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Green Extraction of Anthocyanins from Autumn Leaves: Comparative Evaluation of Food-Grade Solvent Systems

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ABSTRACT

This study investigates the potential of autumn leaves of *Smilax excelsa* L., typically regarded as edible plant residues, for the green extraction of anthocyanin-rich bioextracts. Four food-grade solvent systems - ethanol (EtOH), citric acid (100 mM), EtOH: Citric acid (1:1), and DMSO: EtOH: Citric acid (1:1:1, DES) were evaluated for their extraction efficiency of anthocyanins. The total anthocyanin content was determined using UV-Vis spectrophotometry at 520 nm and expressed as cyanidin-3-glucoside (C-3-G) equivalents based on a calibration curve (R^2 = 0.98994). The highest anthocyanin yield was obtained with the EtOH: Citric acid binary system (11.325 µg), followed by citric acid alone (10.254 µg), DMSO: EtOH: Citric acid (9.479 µg), and ethanol alone (8.736 µg). The EtOH: Citric acid extract also exhibited the strongest antioxidant activity (89.38±0.89 % inhibition), while the ethanol extract showed the lowest (74.2±0.81%). Interestingly, although the DES like ternary system (DMSO: EtOH: Citric acid) demonstrated moderate anthocyanins yield, it displayed rapid free radical scavenging kinetics, suggesting the extraction of additional bioactive compounds beyond anthocyanins. These findings demonstrate that the combination of ethanol and citric acid provides synergistic effects, enhancing both extraction efficiency and antioxidant capacity. The utilization of post-vegetative autumn leaves represents a sustainable approach that aligns with circular economy principles, converting low-value biomass into high-value functional ingredients for the food industry.

1- INTRODUCTION

In contemporary society, there is a growing global preference for natural products across multiple industries, including food production, pharmaceuticals, and textile manufacturing. Natural foods, plant-based colorants, and the use of natural dyes in textile treatment are increasingly favored over synthetic alternatives. This shift is driven not only by consumer demand for healthier and more sustainable products but also by heightened awareness of environmental protection, ecological balance, and long-term economic efficiency. In the development and production of such natural products, it is essential to consider factors such as cost-effectiveness, preservation of ecosystems, and minimization of environmental impact [1], [2].

One of the most actively explored research areas within this framework is the study of medicinal plants. Investigations focus on identifying and analyzing biologically active compounds, essential minerals, and secondary metabolites present in plant materials. These substances are being evaluated for their potential applications as natural dyes, food additives, antioxidants, preservatives, and therapeutic agents. The exploration of plant-based bioactive compounds represents one of the most relevant and rapidly advancing scientific fields of our time [3], [4].

The interest in naturally occurring bioactive compounds has increased significantly in recent years [5]. Among these compounds, anthocyanins occupy a special place—they are flavonoid derivatives that impart red, purple, and blue colors to various plant organs [6]. Anthocyanins possess strong antioxidant, antimicrobial, anti-inflammatory, and anticarcinogenic properties, offering wide application potential in the food, pharmaceutical, and cosmetic industries [7].

Smilax excelsa L. is a perennial climbing plant belonging to the *Smilacaceae family*, widely distributed in the Caucasus region [8]. Various organs of this plant have been used in traditional medicine for the treatment of rheumatism, skin diseases, and inflammatory processes[9]. Recent research has revealed that the dark-red autumn leaves of *Smilax excelsa* L. possess a rich phenolic composition, containing 18 biologically active substances alongside 25 mineral chemical elements [1]. The majority of these compounds exhibit antioxidant, antimicrobial, and antifungal properties [10]. However, systematic studies on the extraction of anthocyanins from *Smilax excelsa* L. leaves and their antioxidant characteristics remain limited.

The most commonly employed method for the quantitative determination of anthocyanins is pH-differential spectrophotometry. This technique is based on the difference in absorbance of anthocyanins at pH 1.0 and pH 4.5. Cyanidin-3-O-glucoside (C-3-G) is the most widespread anthocyanin in nature and is accepted as an equivalent standard in most studies [11], [12].

A significant portion of research focused on the use of environmentally friendly and plant-based consumer products primarily concentrates on the vegetative growth periods of plants, often overlooking other critical developmental stages such as flowering, seed formation, and dormancy. While this focus is typically justified by the higher biomass yield and extractable bioactive compounds available during vegetative phases, it inadvertently narrows the ecological and genetic scope of investigation. Such a limited temporal framework may fail to capture the full spectrum of phytochemical diversity and adaptive traits expressed across the plant life cycle [13].

This approach has broader ecological implications. By prioritizing specific growth stages and, in many cases, a restricted number of commercially valuable species, research and subsequent industrial practices may reinforce monocultural cultivation systems and selective harvesting strategies. These patterns can

intensify pressure on wild plant populations, disrupt natural regeneration cycles, and accelerate habitat homogenization. Over time, such processes contribute to the reduction of plant biodiversity at both species and intra-species levels [14].

Moreover, the systematic preference for particular genotypes associated with high yield, rapid growth, or enhanced bioactive compound production may promote genetic uniformity. This trend increases vulnerability to environmental stressors, pathogens, and climate variability, thereby undermining ecosystem resilience. The resulting erosion of the plant gene pool not only diminishes adaptive capacity but also constrains future opportunities for breeding, conservation, and sustainable resource management. Accordingly, a more integrative research paradigm—one that accounts for multiple developmental stages, diverse taxa, and ecosystem-level interactions—is essential to balance commercial objectives with long-term ecological sustainability [15].

In the context of green chemistry and environmental sustainability, the selection of both the plant material and the extraction method is of paramount importance, particularly when the final extract is intended for human consumption as a food additive or nutraceutical. Conventional extraction methods often rely on organic solvents such as methanol, acetone, or hexane, which are efficient but pose significant health risks due to potential toxic residues in the final product. Furthermore, the traditional sourcing of plant materials can contribute to the overexploitation of natural resources, threatening biodiversity. Therefore, the use of food-grade (edible) solvents and sustainably sourced raw materials presents a critical advantage [16].

An equally important consideration for sustainable practices is the sourcing of plant material. In this study, the plant extracts were obtained from post-vegetative autumn leaves, a biomass that is typically regarded as edible plant residues. Crucially, the collection of these senesced leaves does not harm the plant's biodiversity or its genetic pool, as the material is harvested after the plant has completed its reproductive and vegetative cycle. This approach ensures that the research does not contribute to the overexploitation of medicinal plants or threaten natural populations, aligning perfectly with the principles of environmental sustainability. By utilizing this biological waste material, the study transforms a low-value residue into a high-value functional ingredient, embodying the concept of a circular economy.

Therefore, the present study was designed to evaluate the potential of these sustainably sourced autumn leaves as a novel source of anthocyanin-rich extracts for food applications. Using food-grade solvents (citric, ethanol, and ethyl citric), the study aims to: (I) compare extraction efficiencies for anthocyanins; (II) quantify anthocyanin content; (III) assess antioxidant activity and (IV) demonstrate the potential of these extracts as safe, functional food additives [17]. By combining environmentally sustainable raw material sourcing with green extraction techniques, this research contributes to the circular economy while developing high-value ingredients for the food industry. The combination of sustainable raw material sourcing and green extraction techniques underscores the commitment to environmental sustainability throughout the entire research methodology.

2- MATERIALS AND METHODS

2.1. Collecting and drying the leaves

Autumn leaves of the *Smilax excelsa* L. plant were collected from the garden of Sheki RSC (41.220733 N, 47.175806 E) in December 2025. The leaves, exhibiting a characteristic dark-red coloration indicative of seasonal anthocyanin accumulation, were carefully harvested from the climbing vines. After collection, the leaves were thoroughly washed with distilled water to remove any dust or debris, and then gently air-dried under controlled conditions to preserve their biochemical integrity. Once dried, the leaves

were processed and prepared for extraction to quantify their anthocyanin content.

2.2. Chemicals

To guarantee accurate extraction and analytical measurements, high-purity analytical-grade reagents were employed. Dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and cyanidin-3-glucoside (C-3-G) standards were purchased from Sigma-Aldrich, but ethanol and citric acid were purchased from Aura Chemistry, Turkeywere. DMSO, ethanol and citric acid were employed as a solubilising, free radicals scavenger was measured using DPPH, and anthocyanin quantification was done using C-3-G as a reference standard. Utilising high-purity reagents promoted effective extraction and stability of the bioactive pigments during later studies while reducing analytical interference.

2.3. Preparation of Extraction Solvents

Initially, a 12 M ethanol solution was prepared from 96% ethanol, along with a 100 mM citric acid solution and a 10 M DMSO solution. Four extraction solutions were then prepared by mixing: EtOH, Citric acid, EtOH:Citric acid (1:1), and DMSO:EtOH:Citric acid (1:1:1) [1],[18], [19].

2.4. Extraction of Plant Samples

The dried leaves were ground to a particle size of 0.1–0.5 mm and divided into four portions, each weighing 1 g. The powdered leaf samples were placed into four appropriately sized chemical beakers, and 30 mL of the respective extraction solvents were added to each. Extraction was carried out using a decoction method at 55–60 °C for 120 minutes. At the end of the extraction, the extracts were centrifuged at 3500 rpm.

2.5. UV-Vis Analysis and pH Measurement

The pH of each extract was adjusted to 4.5 using an acetate buffer to provide consistent conditions for spectrophotometric analysis and to maintain the stability of anthocyanin pigments. The maximum absorbance wavelength (A_{max}) of the extracts was determined at 520 nm using a UV-752N Plus spectrophotometer [21]. This wavelength was selected because of anthocyanin compounds commonly exhibit strong visible-light absorption in this region under mildly acidic conditions. Following appropriate dilution, the absorbance values of the extract solutions were measured at different concentrations. The corresponding concentrations (C) were calculated using the Beer–Lambert law, expressed as $A = c\varepsilon l$, where A is absorbance, c is the concentration of the analyte, ε is the molar absorptivity coefficient, and l is the optical path length of the cuvette. The obtained data were used to evaluate the relationship between extract concentration and absorbance and to estimate the anthocyanin content of the samples.

2.6. Quantitative Analysis of Anthocyanins.

An amount of anthocyanins in the extracts obtained from 1 g of dried leaves using four different extraction solvents was determined as cyanidin-3-glucoside (C-3-G) equivalents using a linear regression model. For this purpose, the dependent (y) and independent (x) variables were established for different concentrations of cyanidin-3-glucoside, and the coefficient of determination (R^2) was calculated. All data processing, including the construction of the calibration curve and linear regression analysis, was performed using Origin 8 software. This approach allowed precise quantification of anthocyanin content in each extract and ensured that the measurements were expressed consistently as C-3-G equivalents.

2.7. DPPH radical scavenging activity

A 70 µM solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) was freshly prepared in 96% ethanol. For the assay, 100 µL of each plant extract was added to 2,900 µL of the DPPH

solution, resulting in a total reaction volume of 3 mL. The mixture was gently mixed and the change in absorbance at 517 nm was recorded over a period of 30 minutes using a UV-vis 752N Plus spectrophotometer to monitor the kinetics of the free radical scavenging reaction. The decrease in absorbance indicated the DPPH radical scavenging activity of each extract. All measurements were carried out in triplicate to ensure reproducibility, and the results were expressed as the mean ± standard deviation. This setup allowed accurate comparison of the antioxidant potential of the different extracts over time [22] [23].

$$(\text{inhibition } \%) = [(A_{control} - A_{sample})/A_{control}] \times 100.$$

2.8. Statistical analysis.

All experiments were performed in triplicate or more to ensure reproducibility. The data were analyzed using standard deviation (SD) to assess variability and subjected to analysis of variance (ANOVA) using Origin 8 software to evaluate statistical significance. Differences were considered statistically significant at a probability level of $p \leq 0.05$. This statistical approach ensured the reliability and robustness of the experimental results.

3-RESULTS AND DISCUSSION

L In the research study, the concentrations of anthocyanins in extracts obtained using various food-grade solvents were determined and comparatively evaluated. Based on these concentration values, the total anthocyanin content was calculated and expressed as C-3-G (cyanidin-3-glucoside) equivalents. Furthermore, the Free Radical Scavenging activity of the same extracts was assessed in order to evaluate their antioxidant potential. Initially, a 40 µM stock solution of C-3-G was prepared in ethyl citrate. Serial dilutions were carried out to obtain four calibration points, and UV-Vis spectrophotometric measurements were performed. Using the absorbance values at the corresponding λmax, a linear regression curve was constructed in Origin8. As can be seen from Figure 1, from this calibration curve, the following parameters were obtained: R^2 = 0.98994, slope (a) = 0.84386, and intercept (b) = 0.04616 [24] .

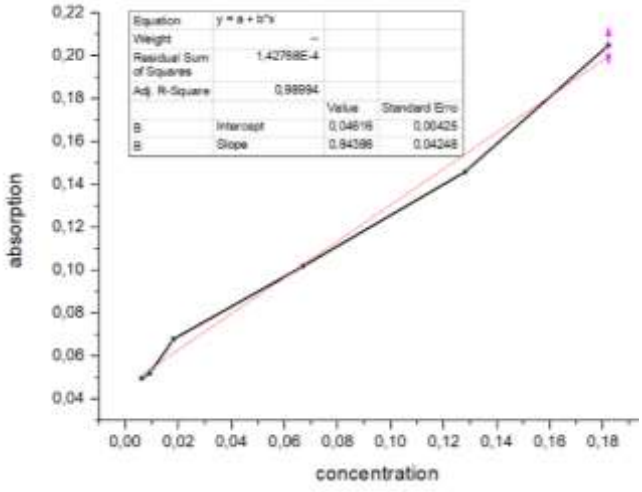

Figure 1: Linear regression curve for different dilutions of C-3-G.

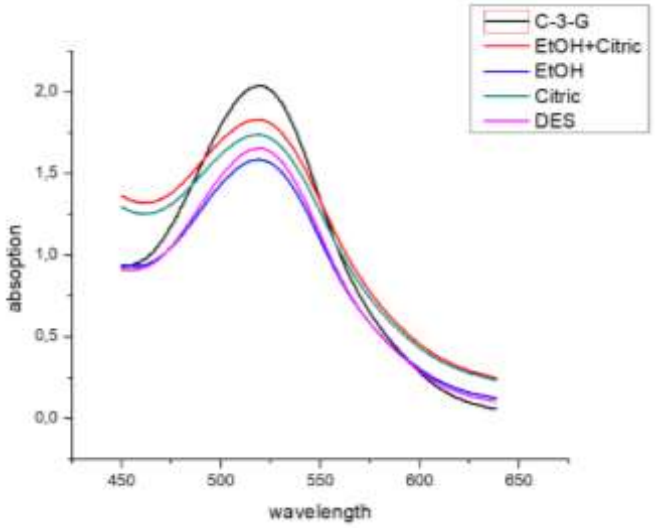

Figure 2. Spectra of anthocyanin-rich extracts obtained in different solvents and C-3-G using a UV-Vis spectrophotometer.

The initially obtained extracts were first cooled to stabilize their chemical composition and prevent potential degradation of thermolabile compounds. Following this, the samples were centrifuged to remove suspended particles and insoluble residues, ensuring clarity and homogeneity. The clarified supernatants were then carefully collected and stored as stock solutions under appropriate conditions until further analysis.

Prior to spectrophotometric measurements, the pH of each extract was adjusted to 4.5 using suitable buffering agents or dilute acid/base solutions. This pH adjustment was performed to standardize the medium and ensure comparability between samples, as anthocyanins are known to exhibit pH-dependent structural transformations that significantly influence their absorbance characteristics.

UV–Vis spectrophotometric analysis was subsequently carried out to evaluate the optical properties of the extracts. Absorbance values were recorded across the wavelength range of 450–650 nm, with measurements taken at 10 nm intervals to obtain detailed spectral profiles. This wavelength region was specifically selected because it encompasses the characteristic absorption maxima of anthocyanins in the visible region.

As illustrated in Figure 2, the maximum absorbance was observed at 520 nm for all extracts, which is consistent with the typical absorption maximum of anthocyanin compounds in mildly acidic conditions. At this wavelength, the absorbance values were determined to be A = 1.839 for the EtOH + citric acid extract, A = 1.741 for the citric acid extract, A = 1.655 for the DES extract, and A = 1.584 for the EtOH extract. These results indicate that the EtOH + citric acid solvent system exhibited the highest extraction efficiency among the tested solvents, as evidenced by its superior absorbance value at the characteristic maximum wavelength. To calculate the anthocyanin yield in all four types of extracts, solutions diluted at four different ratios were prepared, and their absorbance values were recorded and accepted as equivalents. The same solution was diluted at ratios of 1:1, 1:2, 1:3, and 1:4, and the corresponding absorbance values were measured. Using the coefficients obtained from the equation (1) the anthocyanin concentrations

(x) in the extracts at different dilution levels were first determined.

$$x = \frac{y_{(ext)} - b}{a} \qquad (1)$$

Subsequently, considering that the volume of the spectrophotometer cuvette was 3 mL, the total amount of anthocyanins in the extracts was calculated using the formula (2).

$$m = C \times V \qquad (2)$$

Within the framework of this study, the effects of different solvent systems on the extraction efficiency of the target compounds were quantitatively evaluated. As illustrated in Figure 3, the total anthocyanin content varied considerably depending on the chemical nature of the applied solvent and the properties of the extraction medium.

Analysis of the experimental data demonstrated that the highest anthocyanin yield was obtained using the combined ethanol–citric acid solvent system, reaching 11.325 µg. This was followed by the citric acid extract, which contained 10.254 µg of anthocyanins. Deep eutectic solvents (DESs), which have gained increasing attention in recent years the framework of green chemistry principles, enabled the extraction of 9.479 µg of anthocyanins. Among the investigated solvent systems, the lowest anthocyanin content was observed in the pure ethanol extract, with only 8.736 µg determined. The superior performance of the ethanol–citric acid system may be attributed to the synergistic effect between its two components. Anthocyanins are known to exist predominantly in their more stable flavylium cation form under acidic conditions. Citric acid provides an acidic environment that promotes pigment stability, whereas ethanol can enhance cell membrane permeability and facilitate the migration of polyphenolic compounds into the solvent phase. The lower yield obtained with pure ethanol may be associated with the absence of acidification, which can lead to partial degradation of anthocyanins. Although DES showed greater extraction efficiency than pure ethanol, their relatively high viscosity or polarity may have limited mass transfer and prevented them from achieving the selectivity observed in the acidified ethanol system [25] [26], [27]. The extraction efficiency of the investigated solvent systems for anthocyanins decreased in the showing order: EtOH + citric acid>citric acid>DES>EtOH

These findings indicate that the combination of ethanol and citric acid enhances anthocyanin extraction efficiency compared to the individual solvents. The improved performance of the EtOH + citric acid system may be attributed to the synergistic effect of ethanol's solvent capacity and the acidic environment provided by citric acid, which stabilizes the flavylium cation form of anthocyanins and promotes their solubility. In contrast, ethanol alone demonstrated the lowest extraction efficiency, possibly due to the absence of sufficient acidity required for optimal anthocyanin stabilization [28].

Although the DES system showed moderate extraction performance, its efficiency remained lower than that of the acidic solvent systems. This difference may be related to variations in polarity, viscosity, or mass transfer properties of the solvent systems. Overall, the results clearly demonstrate that solvent composition plays a critical role in maximizing anthocyanin yield, with acidified ethanol providing the most favorable extraction conditions among the tested systems.

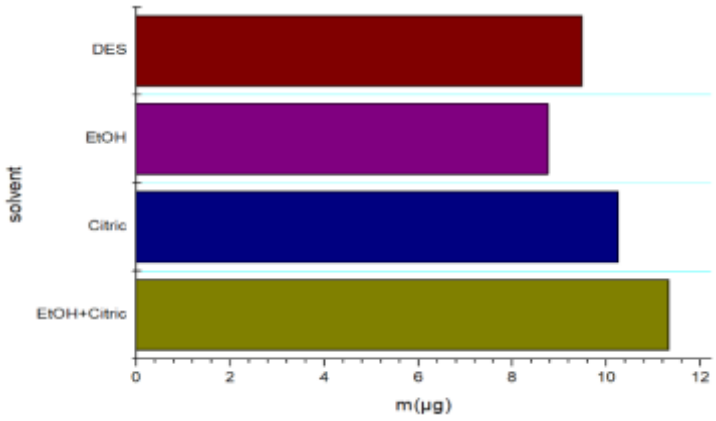

Figure 3. The anthocyanin content of the extracts obtained using different solvents was determined (μg).

Subsequently, the free radical scavenging activity (FRS, %) of the stock solutions was evaluated using an 80 µM DPPH solution with an absorbance of A = 0.911 at 516 nm. For the assay, 2900 µL of DPPH solution was transferred into a 3 mL quartz cuvette, followed by the addition of 100 µL of each stock solution. The reaction kinetics were monitored for 30 minutes, the data were recorded, and the corresponding curves were constructed using Origin 8 software.

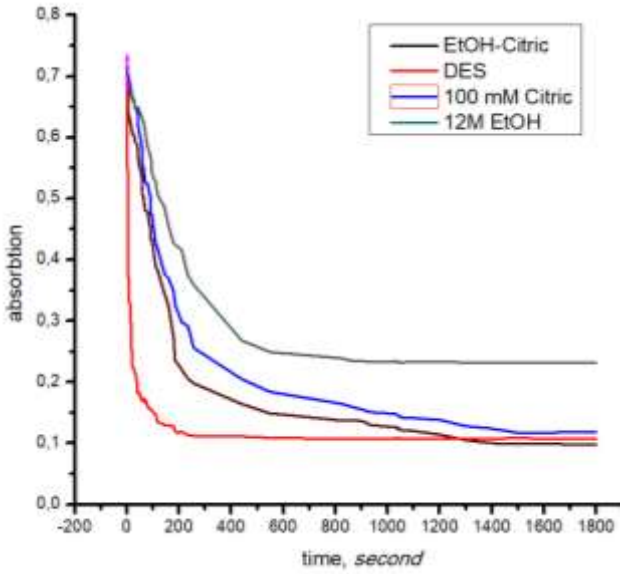

Figure 4. Kinetics of the FRS activity of extracts obtained using different solvents.

As shown in Figure 4, the inhibition process occurred most rapidly in the DES extract, whereas the slowest kinetic behavior was observed in the EtOH extract. Although the DES system exhibited faster reaction kinetics, the highest FRSA value was obtained for the EtOH + citric acid extract, while the lowest antioxidant activity was recorded for the EtOH extract. The comparative order of effectiveness among the extractants was as follows: EtOH + Citric > DES > Citric > EtOH. Interestingly, although the anthocyanin yield in the citric acid

extract was higher than that obtained with DES, the FRSA results showed the opposite trend. This finding may be attributed to the enhanced ability of DES solvents to extract a broader range of bioactive compounds, which collectively contribute to antioxidant activity and the final results are presented in Table 1.

Table 1. Absorbance of extracts after 30 minute and value of FRSA

No	Extract	Absorbance (after 30 min)	FRSA (%)
1	EtOH+Citric	0.098	89.38±0.89
2	Citric	0.124	86.47±0.82
3	DES	0.107	87.99±0.48
4	EtOH	0.231	74.2±0.81

Considering its improved microbial stability, high anthocyanin content, and rich profile of biologically active substances, the bioextract obtained using the binary solvent system appears suitable for application as a natural food additive [29]. Its composition suggests potential benefits for human health, particularly due to the antioxidant properties associated with anthocyanins and related phytochemicals. Although ethanol-citric solution was the best solvent in terms of anthocyanin yield and color, and ethanol-citric solution showed a high percentage in terms of antioxidant activity, DES was more effective over time [30].

Overall, the results clearly demonstrate that the binary extractant system is more efficient and productive than the individual solvents tested [31]. The synergistic effect of citric acid and ethanol enhances both extraction efficiency and storage stability.

4- CONCLUSION

The experimental findings of this study demonstrate that the combination of ethanol and citric acid creates a synergistic effect that significantly enhances anthocyanin extraction compared to individual solvents. This improved performance is attributed to the acidic conditions provided by citric acid, which are essential for stabilizing the flavylium cation form of anthocyanins and maximizing extraction yield. Among the four solvent systems evaluated, the ethanol-citric acid binary mixture proved most effective, yielding the highest anthocyanin content and strongest antioxidant activity. Interestingly, the DES-like ternary system (DMSO: EtOH: Citric) showed rapid antioxidant kinetics despite moderate anthocyanin content, indicating co-extraction of other bioactive compounds beyond anthocyanins. From a sustainability perspective, utilizing post-vegetative autumn leaves transforms edible plant residues into high-value functional ingredients, aligning with circular economy principles and avoiding overexploitation of medicinal plants. The ethanol-citric acid bioextract, characterized by high anthocyanin content and strong antioxidant activity, is suitable for application as a natural food colorant and functional additive, offering a viable pathway for developing safe and eco-friendly products for the food industry. It enables the potential application of the extract as a radioprotective dietary supplement for individuals exposed to ionizing radiation, including patients undergoing radiotherapy and individuals exposed to radiation as a result of radiological contamination [32].

Ethical Approval

This study did not involve human participants or animals. All experimental procedures were conducted in accordance with institutional and laboratory biosafety guidelines.

Conflict of Interest

The authors declare that they have no conflict of interest.

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This research received no external funding.

Data Availability

All data supporting the findings of this study are available within the manuscript.

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