chromatography

Four distinct solvent systems were used to optimize the chromatographic separation of the constituents of the leaf and stem extracts of the two spinach species studied. The chromatograms obtained revealed a series of spots corresponding to the presence of various compounds (Figure 1). These compounds were identified by comparing the Rf values and the colors observed under UV light of the spots appearing on the TLC plates (Table 5).

Flavonoids exhibited characteristic fluorescence patterns under UV/365 nm. Yellow emissions correspond to flavonols and rutin-type derivatives, whereas blue or purple fluorescence indicates methy lated flavones (Dawson *et al*., 1991). Yellow-green fluorescence suggests kaempferol-based flavonols. After revelation with $AlCl_3$, intensified yellow, blue, and green fluorescent spots were observed, confirming complex formation between aluminum ions and flavonoid hydroxyl groups, a reaction widely used for flavonoid detection and quantification (Mammen and Daniel, 2012). The higher fluorescence intensity observed in leaves agrees with previous TLC phytochemical investigations and reflects enhanced phenolic accumulation in photosynthetic tissues (Panche *et al*., 2016).

Coumarins showed intense blue, green, and brown fluorescence under UV/365 nm, consistent with previously described chromatographic behavior (Dawson *et al*., 1991; Wagner and Bladt, 1996). Increased fluorescence after KOH treatment indicates the presence of hydroxycoumarins,

including furanocoumarins and pyranocoumarins. Coumarins are widely recognized for their antimicrobial and antioxidant properties (Venugopala *et al*., 2013), which supports their ecological and physiological relevance in leaf tissues.

Terpenoids displayed blue, green, yellow, and violet fluorescence under UV/365 nm, consistent with triterpene spectral behavior described in TLC atlases (Wagner and Bladt, 1996). Yellow-orange coloration under visible light suggests lupane-type triterpenes. The preferential detection of these compounds in leaves aligns with reports indicating tissue-dependent terpenoid biosynthesis linked to plant defense and oxidative stress responses (Tholl, 2015).

Free quinones were identified by yellow-to-violet chromatic transitions and violet fluorescence after alkaline treatment. Quinones are well-known redox-active phenolic derivatives involved in plant defense and oxidative metabolism (Dong *et al*., 2024). Their exclusive detection in leaves further supports the role of aerial tissues in stress-related metabolite accumulation.

4. Quantification of bioactive compounds

The total polyphenol and total flavonoid contents of the different parts of *S. oleracea* and *C. bonus-henricus* are shown in Table 6. Total polyphenols are expressed in mg of gallic acid equivalents per gram/dry extract (mg GAE/g DE), while total flavonoids are expressed in mg of quercetin equivalents per gram/dry extract (mg QE/g DE).

The results for the total phenolic and flavonoid content of extracts from *S. oleracea* and *C. bonus-henricus* reveal significantly different profiles depending on the plant organ studied. The leaves of *C. bonus-henricus* have the highest ($p < 0.05$) values for total polyphenols (102.9 ± 6.8 mg GAE/g ES) and total flavonoids (74.3 ± 4.7 mg EQ/g DE) compared to *S. oleracea* leaves (92.1 ± 3.84 mg GAE/g DE and 68.5 ± 1.6 mg EQ/g DE, respectively). These quantitative differences are strongly supported by the preliminary phytochemical screening and TLC analysis, which confirmed a massive presence of gallic tannins and intense fluorescence of flavonols in the leaves, whereas these compounds were less dense or limited in the stems.

In contrast, the stems of both species have significantly lower concentrations of these secondary metabolites ($p < 0.001$). This marked tissue-dependent distribution is further validated by TLC profiles, which reveal that certain bioactive classes, such as terpenoids and quinones, are exclusively localized in the leaves and entirely absent in the stems. This pattern is consistent with the known biosynthetic mechanisms of polyphenols and flavonoids, mainly derived from the shikimate and phenylpropanoid pathways. These pathways are highly active in chlorophyll-rich cells and chloroplasts to protect against oxidative stress induced by light (Zhang *et al*., 2023; Zagoskina *et al*., 2023).

Furthermore, the detection of saponins, coumarins, and alkaloids specifically in leaf tissues highlights a broader chemical diversity compared to the stems. Our findings align with recent studies on edible leafy vegetables reporting higher phenolic levels in leaves (Masiala *et al*., 2024), supporting the generality of this phenomenon in *Spinacia* and *Chenopodium*. Comparing our results with the literature, the measured values fall within ranges observed for phylogenetically related species (Kokanova-Nedialkova *et al*., 2021). For example, concentrations between 80 and 120 mg GAE/g ES have been documented for several salad mixtures, while total flavonoids are similar to those reported in high-quality edible leaf extracts (Mazzucotelli *et al*., 2018).

6. DPPH radical scavenging assay

The evaluation of the antioxidant activity of leaf and stem extracts from *S. oleracea* and *C. bonus-henricus*, using the DPPH assay, revealed significant and progressive inhibition. This increased with concentration (25-200 µL/mL), reflecting a dose-dependent relationship typical of antioxidant phenolic compounds (Figure 2). This antiradical power was compared to that of vitamin C (positive control) (Figure 3).Vitamin C exhibits high antioxidant activity with a very low IC50 value (9.4 µg/mL) that is strictly dose-dependent, characterized by a rapid increase in the percentage of inhibition at low concentrations, followed by a plateau at intermediate and high concentrations, with values reaching approximately 99%. This pattern confirms the strong ability of vitamin C to neutralize the DPPH radical. Leaf extracts from both spinach species show high radical activity, significantly higher than that of stem extracts. These results are chemically consistent with the phytochemical screening and supported by TLC analysis, which identified a diverse range of bioactive compounds in the leaves, including flavonoids and quinones, both characterized by their ability to quench fluorescence and neutralize free radicals (Hassanpour and Doroudi, 2023), using various mechanisms, including hydrogen-bond donors and acceptors, and polyanions (Zheng *et al*., 2021). At the highest concentration tested, *C. bonus-henricus* leaves achieve inhibition rates comparable to those of vitamin C (95%) and an

IC50 of 37 µg/mL, indicating very high antioxidant power. TLC revelation confirms a high density of flavonols, which are likely responsible for this efficacy. The leaves of *S. oleracea* also showed strong radical scavenging activity (IC50 = 43.65 µg/mL) with marked inhibition (92%), although significantly less pronounced than *C. bonus-henricus*. In contrast, stem extracts from both species exhibit moderate antioxidant activity, with inhibition percentages generally not exceeding 40-50% and higher IC50 values (192.4 µg/mL for *C. bonus-henricus* and 250 µg/mL for *S. oleracea*), even at the highest concentrations. The phytochemical screening showing fewer phenolic compounds and the TLC profile revealing a total absence of terpenoids and quinones in the stems corroborates this low efficacy. This suggests a lower concentration of active antioxidant metabolites, particularly phenolic compounds, which are traditionally more abundant in leaf tissue. Similar observations have been reported in *S. oleracea*, where the leaves are rich in phenolic compounds that are strongly correlated with high antioxidant activity (Faujan *et al*., 2023). In the case of *C. bonus-henricus*, although this species remains relatively poorly studied, work on related species reveals a richness in phenolic compounds closely associated with marked antioxidant activity, as demonstrated in *C. album* and *C. pumilio* by Kolar et al. (2024).

7. Ferric reducing antioxidant power assay

The antioxidant activity of leaf and stem extracts from both spinach varieties was also evaluated using the FRAP test, in comparison with a Trolox standard curve (Figure 4). Analysis of antioxidant capacity expressed in µmol of Trolox equivalents per gram/dry extract reveals marked variability between the organs and species studied (Table 8).
The leaves showed significantly higher antioxidant capacity values than the stems ($p < 0.001$), regardless of the genotype, indicating a differential distribution of antioxidant potential in favor of leaf tissue. These quantitative findings are strongly supported by the phytochemical screening, which showed a maximum density of gallic tannins and flavonoids in the leaves, and further confirmed by TLC analysis revealing intense blue and yellow-green fluorescence (365 nm). In *S. oleracea*, the antioxidant capacity of the leaves reaches 400.8 ± 10.5 µmol TE/g DE, while the stems are limited to 294.3 ± 9.8 µmol TE/g DE ($p < 0.05$). This difference reflects the preferential accumulation of phenolic acids and flavonoids correlated with photosynthetic activity and UV exposure (Jaiswal *et al*., 2017; Zhang *et al*., 2023). The leaves and stems of wild spinach (*C. bonus-henricus*) exhibit antioxidant capacity superior to that of cultivated spinach (*S. oleracea*), with values of 486.5 ± 14.1 µmol TE/g DE and 352.6 ± 10.5 µmol TE/g DE, respectively ($p < 0.05$). This enhanced reducing power may reflect an abiotic stress adaptation strategy involving constitutive activation of secondary phenolic metabolism (Wang *et al*., 2021; Batool *et al*., 2024). Specifically, the TLC results highlight a unique combination of quinones and terpenoids in *C. bonus-henricus* that contributes to this superior capacity, placing it among the leafy vegetables richest in antioxidants (Eligar, 2022). Furthermore, the presence of coumarins and saponins detected in the screening may act synergistically with the flavonoid content to enhance the overall ferric reducing power of the wild variety compared to its cultivated counterpart.

8. Evaluation of anti-inflammatory activity

The anti-inflammatory activity of *Spinacia oleracea* and *Chenopodium bonus-henricus* extracts was assessed using the BSA thermodenaturation inhibition assay. All extracts inhibited protein denaturation in a clear dose-dependent manner (Figure 5). Comparative analysis revealed that leaf extracts of the wild species *C. bonus-henricus* exhibited the strongest activity, with the lowest IC50 value (216.1 µg/mL), significantly outperforming *S. oleracea* leaves (IC50= 416.1 µg/mL; $p < 0.05$) (Table 9). At 800 µg/mL, *C. bonus-henricus* leaves achieved 81.2% inhibition, approaching the performance of the reference drug Diclofenac (IC50 = 156.6 µg/mL; 91.5%). The dose-dependent inhibition of BSA denaturation observed in this study confirms the anti-inflammatory potential of *S. oleracea* and *C. bonus-henricus*. The mechanism of protein stabilization by plant extracts is generally attributed to the presence of polyphenols capable of creating bridges between polypeptide chains, thereby preventing their unfolding under the effect of heat or denaturing agents. Flavonoids are known to stabilize proteins by binding to hydrophobic pockets, thereby preventing the unfolding induced by thermal stress (Fu *et al*., 2016). In agreement with this, Mroczek (2015) reported a high affinity of Amaranthaceae metabolites for albumin binding sites, enhancing its thermal stability.The enhanced performance of the wild species aligns with Mishra et al. (2021), who demonstrated that wild plants often exhibit more complex biosynthesis of glycosylated flavonoids in response to environmental stresses. Our TLC results support this, showing an intense and complex yellow-green fluorescence in *C. bonus-henricus* leaves, characteristic of a diverse array of

flavonols that may have been partially depleted in cultivated spinach through selective breeding for yield traits. A marked tissue-dependent effect was also observed, as stems exhibited significantly higher IC50 values (634.7µg/mL for *C. bonus-henricus* and 767.6 µg/mL for *S. oleracea*; $p < 0.001$). This is chemically validated by TLC profiles, which showed a total absence of terpenoids and quinones in the stems, contrasting with the rich diversity of fluorescent bands in the leaves. This distribution is consistent with the activation of the phenylalanine ammonia-lyase (PAL) pathway in leaf tissues stimulated by solar exposure (Belew *et al*., 2025). Notably, the 81.2% inhibition achieved by *C. bonus-henricus* leaves places this extract within a performance range comparable to pharmacological standards. The presence of saponins and coumarins, detected specifically in the wild variety, likely acts synergistically with flavonoids to enhance protein protection. It has been reported that plant extracts rich in terpenoids, flavonoids, and tannins can act as dual inhibitors of COX-2 and 5-LOX, leading to a reduction in prostaglandin and leukotriene synthesis and thus helping to modulate inflammatory responses. This dual inhibition is consistent with the decreases in inflammatory markers observed *in vitro* and also corresponds to the protein-stabilizing effects observed in inflammatory conditions (Weletnsae *et al*., 2024). As suggested by Shrivastava et al. (2023), inhibition levels exceeding 80% represent a strong indicator of potential systemic efficacy, highlighting wild spinach as a promising natural source of anti-inflammatory agents.

Table 1. *Qualitative phytochemical screening tests according to Terease and Evans (1989), Harborne (1998), and Bruneton (1999).*

Metabolite	Extract Amount	Reagents/Conditions	Result
Flavonoids	5 mL methanolic extract	1 mL concentrated HCl + 0.5 g magnesium	Pink to purple-red coloration
Gallic tannins	1 mL methanolic extract	2 mL distilled water + 2–3 drops diluted $FeCl_3$	Blue-black coloration
Catechic tannins	1 mL methanolic extract	2 mL distilled water + 2–3 drops diluted $FeCl_3$	Blue-green coloration
Coumarins	5 mL methanolic extract	0.5 mL 10% NH_4OH; observe under UV at 366 nm	Blue or intense fluorescence
Alkaloids	2 mL methanolic extract	2 mL 10% H_2SO_4; shake and filter; divide filtrate; add 5 drops Mayer reagent (Tube 1) or Wagner reagent (Tube 2)	White/brown precipitate or turbidity
Anthocyanins	2 mL aqueous infusion	2 mL 2N HCl; then ammonia	Pink-red coloration turning bluish-violet
Carotenoids	Dry methanolic extract	Dissolve in 1 mL $CHCl_3$ + 2-3 drops saturated $SbCl_3$	Blue coloration turning to red
Saponosides	2 mL aqueous extract	Dilute with distilled water + vigorous shaking; rest 20 min; measurement from height	Height: <1 cm (weakly positive); 1-2 cm (positive); >2 cm (strongly positive)

Table 2. *Phytoconstituents and their corresponding migration solvents and specific detectors according to Wagner et al. (1996).*

Phytoconstituents	Solvent system	Detection reagent
Coumarins	AcOEt-HCOOH-AcOH-H_2O (100 :11:11:26)	KOH
Flavonoids	AcOEt-HCOOH-AcOH-H_2O (100 :11:11:26)	Neu
Quinones	AcOEt-MeOH-H_2O (100:17:13)	KOH
Terpenoids	CHCl3-MeOH-H_2O-AcOH (60 :32:7:1)	Vanillin-sulfuric acid

Table 3. *Extraction yields of methanolic extracts: leaf vs. Stem.*

Spinach species	Part	Yield (%)
S. oleracea	Leaf	8.3

	Stem	4.1
C. bonus-henricus	Leaf	8.6
	Stem	4.7

Table 4. *Phytochemical screening of S. oleracea and C. bonus-henricus: leaf vs. stem.*

Compounds	***S. oleracea***		***C. bonus-henricus***	
	Leaf	**Stem**	**Leaf**	**Stem**
Flavonoids	+++	++	+++	++
Catechic tannins	-	-	-	-
Gallic tannins	+++	+	+++	+
Coumarins	++	±	++	+
Alkaloids	+	-	+	-
Anthocyanins	±	+	+	+
Carotenoids	+++	++	+++	++
Saponins	++	+	++	+

The presence of phytochemicals is indicated as follows:
+++ (high presence), ++ (moderate presence), + (low presence), ± (traces), and - (absent).

Table 5. *TLC profile of phytoconstituents detected in leaves and stems.*

Phytoconstituents	***S. oleracea***		***C. bonus-henricus***		**Detection**	
	Rf		***Rf***		**Before revelation**	**After revelation**
	Leaf	**Stem**	**Leaf**	**Stem**		
Flavonoids	0.22 0.32 0.42	0.22 0.32 0.42	0.22 0.32 0.42	0.22 0.32 0.42	-at 254nm: quenching -at 365nm: yellow to yellow-green fluorescence	-Visible: yellow coloration -Under UV light at 365 nm: intense blue to yellow-green fluorescence
Terpenoids	0.16 0.64 0.82	/	0.17 0.68 0.83	/	-at 254nm: quenching -at 365nm: yellow to brown fluorescence	-Visible: yellow-green coloration -at 365nm: red to brown fluorescence
Coumarins	0.12 0.33 0.46	0.12	0.17 0.33 0.46	0.16	-at 254nm: quenching -at 365nm: intense blue to yellow/brown fluorescence	-Visible: yellow coloration -at 365nm: yellow / green to blue-green fluorescence
Quinones	0.19 0.36	/	0.19 0.36	/	-at 254 nm: quenching -at 365nm: yellow to reddish-brown fluorescence	-Visible: yellow to green coloration -A 365nm: red to violet fluorescence

Table 6. *Total polyphenols and flavonoids quantification results.*

Spinach species	**Part**	**Total polyphenol mg GAE/g DE**	**Flavonoides mg QE/g DE**

S. oleracea	Leaf	92.1 ± 3.84[a]	68.5 ± 1.6[a]
	Stem	37.8 ± 3.6[b]	30.9 ± 4.3[b]
C. bonus-henricus	Leaf	102.9 ± 6.8[c]	74.3 ± 4.7[c]
	Stem	41.4 ± 3.9[b]	37.75 ± 3.4[b]

Values are presented as mean ± standard deviation (n = 3). Different letters within the same column indicate statistically significant differences ($p < 0.05$, ANOVA followed by Tukey's test).

Table 7. *Comparative antioxidant activity (IC_{50} values).*

IC_{50} (µg/mL)	**Vitamin C**	***S. oleracea***		***C. bonus-henricus***	
		Leaf	Stem	Leaf	Stem
	9.40	43.65	250	37	192.44

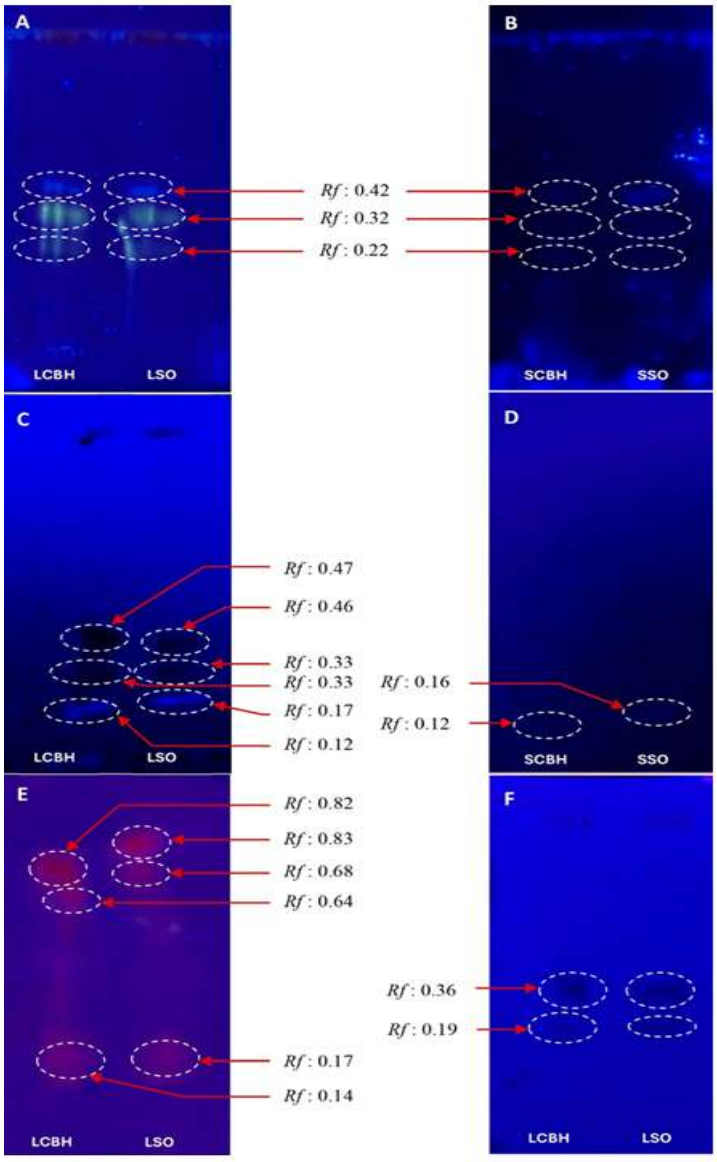

Figure 1. *Thin-layer chromatography of key phytoconstituents.*
*S. oleracea: leaves (**LSO**) vs. stems (**SSO**) and C. bonus-henricus: leaves (**LCBH**) vs. stems (**SCBH**).*
*(**A**) Flavonoid chromatogram profile in leaves, (**B**) Flavonoid chromatogram profile in stems, (**C**) Coumarin chromatogram profile in leaves, (**D**) Coumarin chromatogram profile in stems, (**E**) Terpenoid chromatogram profile in leaves, (**F**) Quinone chromatogram profile in leaves.*

Table 8. *Antioxidant capacity of S. oleracea and C. bonus-henricus extract.*

Spinach species	Part	µmol TE/g DE
S. oleracea	Leaf	400.8 ± 10.5[a]
	Stem	294.3 ± 9.8[b]
C. bonus-henricus	Leaf	486.5 ± 14[c]
	Stem	352.6 ± 10.5[d]

TE: *Trolox Equivalents,* ***DE:*** *Dry Extract. Values are presented as mean ± standard deviation (n = 3). Different letters within the same column indicate statistically significant differences (p < 0.05, ANOVA followed by Tukey's test).*

Table 9. *Comparative anti-inflammatory activity (IC_{50} values).*

IC_{50} (µg/mL)	Diclofenac	*S. oleracea*		*C. bonus-henricus*	
		Leaf	Stem	Leaf	Stem
	156.6	416.1	767.6	216.1	634.7

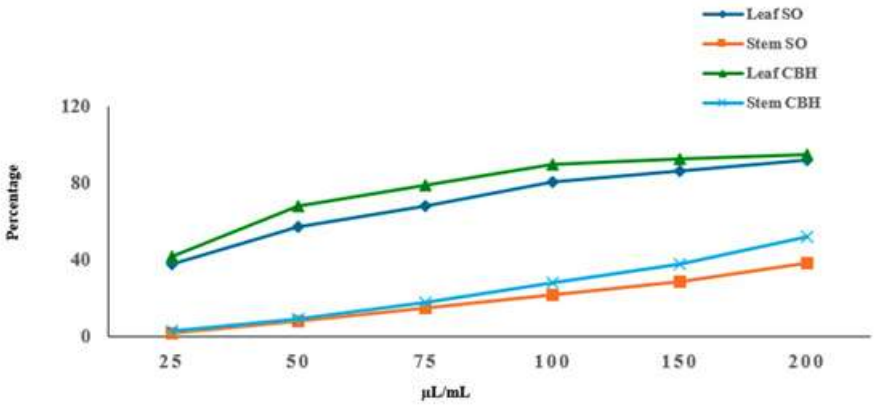

Figure 2. *Concentration-dependent DPPH inhibition by leaf and stem extracts.*

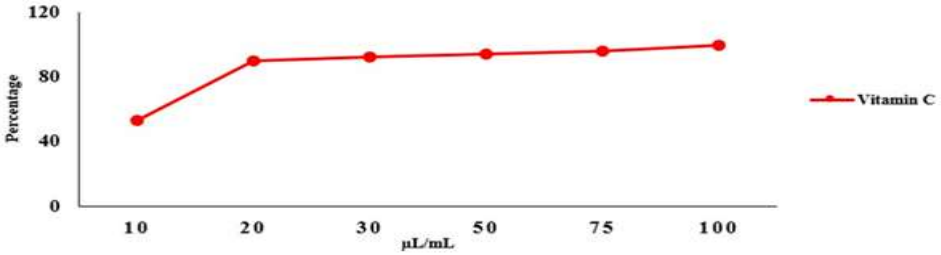

Figure 3. *Concentration-dependent DPPH inhibition by vitamin C.*

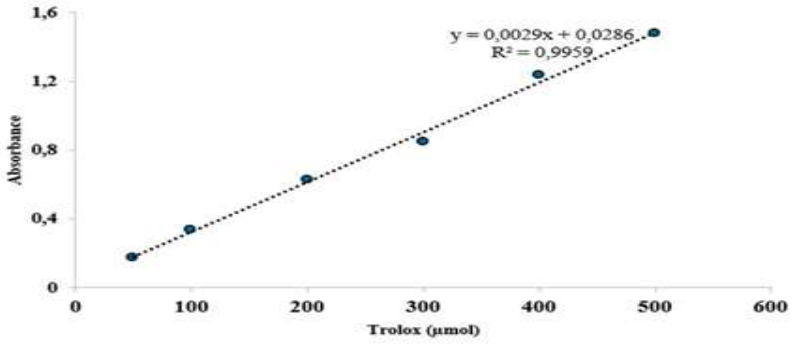

Figure 4. *Trolox standard curve.*

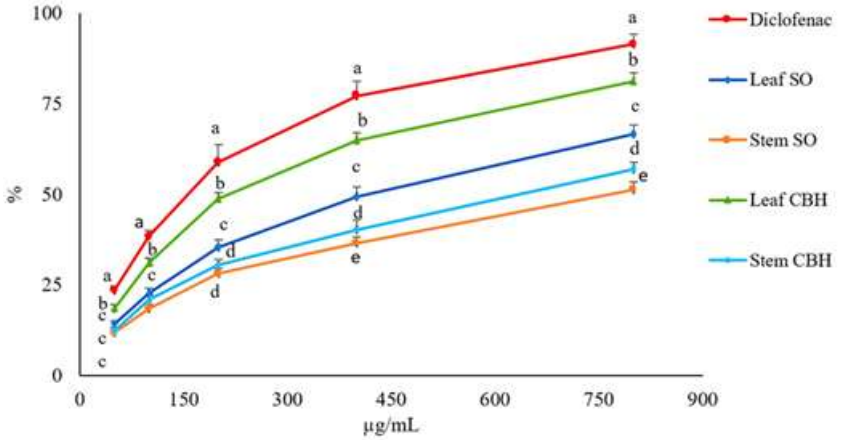

Figure 5. *Percentage inhibition of BSA denaturation by S. oleracea and C. bonus-henricus extracts compared to Diclofenac.Values are presented as mean ± standard deviation (n = 3). Different letters within the same column indicate statistically significant differences (p < 0.05, ANOVA followed by Tukey's test).*

9. Conclusions

This comparative study provided an in-depth phytochemical and functional characterization of the cultivated spinach S. oleracea and the wild species C. bonus-henricus. The results of phytochemical and TLC profiles highlight that both tissue and genotypic distribution of secondary metabolites, with leaves, particularly those of the wild species, exhibiting the highest concentrations of polyphenols and flavonoids. This phytochemical richness leads to enhanced biological performance, as shown by low IC_{50} values obtained in DPPH radical scavenging and BSA denaturation inhibition tests, as well as high reducing capacity (FRAP), confirming a significantly higher antioxidant and anti-inflammatory potential in C. bonus-henricus. The convergence of results from different tests suggests that redox-active phenolic compounds play a central role in the biological mechanisms observed. The clearly established functional gradient, with wild species superior to cultivated species and leaves superior to stems, supports the hypothesis that a compromised balance may accompany domestication between agronomic yield and bioactive compound density. Beyond this comparative interest, the bioactive profile of C. bonus-henricus makes it an under-exploited bioresource with strong potential for the development of natural functional, nutritional, and phytopharmaceutical products. Further work, combining advanced metabolomic approaches (HPLC-MS/MS) and in vivo validation, will confirm the therapeutic relevance and translational applicability of these observations.

Author Statements:

- **Ethical approval:** The conducted research is not related to either human or animal use.
- **Conflict of interest:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper
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- **Data availability statement:** The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.
- **Use of AI Tools:** The author(s) declare that no generative AI or AI-assisted technologies were used in the writing process of this manuscript.

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