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Research Article

An insight into the biochemical content of *Colchicum autumnale* L. throughout its ontogenetic cycle

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Abstract

Meadow saffron (*Colchicum autumnale* L.) is a poisonous perennial species with an unusual ontogenetic cycle. This study investigated its biochemical composition during three key developmental phases: growth, fruiting, and flowering. Aqueous and alcoholic extracts from different plant organs were analyzed using UPLC and spectrophotometry to evaluate the influence of developmental stage, extraction method, and solvent on secondary metabolite accumulation, with implications for extract potency and pharmacological properties. Colchicine and phenolic compounds were detected in all organs, though their concentrations varied with extraction technique and harvest time. Flowers contained the highest colchicine levels (0.7815 mg ml^{-1} in ethanol, 0.1227 mg ml^{-1} in water), while bulbs accumulated lower amounts that decreased from spring to autumn. In general, alcoholic extracts yielded more bioactive compounds than aqueous ones. Flowers and leaves also contained significant levels of polyphenols, with ethanol extracts showing markedly higher concentrations than water extracts. Antioxidant assays revealed strong activity in all alcoholic extracts (79.8 - 90.7 mg ml^{-1} eq. ascorbic acid), except for spring bulbs (57.2 mg ml^{-1}), whereas aqueous extracts exhibited negligible antioxidant capacity (0.07 - 0.3 mg ml^{-1}). These results highlight the dynamic phytochemistry of *C. autumnale*, demonstrating that the concentration of bioactive compounds is strongly influenced by plant organ, developmental stage, extraction method, and solvent. Such variability should be considered in future research and practical applications, including ecological studies, weed management, and standardization of extracts for medicinal use.

Keywords: antioxidant activity; colchicine; flavonoids; polyphenols; Soxhlet

Introduction

Colchicum autumnale, a member of the Colchicaceae family, is a toxic bulbous perennial plant typically found in mountain grasslands (Pop *et al*., 1983). This species thrives in various damp grassland environments, from hilly to mountainous regions, and it is widely distributed, particularly in Europe, being considered abundant (Chadburn, 2014).

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The plant features a dense tuber that produces an axillary bud, which gives rise to one to three violet flowers in autumn (Ștefan and Ivănescu, 2002). The leaves are basal and originate from the primordia of the underground bulb disc. The flower consists of a perigone with six pinkish-purple tepals, six stamens, and an extended perigone tube that forms on the bulb disc alongside the ovary, shielded by bulb cataphylls (Pop *et al.*, 1983). In spring, the fruit emerges aboveground and is encircled by 3-4 oblong-lanceolate leaves (Ștefan and Ivănescu, 2002). The fruit is a trilocular capsule containing colchicine-rich seeds (Pop *et al.*, 1983).

The plant's distinctive ontogenetic cycle begins with autumn flowering, followed by a dormant winter period during which the fruit remains underground. Spring marks the peak of photosynthetic activity, with fruits and leaves appearing aboveground. Plants enter a period of inactivity after fruit ripening (Jung *et al.*, 2011) (Figure 1).

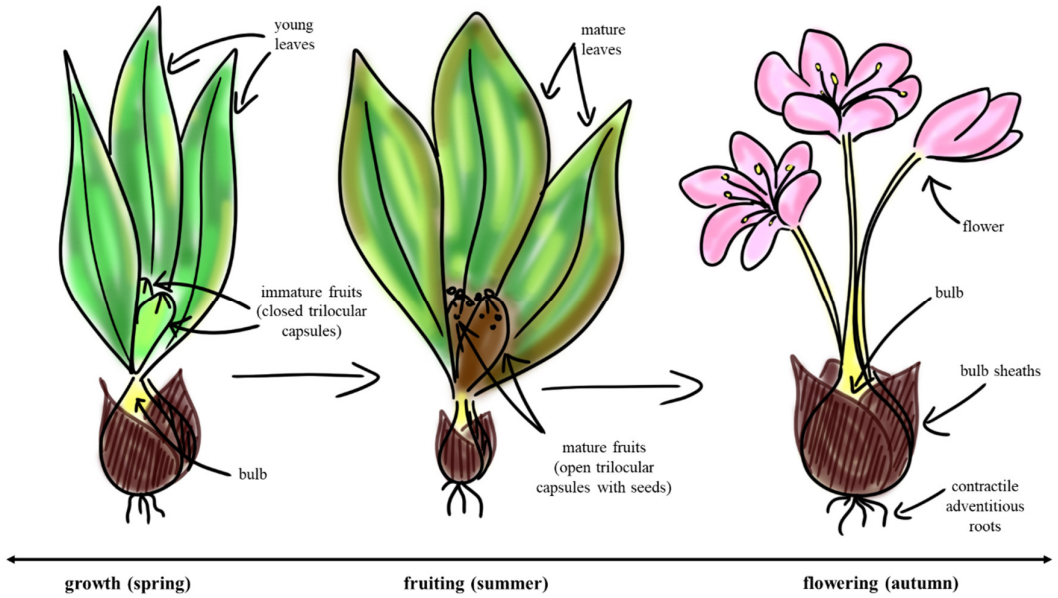

Figure 1. Schematic representation of *Colchicum autumnale* plants' ontogenetic cycle

Research on *C. autumnale* spans pharmacology, ecology, and agriculture; however, several important gaps remain. Recent studies have highlighted organ-specific and environment-dependent variations in metabolite content (Boboev *et al.*, 2023; Dincheva *et al.*, 2025). However, systematic studies on how environmental factors, developmental stages, and plant parts influence the phytochemical spectrum are lacking. Although optimized methods for colchicine extraction exist, there is limited research on efficient and standardized extraction and quantification protocols for phytochemicals in *C. autumnale* (Çankaya *et al.*, 2018).

According to Burzo *et al.* (2005), aboveground organs contain starch, sucrose, lipids, phytosterols, benzoic acid, salicylic acid, alkaloids, and tannins. The alkaloids present in underground organs are colchicine, 2-demethylcolchicine, colchicoside, 2-desmethyldeacetylcolchicine, demecolcine, thiocolchicoside, 3-demethylcolchicine, 3-methylcolchicine, 3-demethyl-β-lumicolchicine, α-lumicolchicine, β-lumicolchicine, γ-lumicolchicine, colcamine, colchicene, colchicerine, N-methyldesacetylcolchicine, N-deacetyl-N-methylcolchicine, N-formyl-deacetylcolchicine, and O-demethyl-N-deacetylcolchicine. Aerial organs contain salicylic acid, chelidonic acid, and alkaloids. The alkaloids found in the above-ground organs of plants belonging to this species are 2-acetyl-2-demethylcolchicine, 2-acetyl-3-methylcolchicine, colchifoline, demecolceine, demecolcine, N-acetyl-demecolcine, and O-acetylcolchiceine and apigenin.

Colchicine ($C_{22}H_{25}O_6N$, N-[(7S)-1,2,3,10-tetramethoxy-9-oxo-6,7-dihydro-5H-benzo[a]heptalen-7-yl] acetamide), the primary bioactive component extracted from *C. autumnale*, is an alkaloid that inhibits mitosis by attaching to tubulin and preventing its polymerization, resulting in autopolyploidization. Utilizing the antimitotic property of colchicine, researchers have developed plants with enhanced traits (compared to their diploid counterparts) for commercial applications (Manzoor *et al*., 2019). Colchicine has been widely used in the medical field to address a range of ailments, such as gout, familial Mediterranean fever, skin vasculitis, and Paget's and Behçet's diseases (Nerlekar *et al*., 2014). Recently, colchicine has garnered attention in clinical trials for the treatment of pericarditis (Shah *et al*., 2016), cancer (Zhang *et al*., 2019), and COVID-19 (Lopes *et al*., 2021).

In addition to colchicine and its derivative compounds, *C. autumnale* contains other secondary metabolites that confer protection throughout its ontogenetic cycle. Polyphenols are important biochemical compounds in plants that are involved in the oxidative stability of different parts of the plant and in chemical defense mechanisms, such as allelopathy (Li *et al*., 2014; Singh *et al*., 2021; Zagoskina *et al*., 2023). They function by preventing or inhibiting the generation of reactive oxygen species (ROS) (Agati and Tattini, 2010; Singh *et al*., 2021; Dini and Grumetto, 2022; Zagoskina *et al*., 2023). Polyphenols contribute to the thickening of the secondary cell wall, conferring mechanical resistance and rigidity that prevent the destruction of healthy tissues in the vicinity of the affected tissues (Gunnaiah *et al*., 2012; Singh *et al*., 2021), and play a role in promoting tissue sclerification (Di Ferdinando *et al.*, 2014). Moreover, they modulate plant growth, development, and signaling pathways, influencing processes such as cell division and hormone activity (Dini and Grumetto, 2022; Zagoskina *et al*., 2023).

Flavonoids are polyphenols that play crucial roles in seed germination, plant growth, and development (Wang *et al*., 2022; Zhuang *et al*., 2023). These compounds protect plants against biotic and abiotic stress (Shomali *et al*., 2022; Zhuang *et al*., 2023), serve as significant signaling molecules (Mathesius, 2018; Shah and Smith, 2020; Wang *et al*., 2022; Kumar *et al*., 2024), and function as allelopathic compounds (Mathesius, 2018; Shah and Smith, 2020; Zhuang *et al*., 2023), phytoalexins (Mathesius, 2018; Wang *et al.*, 2022; Zhuang *et al*., 2023), detoxifying (Dias *et al*., 2021; Zhuang *et al*., 2023), and antimicrobial agents (Pollastri and Tattini, 2011; Agati *et al*., 2012; Shah and Smith, 2020; Wang *et al*., 2022). Flavonoids interact with membrane phospholipids, thereby protecting chloroplast membranes against photooxidation (Agati *et al*., 2013; Ferreyra *et al*., 2021; Laoué *et al*., 2022) and inhibiting singlet oxygen ($^1O_2$) (Agati *et al*., 2007). Furthermore, flavonoids can facilitate the morpho-anatomical adaptation of plants to stress conditions (Agati and Tattini, 2010; Agati *et al*., 2013; Buer *et al*., 2013; Shah and Smith, 2020; Shomali *et al*., 2022; Zhuang *et al*., 2023).

Studying and comparing alcoholic and aqueous extracts of *C. autumnale* organs at different ontogenetic (developmental) stages is important because the extraction method, plant organ, and developmental stage significantly influence the chemical composition and biological activity of the extracts. Alcoholic (ethanolic) and aqueous extracts yield different profiles of bioactive compounds. For example, ethanolic extracts of *C. autumnale* leaves showed much higher acaricidal activity than aqueous extracts, indicating that ethanol extracts contain more potent or different active compounds from the leaves than water (Norouzi *et al*., 2020). The concentration and types of alkaloids and other bioactive compounds in *C. autumnale* vary significantly depending on the plant developmental stage. Although studies have focused on the organ and solvent, it is well established in phytochemistry that the ontogenetic stage impacts secondary metabolite content, which in turn affects extract potency and pharmacological properties (Ellington *et al*., 2003).

This study aimed to perform a comparative analysis of the aqueous and alcoholic extracts of *C. autumnale*, focusing on their phytochemical composition and antioxidant potential. Specifically, the objectives were to quantify and compare the contents of colchicine, total polyphenols, and flavonoids in aqueous and alcoholic extracts; evaluate and compare the antioxidant activity of the extracts and examine its correlation with polyphenol and flavonoid levels; investigate the distribution of these bioactive compounds across different

plant organs; assess the influence of ontogenetic stage on colchicine accumulation, phenol content, and antioxidant potential; and identify the extraction method, plant organ, and developmental stage that maximizes the yield of pharmacologically relevant compounds, providing insight into potential applications in medicine and phytotherapy. An analysis of the biochemical composition of a plant species may also provide insights into the mechanisms utilized to adapt to stress induced by interspecific competition, herbivores, pathogens, pests, and adverse environmental conditions. By comparing extracts from different organs and developmental stages, researchers can identify the optimal combination for maximum yield of desired compounds. Understanding these differences will facilitate the development of more effective medicines and biopesticides.

Materials and Methods

Plant material collection

The plant material comprising bulbs, leaves, and immature fruits (Figures 2 and 4) of *C. autumnale* was collected on 15.05.2021 (with the voucher specimen deposited in the Herbarium of the Faculty of Biology of the "Alexandru Ioan Cuza" University in Iasi, I186.542), which corresponds to the active growth period in the ontogenetic cycle of this species. Leaves and mature fruits (Figure 3) were obtained during the fruiting period, on 8.07.2021 (I186.555), and bulbs and flowers (Figure 5) were collected on 5.11.2021, during the flowering period (I186.556) from a meadow in Voroneț, Suceava County (Figure 6A and 6B). Plant material from 32 individual plants was collected at each ontogenetic stage (96 plants in total). The coordinates were recorded using GPSEssentials: latitude 47.493889° longitude 25.885833° and imported into the NASA Worldview Map (*https://worldview.earthdata.nasa.gov*) (Figure 7).

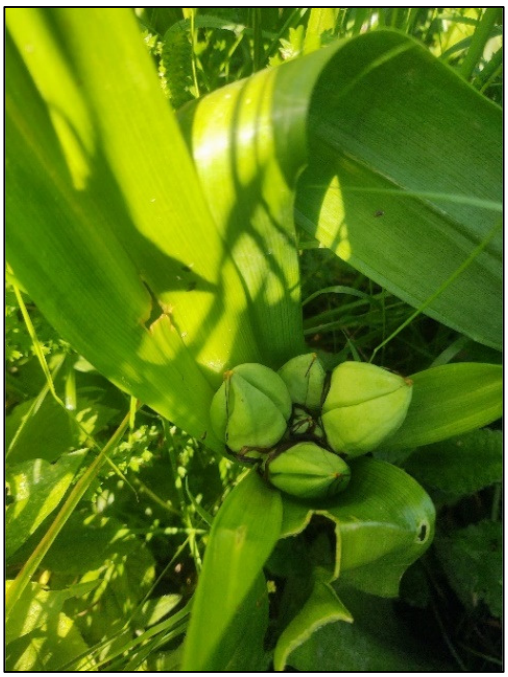

Figure 2. Immature *C. autumnale* fruits (closed trilocular capsule), photographed and collected on 15.05.2021

Figure 3. Mature *C. autumnale* fruits (open trilocular capsule with seeds), photographed and collected on 8.07.2021

Figure 4. Young *C. autumnale* plants with leaves, immature fruits, bulbs, and roots, collected on 15.05.2021

Figure 5. *C. autumnale* plants, showing only flowers, bulbs, and roots, collected during the flowering stage, on 5.11.2021

Soil samples were collected from the plant material harvest point, dried, and ground into fine powder. Five grams of soil powder was weighed and dissolved in 12.5 mL of distilled water (1:2.5 m/V). The solution thus obtained was analyzed using a pH meter, according to the method described by Blakemore *et al*. (1987), recommended by the Food and Drug Administration (FDA), and the results were confirmed by comparison with those obtained using pH indicator paper.

Data on the average temperature and relative humidity were obtained from the NASA POWER Daily API (*https://power.larc.nasa.gov*). The soil type was identified according to the World Reference Base (2006) Soil Groups (*https://soilgrids.org*) for the plant material collection point.

(A) **(B)**

Figure 6. The habitat from which the individuals of *C. autumnale* were collected on 15.05.2021 (A) and in 5.11.2021 (B)

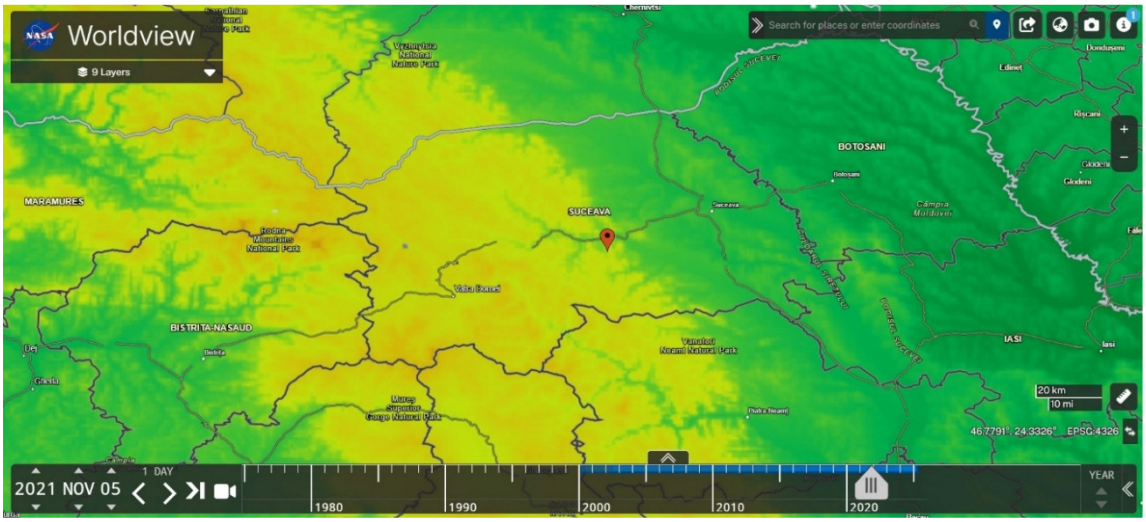

Figure 7. *C. autumnale* individuals harvest point (latitude 47.493889° longitude 25.885833°) (*https://worldview.earthdata.nasa.gov*)

Plant material preparation

The collected plant material was subjected to oven treatment at 65 °C for 12 h to inhibit enzymatic reactions, followed by desiccation in the dark at ambient temperature (23 ± 2 °C) for 7 days (Figure 8A and 8B).

(A) **(B)**

Figure 8. Dried plant parts of *C. autumnale*: leaves and fruits (A); bulb sheaths, bulb fragments, and flowers (B)

Extract preparation

Methanol and ethanol are the most effective solvents for extracting colchicine from *Colchicum autumnale*, with methanol generally yielding the highest extraction efficiency, followed closely by ethanol. In general, aqueous and ethanol extracts are also used for broader phytochemical and biological activity studies, however, for maximum colchicine yield, methanol and ethanol are preferred (Çankaya *et al*., 2018; Norouzi *et al*., 2020)

Alcoholic extracts were prepared using a Soxhlet apparatus by extracting 5 g of plant material from each organ in absolute methanol, which was recirculated for 8-10 hours (method adapted from Rocchetti *et al.*, (2019). The solvent was evaporated to dryness using a rotary evaporator (IKA RV3, Staufen, Germany), and the dry extract was re-dissolved in 50 mL of 70% ethanol.

Aqueous extracts were prepared by infusing 5 g of plant material from each collected organ in distilled water (Figure 9) for 24 h on a shaker at 25 °C, followed by filtration through filter paper. The solvent was evaporated to dryness using a lyophilizer (Christ Alpha 3-4, Osterode am Harz, Germany), and each dry extract was re-dissolved in 50 mL of distilled water.

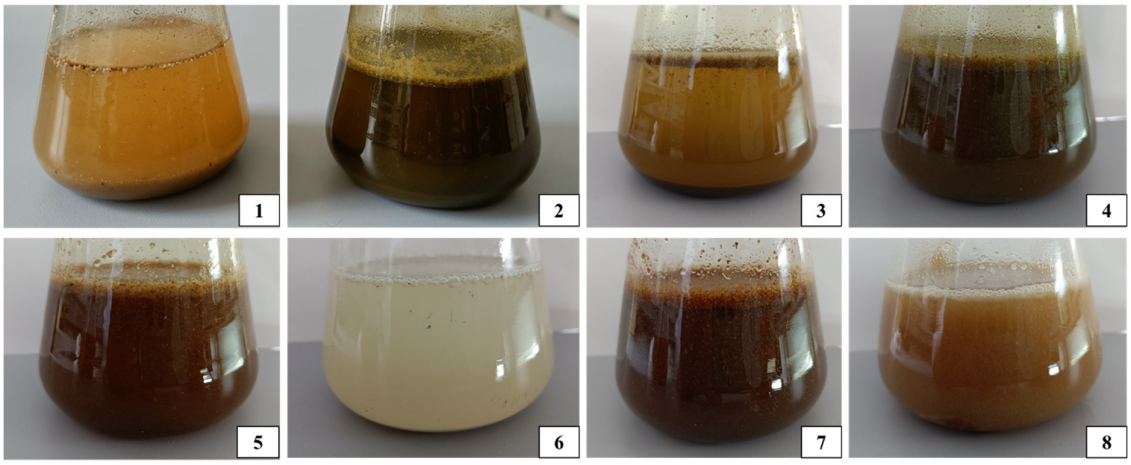

Figure 9. Aqueous extracts from *C. autumnale* organs before filtration
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract

Finally, eight alcoholic and eight aqueous extracts were obtained from the same mass of plant organ powder (5 g) and concentrated using 50 mL of solvent. The extracts were annotated as follows: 1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract.

Extract content analysis

The main bioactive compounds from the obtained extracts were identified and quantified through reversed-phase ultra-performance liquid chromatography (RP-UPLC), using a Shimadzu Prominence UPLC system (2 LC20AD pumps, SIL20AC autosampler, oven CT20AC, SPD M20A DAD detector, RF 20A XS florescence detector) coupled to a Zorbax Eclipse XDB - C18 column (length 250 mm, particle size 3 microns). Colchicine, colchicine, demecolcine, apigenin (purity ≥ 95%), and salicylic acid (purity ≥ 99%) were purchased from Sigma Aldrich (sigmaaldrich.com) and used for the construction of calibration curves in the range of 0.312-2.5 μg ml^{-1} for colchicine, 0.025 - 1.6 μg ml^{-1} for colchiceine, 0.05-0.5 μg ml^{-1} for demecolcine, 0.025 - 2 μg ml^{-1} for apigenin, and 0.04 - 4 μg ml^{-1} for salicylic acid.

For the detection of colchicine, colchicine, demecolcine, and apigenin, acetonitrile was used as mobile phase A, and 3% acetic acid was used as mobile phase B (Sigma Aldrich, Germany), according to the method used by Alali *et al.* (2004), which has also been used in previous studies (Moroșan *et al.*, 2022). Elution was performed at a flow rate of 1 ml/min using the following program: 0-3 min 90% B isocratic, 3-11 min 90-40% B gradient, 11-12 min 40% B isocratic, 12-13 min gradient 40-90% B, 13-20 min 90% B isocratic.

For the detection of salicylic acid, the method of Toiu *et al.* (2011) was adapted, using 0.85% orthophosphoric acid as mobile phase A and acetonitrile as mobile phase B. Elution was performed at 1 ml/min

using the program: 0-2 min 5% B isocratic, 2-5 min 5-80% B gradient, 5-10 min 80-100% B gradient, 10-12 min 100% B isocratic, 12-18 min 5% B isocratic.

Colchicine (Figure 10), colchiceine (Figure 11), and apigenin (Figure 12) were detected at 245, 254, and 350 nm, respectively, and eluted at 13.4 ± 0.08 min. Demecolcine was not detected in any of the extracts. Salicylic acid (Figure 13) was detected using a fluorescence detector at excitation and emission wavelengths of 310 and 450 nm, respectively. Chromatographic data were acquired using Shimadzu LC Software and interpreted manually by comparing the retention times and detection spectra of the standard compounds with those of the samples (extracts).

A volume of 20 µL was injected to detect compounds in standard solutions and extract samples for five technical replicates. The concentrations of the compounds in the extracts were quantified according to the area of the detected peaks and calculated according to the standard curves (Figure 10, 11, 12 and 13).

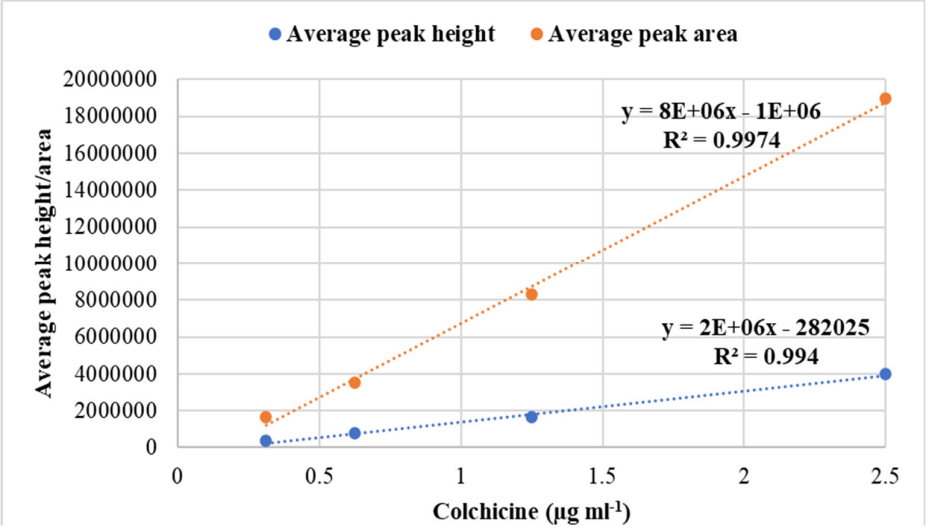

Figure 10. Standard curve used for the quantification of colchicine (µg ml^{-1}) content in *C. autumnale* extracts

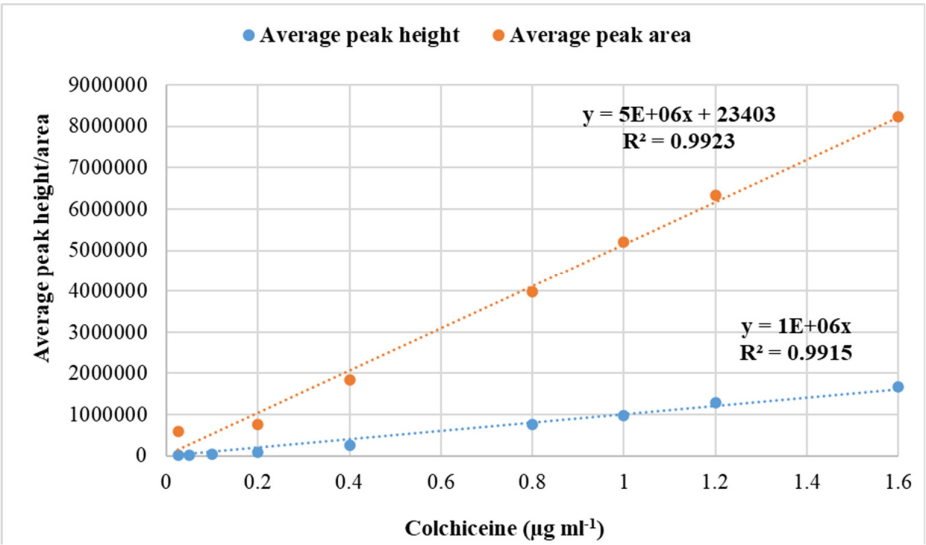

Figure 11. Standard curve used for the quantification of colchiceine (µg ml^{-1}) content in *C. autumnale* extracts

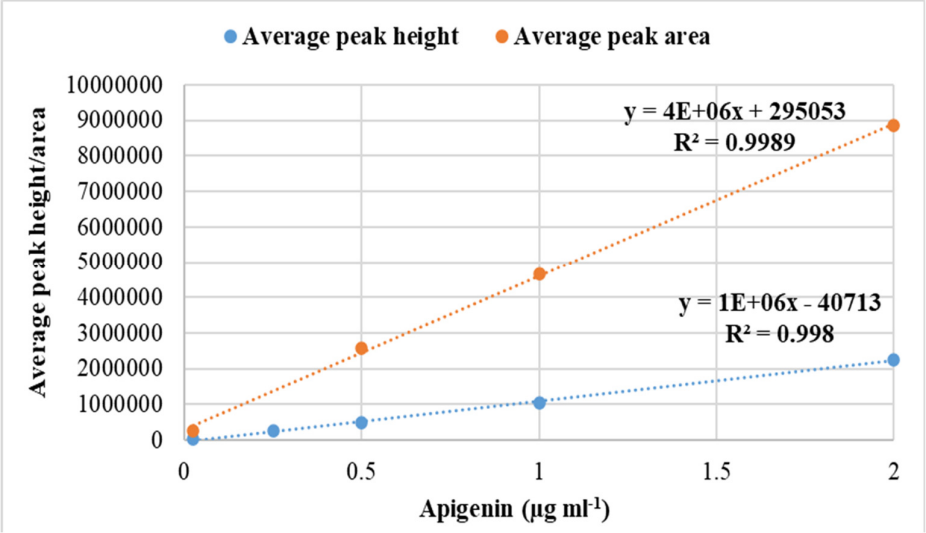

Figure 12. Standard curve used for the quantification of apigenin (µg ml^{-1}) content in *C. autumnale* extracts

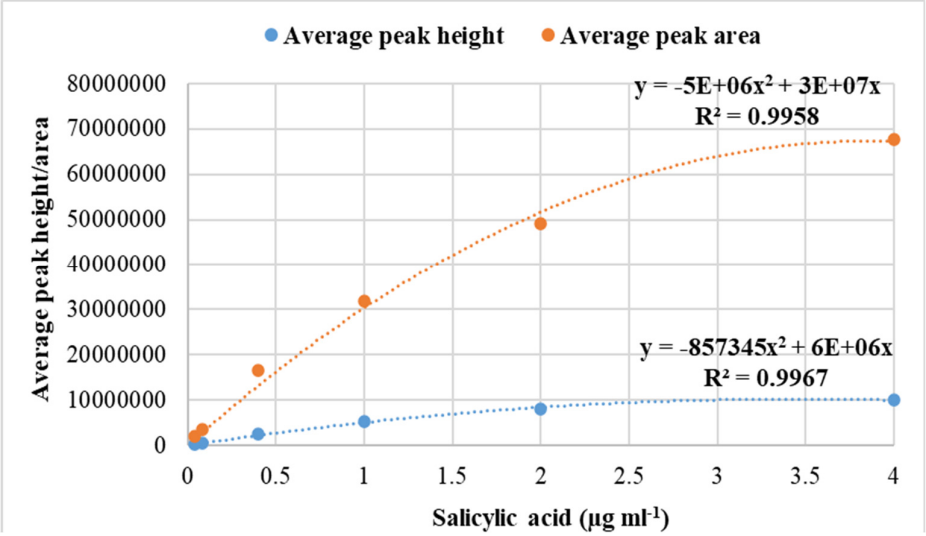

Figure 13. Standard curve used for the quantification of salicylic acid (µg ml^{-1}) content in *C. autumnale* extracts

The total polyphenol content was determined using the conventional method described by Singleton and Rossi (1965) in Herald *et al.* (2012). The standard curve was obtained by preparing standard solutions using gallic acid at concentrations of 0-400 µg ml^{-1}. The flavonoid content was determined using the conventional colorimetric method based on aluminum chloride, according to Herald *et al.* (2012), adapted from Zhishen *et al.* (1999). To determine the standard curve, standard solutions were prepared using quercetin at concentrations of 0-500 µg ml^{-1}. The concentrations of polyphenols and flavonoids in the extracts were calculated using the standard curve formula by calculating the mean values obtained by analyzing 3 biological replicates (n = 3) (Figure 14 and 15).

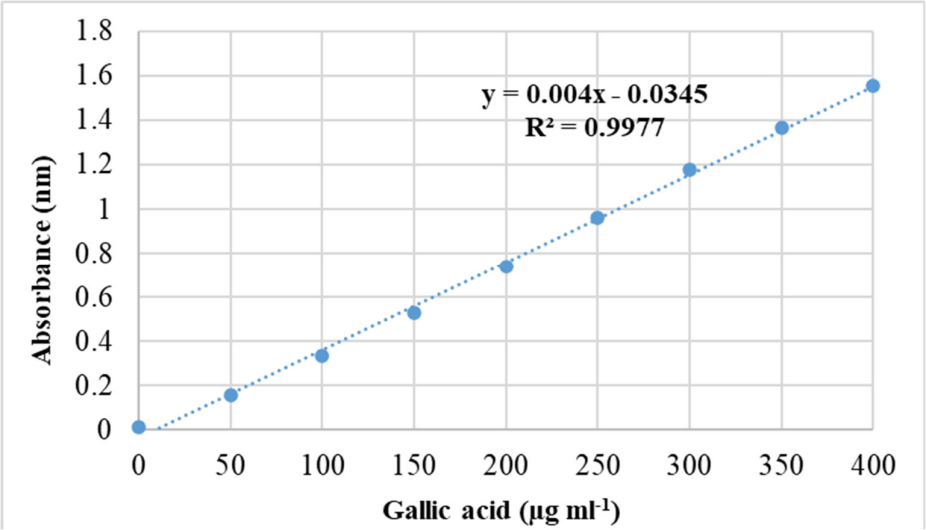

Figure 14. Standard curve used for the quantification of polyphenols (µg eq. gallic acid ml^{-1}) content in *C. autumnale* extracts

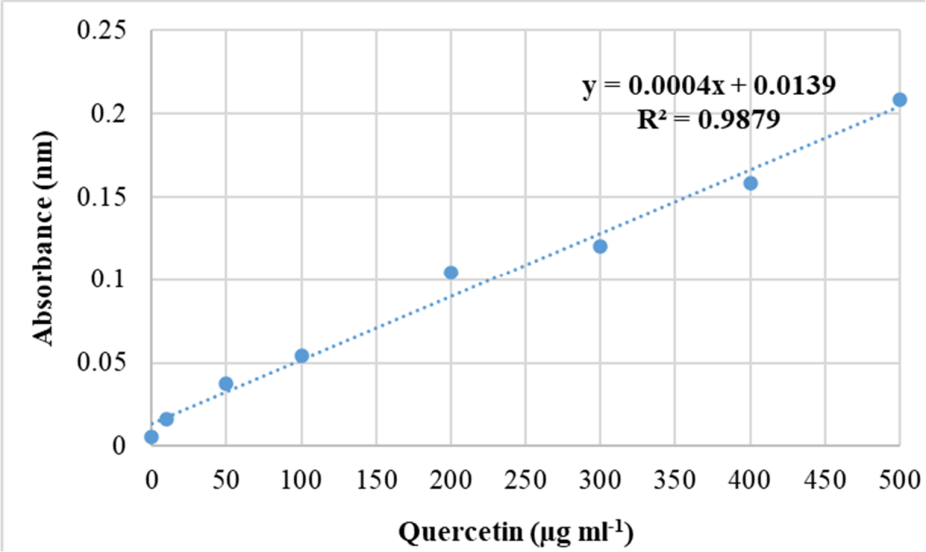

Figure 15. Standard curve used for the quantification of flavonoids (µg eq. quercetin ml^{-1}) content in *C. autumnale* extracts

The antioxidant activity of the *C. autumnale* extracts (n = 3) was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method described by Herald *et al.* (2012), Thaipong *et al.* (2006), and Brand-Williams *et al.* (1995). To obtain a standard curve, standard solutions were prepared using ascorbic acid at concentrations of 0-200 µg ml^{-1}. Antioxidant activity was expressed as equivalent mg ascorbic acid per gram of fresh plant material, calculated according to the standard curve formula (Figure 16).

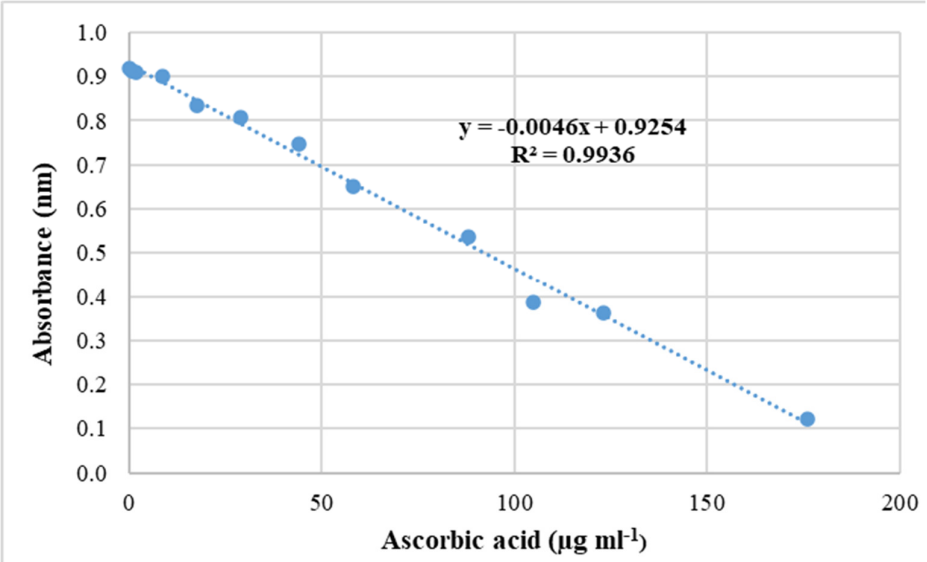

Figure 16. Standard curve used for the quantification of antioxidant activity (µg eq. ascorbic acid ml^{-1}) of *C. autumnale* extracts

Statistical analysis

For the interpretation of the results obtained from the biochemical analyses of the extracts, the two-way ANOVA statistical test, Tukey test for multiple comparisons (post-hoc), and Pearson's correlation coefficient were applied using GraphPad Prism 9.5.1 software. For UPLC analysis, the Relative Standard Deviation (%RSD), Limit of Detection (LOD), and Limit of Quantification (LOQ) are presented in Table 1.

Table 1. Relative Standard Deviation (%RSD), Limit of Detection (LOD), Limit of Quantification (LOQ) values for colchicine, colchiceine, apigenin and salicylic acid detected through UPLC

Parameters	Colchicine		Colchiceine		Apigenin		Salicylic acid	
	Peak height	Peak area	Peak height	Peak area	Peak height	Peak area	Peak height	Peak area
%RSD	1.047501	2.874768	1.406537	1.656845	2.449800	2.384739	1.546535	2.759667
LOD	0.000002	0.000000	0.000003	0.000001	0.000001	0.000000	0.000001	0.000000
LOQ	0.000006	0.000001	0.000009	0.000002	0.000003	0.000001	0.000004	0.000001

The values presented in the graphs and tables represent the mean ± standard error of the mean. Significant differences are marked in the graphs and tables with different letters ($p < 0.05$). Pearson's correlation coefficient was determined by comparing the biochemical analysis results of the alcoholic extracts with those of the aqueous extracts and by comparing the antioxidant activity of the extracts against the polyphenol and flavonoid content.

Results and Discussions

Influence of environmental conditions on secondary metabolites synthesis

Soil pH indirectly affects plants through nutrient availability, microbial activity, and metal solubility. Most plants thrive in slightly acidic to neutral soils (pH 6-7), where essential nutrients are most accessible and toxic metals are less soluble. At low pH, some nutrients become less available, whereas toxic metals can increase (Mróz, 2011).

According to the World Reference Base (2006) Soil Groups (*https://soilgrids.org*), the soil at the plant collection point is Cambisol, which usually contains moderate organic matter, sand, silt, and clay, is rich in essential nutrients, has good water retention, and has a slightly acidic to neutral pH (Twaij and Hasan, 2022).

Soil pH analysis at the collection site indicated that *C. autumnale* plants grow indicated an acidic pH of 5.8. *C. autumnale* grows ideally in light, nutritious, well-drained soils with a pH around 6. Poorly drained or highly acidic/alkaline soils can reduce plant size, flowering duration, and corm productivity, whereas neutral pH soils promote better vegetative growth, flowering, and seed production (Kaysarov and Akhmedov, 2021). Soil pH influences the uptake of nutrients in *C. autumnale* plants, which may explain the differences in plant performance and colchicine content in corms and other plant organs (Mróz, 2011).

The annual average temperature at the plant material collection point in 2021 was 6.3 °C and the annual relative humidity was 81.5%. The average temperature in spring was 4.24 °C, and the relative humidity was 82.73%; in summer, the temperature rose to an average of 17.54 °C, and the %RH (relative humidity) lowered to 75.73%; and during autumn, the average temperature was 6.77 °C, while %RH remained approximately constant at 75.52%. During winter (before the collection of plant material), the average temperature was -3.33 °C, with %RH of 90.9%. *C. autumnale* is adapted to temperate climates and can initiate growth at minimum positive air temperatures of 3-5 °C, with the growing season beginning shortly after snowmelt. High relative humidity (e.g., 81.5%) helps maintain soil moisture, which is beneficial for growth, improves plant vigor, and prolongs flowering duration. Seasonal and environmental factors, including temperature and humidity, influence the biosynthesis of secondary metabolites, with higher phenolic and flavonoid contents often linked to stress conditions (Davoodi *et al*., 2021).

Importance of choosing the right extraction methods and solvents

Soxhlet extraction is generally more effective than infusion and other conventional methods for extracting colchicine and other compounds from *C. autumnale* plants; however, advanced modern techniques may offer even higher yields and efficiency. In general, the optimal extraction method depends on the specific plant material, target compounds, and intended application (Wang and Weller, 2006; Danlami *et al*., 2014; Jibhkate *et al*., 2023). Conventional extraction methods include maceration, reflux, and Soxhlet extraction using solvents of varying polarities, such as methanol, ethanol, chloroform, and acetone. According to Abidin *et al*. (2015), Soxhlet extraction with methanol provides the highest colchicine yield (3.49% w/w from seeds), followed by reflux and maceration, with methanol being the most effective solvent.

Advanced extraction methods, such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and homogenizer-assisted extraction (HAE), generally achieve higher extraction yields and shorter processing times than conventional methods, such as Soxhlet and infusion (Chuo *et al*., 2020; Jha and Sit, 2021). For example, optimized UAE conditions (ultrasonication power: 602.4 W, time: 42 min, temperature: 64 °C) yielded 0.238% colchicine from bulbs (Çankaya *et al*., 2018), whereas SFE achieved >98% recovery in 110 min and matched the yield of conventional methods (Ellington *et al*., 2003). In addition, ultrasonication-based extraction (UBE) outperforms traditional extraction methods in terms of colchicine content and efficiency (Alqarni *et al*., 2022). The advantages of advanced extraction methods include shorter extraction times, lower temperatures, reduced solvent use, and eco-friendliness compared to conventional methods (Ellington *et al*., 2003; Çankaya *et al*., 2018; Alqarni *et al*., 2022).

However, these methods may not always be accessible in all settings. Soxhlet extraction can provide high yields of certain compounds, such as polyphenols, flavonoids, and antioxidants, and is sometimes superior to maceration or UAE for specific plant materials (Putra *et al*., 2022; Mokaizh *et al*., 2024). Infusion and decoction are simple, water-based methods suitable for extracting polar, water-soluble compounds; but they are less efficient for non-polar or less soluble bioactives and generally yield less extract than Soxhlet or advanced techniques (Abubakar and Haque, 2020).

The study of Abidin *et al*. (2015) that compares extraction methods for *Colchicum autumnale* seeds found that Soxhlet extraction produced the highest colchicine yield, especially when using methanol as the solvent, outperforming both the maceration and reflux methods. For related species (*Colchicum triphyllum*), both Soxhlet and infusion methods effectively extract alkaloids and polyphenols; however, Soxhlet extraction with methanol often yields higher concentrations of bioactive compounds, including colchicine derivatives. Infusion is effective for some phenolics and alkaloids, but generally yields less than Soxhlet extraction (Senizza *et al*., 2020).

Soxhlet extraction is a reliable and accessible method for extracting colchicine and polyphenols from *Colchicum autumnale*, offering high yields of these compounds, but it has both notable advantages and limitations. The positive aspects of Soxhlet extraction include high extraction efficiency, accessibility, and reproducibility. Soxhlet extraction yielded higher amounts of colchicine than maceration and was comparable to or slightly better than reflux extraction, especially when methanol was used as a solvent. This method is straightforward, does not require advanced equipment, and is suitable for settings lacking modern extraction technologies. Soxhlet extraction provides consistent results because of its continuous solvent cycling, ensuring the thorough extraction of target compounds. The disadvantages of Soxhlet extraction include high solvent and energy consumption, thermal degradation risks, and long extraction time (Abidin *et al*., 2015; Sridhar *et al*., 2021).

Recent research highlights that ultrasound-assisted extraction (UAE), especially when combined with deep eutectic solvents (DES), is one of the most effective and sustainable methods for extracting polyphenols from plant matrices. These approaches outperform conventional methods (such as maceration or Soxhlet extraction) in terms of yield, selectivity, reduced solvent use, and lower energy consumption (Liu *et al*., 2022; Wang *et al*., 2023; Aktaş and Kurek, 2024; Palos-Hernández *et al*., 2024; Szopa *et al*., 2024), yet Soxhlet extraction may provide a reliable extraction method when these advanced techniques are not available.

Biochemical content of C. autumnale in different ontogenetic stages

Regarding the biochemical content of meadow saffron plants, according to Wildman and Pursey (1960), all organs of *C. autumnale* accumulate colchicine, which is accompanied by several alkaloids in small amounts. Colchicine and other alkaloids are abundant in the endosperm, cotyledons, seeds, perisperm, testa, and fused pericarp (Wildman and Pursey, 1960). Preliminary phytochemical studies conducted by Davoodi *et al*. (2021) on *C. autumnale* reported the presence of alkaloids, phenolic compounds, tannins, flavonoids, coumarins, saponins, terpenoids, steroids, and glycosides in alcoholic extracts. The total tropolone alkaloid content was 9.8 ± 0.3 mg of colchicine per gram of plant material (bulbs), the polyphenol content was 5.6 ± 0.4 mg of gallic acid per gram of plant material, and the flavonoid content was 3.7 ± 0.4 g of quercetin per gram of plant material, using methanol and water at an 80:20 ratio as the extraction solvent. The total colchicine content was 4.4592 ± 0.0109 mg per gram of plant material represented by the bulbs (Davoodi *et al*., 2021).

In the present study, the colchicine content in bulbs was 1.328 ± 0.003 mg per gram of plant material collected during the growth period (spring) and 0.617 ± 0.002 mg per gram of bulbs collected during the flowering period (autumn), using a 70:30 ratio of ethanol to water. By comparing with the results obtained by Davoodi *et al*. (2021), methanol may be a more suitable solvent for colchicine, and this compound has a better solubility at higher methanol concentrations. When comparing the results of various studies, it is essential to consider several factors, including the environmental conditions experienced by *C. autumnale* plants, the neighboring plant species that compete with meadow saffron for nutritional resources, the availability of soil nutrients, and the extraction method employed. Overall, the content of bioactive compounds was higher in alcoholic extracts than in aqueous extracts prepared from the same plant material (using the same mass of powdered plant material in the preparation of the extracts, namely 5 g), except for the extract from bulbs collected during the flowering period (autumn).

UPLC analysis revealed that the highest concentration of colchicine, the primary bioactive compound, was found in the alcoholic and aqueous extracts obtained from flowers (8), whereas the lowest concentration was detected in the extracts from the bulb sheaths (7) (Table 2). Colchicine, like many other secondary metabolites, contributes to plant survival through its toxic effects on herbivores and pathogens (Ghosh and Jha, 2008). This may explain the higher colchicine content in the aboveground organs of meadow saffron compared to the underground organs. Pearson's correlation coefficient analysis indicated no correlation between the colchicine content of the alcoholic and aqueous extracts of *C. autumnale* ($p = 0.165$).

Table 1. Annotation of extracts, data on the plant material and compounds detected by RP-UPLC in alcoholic and aqueous extracts obtained from *C. autumnale* organs

Ontogenetic stage	Extract number	Collection date	Voucher specimen	Plant organ	Solvent	Content (mg ml^{-1})			
						Colchicine	Colchiceine	Apigenin	Salicylic acid
Growth	1	15.05.2021	I186.542	bulbs	EtOH 70%	**0.1328 ± 0.0003** ^{e}	0.0011 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.1204 ± 0.0003 ^{e}
					H_2O	**0.0885 ± 0.0015** ^{d}	0.0002 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
	2	15.05.2021	I186.542	leaves	EtOH 70%	**0.4461 ± 0.0004** ^{h}	0.0375 ± 0.0128 ^{d}	0.0184 ± 0.0001 ^{b}	0.1547 ± 0.0001 ^{f}
					H_2O	**0.1492 ± 0.0004** ^{e}	0.0184 ± 0.00 ^{b}	0.00 ± 0.00 ^{a}	0.0296 ± 0.0041 ^{b}
	3	15.05.2021	I186.542	fruits	EtOH 70%	**0.4308 ± 0.0008** ^{h}	0.0179 ± 0.0005 ^{b}	0.00 ± 0.00 ^{a}	0.0714 ± 0 ^{c}
					H_2O	**0.2624 ± 0.0046** ^{f}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
Fruiting	4	8.07.2021	I186.555	leaves	EtOH 70%	**0.204 ± 0.0006** ^{f}	0.0042 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.2026 ± 0.0007 ^{g}
					H_2O	**0.0559 ± 0.0003** ^{c}	0.00 ± 0.00 ^{a}	0.0004 ± 0.0001 ^{a}	0.0186 ± 0.0001 ^{b}
	5	8.07.2021	I186.555	fruits	EtOH 70%	**0.3993 ± 0.0005** ^{g}	0.0257 ± 0.0006 ^{c}	0.00 ± 0.00 ^{a}	0.1342 ± 0.0008 ^{e}
					H_2O	**0.0911 ± 0.0035** ^{d}	0.0005 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
Flowering	6	5.11.2021	I186.556	bulbs	EtOH 70%	**0.0617 ± 0.0002** ^{c}	0.0024 ± 0.0014 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
					H_2O	**0.0854 ± 0.0013** ^{d}	0.00003 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
	7	5.11.2021	I186.556	bulb sheats	EtOH 70%	**0.0305 ± 0.0001** ^{b}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.0915 ± 0 ^{d}
					H_2O	**0.0095 ± 0.0003** ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
	8	5.11.2021	I186.556	flowers	EtOH 70%	**0.7815 ± 0.003** ^{g}	0.0032 ± 0.0001 ^{a}	0.3765 ± 0.0005 ^{c}	0.327 ± 0.0142 ^{h}
					H_2O	**0.1227 ± 0.0001** ^{e}	0.0048 ± 0.0001 ^{a}	0.003 ± 0.0001 ^{a}	0.066 ± 0.00005 ^{c}

Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

Comparing the content of aqueous and alcoholic extracts, it was found that a lower amount of colchicine was obtained by extraction in water than by extraction in methanol performed with Soxhlet, except for the extract from bulbs collected in autumn (6), in which a higher concentration of colchicine was identified than in the alcoholic extract prepared from the same plant material. Wildman and Pursey (1960) reported that the colchicine content in bulbs was 3 times lower in the autumn period than in spring. However, in the present study, similar concentrations of colchicine were identified in the aqueous extracts of the bulbs (1 and 6 in Table

2). In contrast, by analyzing the alcoholic extracts, it was found that this compound was present in a quantity two times higher in bulbs during the growth period (spring) than in the flowering period (autumn). Compared to the results of previously conducted research (Moroșan *et al*., 2022), in which extracts were prepared from plant material (bulbs and flowers) collected from the same meadow (in October 2019), with colchicine concentrations detected by the same method (0.119 ± 0.007 mg ml^{-1} in the bulb extract and 0.286 ± 0.015 mg ml^{-1} in the flower extract), the colchicine concentration in the extracts obtained from the material collected in 2021 was two times lower in the bulbs and approximately three times higher in the flower extract. These differences may be due to environmental conditions, time of collection, total biomass of the harvested plant material, solvent used for extraction, and extraction time.

In the present study, colchiceine (Table 2) was identified at low concentrations in extracts from immature leaves (2) and in alcoholic extracts prepared from fruits (3 and 5), and at very low concentrations in extracts from bulbs (1 and 6), mature leaves (4), and flowers (8). This compound was not detected in the aqueous or ethanolic extracts of bulb sheaths (7). Colchiceine is considered the main degradation product of colchicine (in addition to lumicolchicines) (Kurek and Barczyński, 2016); thus, it is used for quality control of plant extracts. Pearson's correlation coefficient analysis indicated no correlation between the colchiceine content of the alcoholic and aqueous extracts ($p = 0.051$).

Although the presence of demecolcine has been reported in several studies on the biochemical composition of *C. autumnale* species (Malichová *et al*., 1979; Yoneda *et al*., 1984; Herbert *et al*., 1990; Davoodi *et al*., 2021), it was not detected in the extracts prepared in this study. This may indicate that the substrate in the area from which the plant material was collected, as well as the environmental or stress conditions experienced by *C. autumnale* individuals before collection, influenced the chemical composition of these plants, as revealed by the analysis of the alcoholic and aqueous extracts (Table 2).

Apigenin (Table 2) has been identified in the aqueous extracts of flowers (8) and mature leaves (4). Apigenin is a flavone with antioxidant properties that protects plant cells from oxidative stress, which is crucial for preventing damage caused by reactive oxygen species (Madunić *et al*., 2018; Azeem *et al*., 2024) and UV-B rays (Righini *et al*., 2018). As a secondary metabolite, apigenin contributes to plant defense against pathogens and environmental stressors (Mushtaq *et al*., 2023; Azeem *et al*., 2024). Comparing these results with the data from the literature (Burzo *et al*., 2006), it was confirmed that apigenin is present in the flowers of *C. autumnale*. Apigenin has also been identified in very small amounts in ethanolic extracts prepared from young leaves (2) and in aqueous extracts from mature leaves (4). Pearson's correlation coefficient analysis indicated a correlation between the apigenin content in alcoholic and aqueous extracts ($p = 0.000003$), indicating that both water and alcohol efficiently extract this compound.

Salicylic acid (Table 2) was identified at the highest concentration in both alcoholic and aqueous extracts of flowers (8) and leaves (2 and 4); however, the use of Soxhlet and methanol as extraction solvent made it possible to extract this compound from other organs, such as bulbs collected in spring (1), fruits (3 and 5), and bulb sheaths (7). Pearson's correlation coefficient analysis indicated a correlation in the salicylic acid content between alcoholic and aqueous extracts ($p = 0.004$), a compound that can be successfully extracted using either alcohol or water as solvents. Salicylic acid is involved in the flowering process and plays an important role in protecting plants against biotic and abiotic stress (Wani *et al*., 2017). It is also involved in the regulation of physiological and biochemical processes throughout the plant life cycle, thereby influencing its growth and development (Vicente and Plasencia, 2011; Koo *et al*., 2020).

According to a study by Jung *et al*. (2011), *C. autumnale* has a life cycle that begins with the flowering stage, which falls in the autumn period, in which the flowers appear above the ground, unaccompanied by leaves, with the gynoecium buried in the soil and protected by the bulb, the anthers are exposed in the above-ground area, and adventitious roots begin to appear on the young bulb disc (developed during summer). Flowering ends with a short period in which the fruits and leaves begin to develop underground, consuming part of the nutrient reserves in the bulb. This period is followed by dormancy during winter, during which the

fruits and leaf primordia are withdrawn into the soil by contractile adventitious roots, thus being protected from low temperatures. In spring, the fruits begin to appear above the ground surrounded by leaves, with a light green color and white developing seeds, whereas starch reserves in the old bulb are progressively depleted. As the old bulb shrinks, the new bulb begins to grow and accumulate starch. During summer, the fruits mature and open, with a brownish color, releasing blackish-brown seeds, and the leaves approach senescence. The plant has a small bulb (the old bulb), which enters the maceration process simultaneously with the development of the new bulb, while the aboveground organs begin to dry out. At that time, the plants consisted only of underground bulbs surrounded by sheaths that represented the leaves that had dried up, surrounded the bulb, and appeared as a brown tunic. The period of inactivity follows, and flowering resumes (Figure 1). Thus, we chose a different approach to study *C. autumnale*'s ontogenetic stages by starting with the growth period (in spring) because it is the first true stage of new metabolic activity within the plant, and the flowering period (in autumn) relies on reserves from the previous cycle.

During the growth period, which continued into the fruiting period, the colchicine and salicylic acid content in the leaves remained relatively constant (then decreased towards senescence in the bulb sheaths), whereas in the fruits, it increased during maturation. The colchicine content in the underground organs (bulbs) during the growth period was twice as high as that during the flowering period, which can be explained by the progressive reduction in bulb biomass.

Polyphenols are compounds with multiple and varied functions that contribute to the resistance and adaptation of plants to the environment, being essential in protection against stress, defense against herbivores, antioxidant activity, and regulation of growth and development processes (Sharma *et al.*, 2019; Singh *et al.*, 2021; Šamec *et al.*, 2021; Pinto *et al.*, 2021; Zagoskina *et al.*, 2023).

In the analyses of the available plant material, it was found that during the vegetative stage, the highest polyphenol content was found in the aboveground organs, especially in the leaves (2, 4) and flowers (8), and the polyphenol content determined was higher in the bulbs collected during the spring (1) than in the bulbs collected in the fall (6). In contrast, the analysis of the alcoholic extracts suggested that the accumulation of polyphenols in the fruits (3 and 5) would occur during maturation; however, in the aqueous extracts, this content remained approximately constant (Figure 17). Pearson's correlation test revealed a correlation between the polyphenol content of alcoholic and aqueous extracts ($p = 0.007$). This suggests that both ethanol/methanol and water can extract these compounds in a comparable manner, yet alcoholic solvents yield better results.

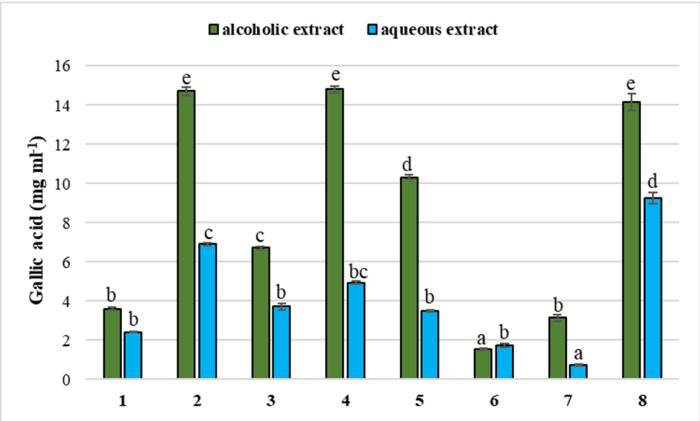

Figure 17. Polyphenols (mg eq. gallic acid ml^{-1}) content in alcoholic and aqueous *C. autumnale* extracts
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract
Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

Similarly to the analysis of the polyphenol content in alcoholic and aqueous extracts from plant organs of *C. autumnale*, it was found that during the growth stage (spring period) the highest flavonoids content is found in the aboveground organs, especially in leaves (2, 4), flowers (8) and fruits (3, 5). Flavonoids are phenolic compounds that regulate plant development and contribute to the pigmentation of flowers and fruits, which are essential characteristics for attracting pollinators (Mierziak *et al*., 2014; Mathesius, 2018), which explains their abundant presence in these organs. Moreover, these compounds accumulate in the epidermis and protect plants from harmful solar radiation (Shah and Smith, 2020; Ferreyra *et al*., 2021). Flavonoids exhibit strong antioxidant properties, helping to eliminate reactive oxygen species (ROS), and thus play a significant role in plant stress tolerance (Khalid *et al*., 2019; Dias *et al*., 2021; Shomali *et al*., 2022), as well as against pathogens and herbivores, increasing their resistance to biotic stress (Mathesius, 2018; Alseekh *et al*., 2020; Shah and Smith, 2020). Moreover, flavonoids are involved in cellular signaling processes, such as root-rhizosphere interactions, an interaction in which they can stimulate or inhibit microbial activity, affect nutrient uptake, and mediate, influence, or even determine allelopathic interactions (Hassan and Mathesius, 2012; Shah and Smith, 2020), which explains their presence, even in lower quantities, in underground organs.

The flavonoid content determined in the present study was higher in the alcoholic extract from mature fruits (5) than that prepared from immature fruits (3), whereas in the homologous aqueous extracts, it was similar in both extracts. Simultaneously, the flavonoid content in the leaves was similar in the alcoholic extracts (2 and 4), but higher in the aqueous extract prepared from leaves collected during the growth period (2) than in the aqueous extract from mature leaves (4). In addition, the flavonoid content was much higher in bulbs collected during spring (1) than in bulbs collected in autumn (6), an observation resulting from the analysis of both types of extracts (aqueous and alcoholic) (Figure 18). Pearson's test revealed a correlation between the flavonoid content of the alcoholic and aqueous extracts ($p = 0.00002$).

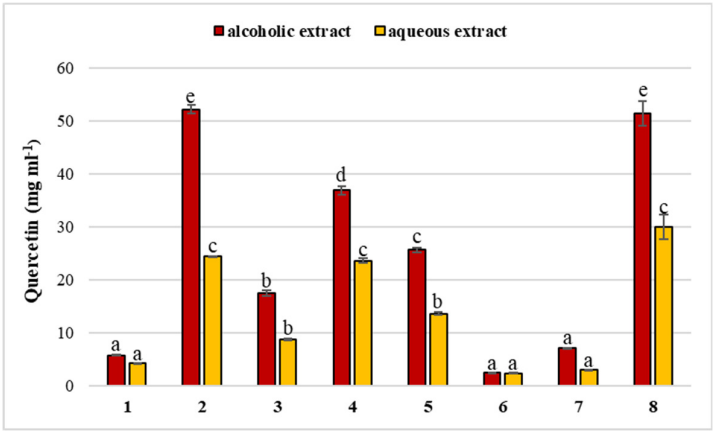

Figure 18. Flavonoids (mg eq. quercetin ml^{-1}) content in alcoholic and aqueous *C. autumnale* extracts
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract; Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

The polyphenol and flavonoid contents remain relatively constant in the leaves during the growth period towards fruiting and are also the most abundant in the leaves and flowers compared to the fruits, bulb sheaths, and bulbs. In the bulbs, this content decreases or remains relatively constant, and in the fruits, it increases significantly during maturation, providing additional protection against stress, pests, and herbivores.

The antioxidant activity (expressed as mg of ascorbic acid per gram of dry plant material) was considerably higher in alcoholic extracts prepared from the vegetative and reproductive organs of *C. autumnale* plants than in aqueous extracts (Figures 17 and 18). All meadow saffron organs exhibited high antioxidant

activity, except for the extract from bulbs collected in spring. In the case of aqueous extracts, the detected antioxidant activity was weak, and no significant variations were observed between the extracts. Pearson's correlation coefficient analysis indicated no correlation between the antioxidant activities of the alcoholic and aqueous extracts ($p = 0.162$).

The highest antioxidant activity was detected in alcoholic extracts from fruits (3 and 5), bulbs collected in autumn (6), flowers (8E), and leaves (2, 4, and 7), without significant variations (Figure 17). However, polyphenols and flavonoids were detected in extracts from flowers (8), leaves (2 and 4, but not 7), and fruits (3 and 5). Flavonoids may be the main compounds contributing to the antioxidant activity in the aboveground organs of meadow saffron, although there are significant differences in flavonoid content between the alcoholic extracts prepared from these organs (Figure 19 and 20).

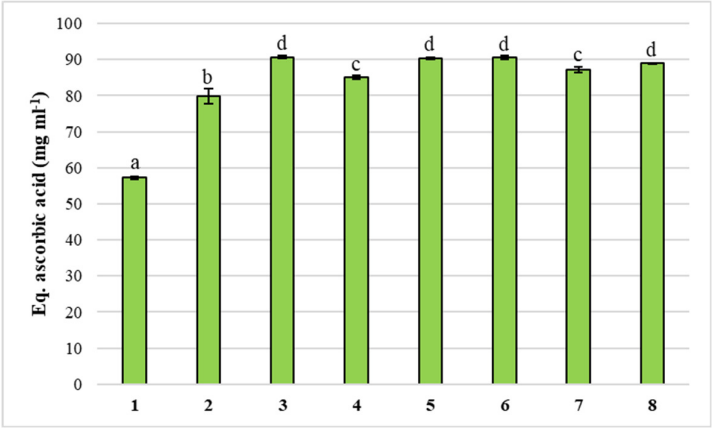

Figure 19. Antioxidant activity (mg eq. ascorbic acid ml^{-1}) of alcoholic extracts
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract; Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

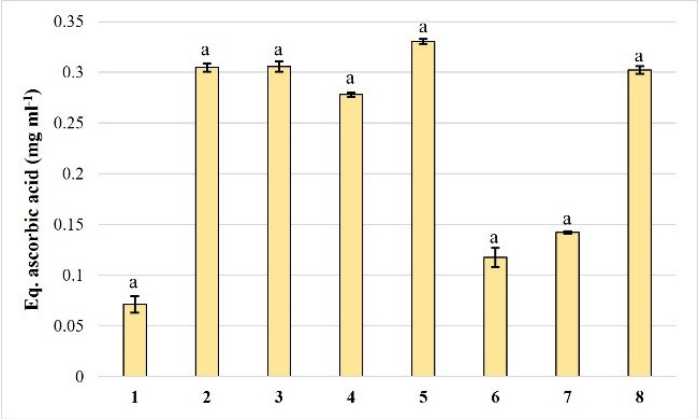

Figure 20. Antioxidant activity (mg eq. ascorbic acid ml^{-1}) of aqueous extracts
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract; Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

Pearson's correlation test also revealed a strong correlation between polyphenol and flavonoid content (as expected) in both extract types (alcoholic: $p = 0.0007$ and aqueous: $p = 0.0002$). When the polyphenol and

flavonoid contents of the alcoholic extracts were compared to the antioxidant activity of the extracts, no significant correlations were observed (polyphenols vs. DPPH: $p = 0.342$; flavonoids vs. DPPH: $p = 0.332$). Only the polyphenols and flavonoids from the aqueous *C. autumnale* extracts displayed a strong correlation with the antioxidant activity of the extracts (polyphenols vs. DPPH: $p = 0.034$; flavonoids vs. DPPH: $p = 0.019$), although it was weak, and there were no significant differences identified between the antioxidant activities of the aqueous extracts (Figure 20). The low antioxidant activity of the aqueous extracts may indicate the presence of compounds with higher solubility in alcohol or compounds that are either insoluble or less soluble in water. In addition, the polyphenol and flavonoid content detected in alcoholic extracts from bulbs collected in autumn (6) was very low, yet its antioxidant activity increased (Figure 19). This may provide clues regarding the presence of other compounds in this extract that contribute to the increased antioxidant activity.

The findings of this study confirm that all plant organs of *C. autumnale* accumulate colchicine and suggest that the time of collection, extraction method, and solvents significantly affect the yield of key bioactive compounds in *C. autumnale*. The alcoholic extracts were richer in secondary metabolites than their homologous aqueous extracts (except for the autumn bulb extracts, where the aqueous extract had a higher concentration). Apigenin and salicylic acid were detected at their highest concentrations in the flower and leaf extracts, respectively, indicating their roles in flowering and plant defence. Overall, the highest concentrations of polyphenols and flavonoids were observed in the aboveground organs during the growth stage, particularly in the leaves and flowers. Moreover, the antioxidant activity of the alcoholic extracts was significantly higher than that of the aqueous extracts, particularly in the extracts from the leaves, fruits, and flowers. This underscores the need for carefully planned and executed collection and processing protocols to maximize the extraction of desired substances from *C. autumnale*.

Conclusions

Soxhlet extraction is a reliable and accessible method for extracting colchicine and polyphenols from *C. autumnale* plants, with high efficiency, accessibility, and reproducibility. It provides consistent results owing to continuous solvent cycling, ensuring thorough extraction of target compounds when modern extraction techniques are unavailable. Usually, in extract preparation, the evaporation of solvents using a rotary evaporator is ideal for obtaining a more concentrated alcoholic extract, whereas lyophilization is better for aqueous extracts because it preserves the extract's integrity, given that many bioactive secondary metabolites (such as polyphenols and alkaloids) are thermolabile, thus avoiding thermal degradation of compounds. This is especially applicable to colchicine, which is a thermolabile compound in aqueous solutions but is more stable in ethanol.

This study revealed how colchicine and phenol concentrations vary across different developmental stages (growth, flowering, and fruiting) in various plant organs, providing new insights into the dynamic accumulation patterns throughout the plant's ontogenetic cycle. Flowers contain the highest colchicine concentrations, whereas bulbs and other organs exhibit varying levels depending on the season, emphasizing the influence of both plant organ and harvest time on bioactive compound content. Alcoholic extracts generally yield higher amounts of colchicine and phenols than water extracts, except in certain cases, such as bulbs during flowering, highlighting the importance of solvent choice in maximizing bioactive compound extraction. By analyzing environmental parameters, ontogenetic factors, and extraction methods, this study provides a comprehensive understanding that can inform optimized harvesting and processing strategies for medicinal and phytotherapeutic applications. The application of UPLC and spectrophotometry can offer precise quantification of secondary metabolites, setting a methodological precedent for future phytochemical studies in this species and potential applications in other plants with similar properties.

The biochemical diversity in *C. autumnale*, especially its high alkaloid content (notably colchicine), plays a key role in plant defense against herbivores and pathogens and supports survival during dormancy. Metabolite distribution, such as the abundance of colchicine and polyphenols in flowers, fruits, and leaves, reflects adaptation to ecological pressures and reproductive strategies. These compounds also contribute to stress tolerance and energy storage, influencing the ecological interactions and fitness of plants. Variations in the secondary metabolite content between plant parts are critical for pharmacognostic identification and safety, as colchicine has a narrow therapeutic index.

Authors' Contributions

Conceptualization: ICM, MMZ; Data curation: ICM, MM; Formal analysis: ICM, MM; Funding acquisition: MM, MMZ; Investigation: ICM, MM; Methodology: ICM, MM; Project administration: MMZ; Resources: MMZ, MM; Software: ICM, MM; Supervision: MMZ, MM; Validation: MMZ, MM; Visualization: ICM, MM, MMZ; Writing - original draft: ICM; Writing - review and editing: ICM, MM, MMZ.

All authors read and approved the final manuscript

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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An insight into the biochemical content of *Colchicum autumnale* L. throughout its ontogenetic cycle

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Abstract

Meadow saffron (*Colchicum autumnale* L.) is a poisonous perennial species with an unusual ontogenetic cycle. This study investigated its biochemical composition during three key developmental phases: growth, fruiting, and flowering. Aqueous and alcoholic extracts from different plant organs were analyzed using UPLC and spectrophotometry to evaluate the influence of developmental stage, extraction method, and solvent on secondary metabolite accumulation, with implications for extract potency and pharmacological properties. Colchicine and phenolic compounds were detected in all organs, though their concentrations varied with extraction technique and harvest time. Flowers contained the highest colchicine levels (0.7815 mg ml^{-1} in ethanol, 0.1227 mg ml^{-1} in water), while bulbs accumulated lower amounts that decreased from spring to autumn. In general, alcoholic extracts yielded more bioactive compounds than aqueous ones. Flowers and leaves also contained significant levels of polyphenols, with ethanol extracts showing markedly higher concentrations than water extracts. Antioxidant assays revealed strong activity in all alcoholic extracts (79.8 - 90.7 mg ml^{-1} eq. ascorbic acid), except for spring bulbs (57.2 mg ml^{-1}), whereas aqueous extracts exhibited negligible antioxidant capacity (0.07 - 0.3 mg ml^{-1}). These results highlight the dynamic phytochemistry of *C. autumnale*, demonstrating that the concentration of bioactive compounds is strongly influenced by plant organ, developmental stage, extraction method, and solvent. Such variability should be considered in future research and practical applications, including ecological studies, weed management, and standardization of extracts for medicinal use.

Keywords: antioxidant activity; colchicine; flavonoids; polyphenols; Soxhlet

Introduction

Colchicum autumnale, a member of the Colchicaceae family, is a toxic bulbous perennial plant typically found in mountain grasslands (Pop *et al*., 1983). This species thrives in various damp grassland environments, from hilly to mountainous regions, and it is widely distributed, particularly in Europe, being considered abundant (Chadburn, 2014).

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The plant features a dense tuber that produces an axillary bud, which gives rise to one to three violet flowers in autumn (Ștefan and Ivănescu, 2002). The leaves are basal and originate from the primordia of the underground bulb disc. The flower consists of a perigone with six pinkish-purple tepals, six stamens, and an extended perigone tube that forms on the bulb disc alongside the ovary, shielded by bulb cataphylls (Pop *et al.*, 1983). In spring, the fruit emerges aboveground and is encircled by 3-4 oblong-lanceolate leaves (Ștefan and Ivănescu, 2002). The fruit is a trilocular capsule containing colchicine-rich seeds (Pop *et al.*, 1983).

The plant's distinctive ontogenetic cycle begins with autumn flowering, followed by a dormant winter period during which the fruit remains underground. Spring marks the peak of photosynthetic activity, with fruits and leaves appearing aboveground. Plants enter a period of inactivity after fruit ripening (Jung *et al.*, 2011) (Figure 1).

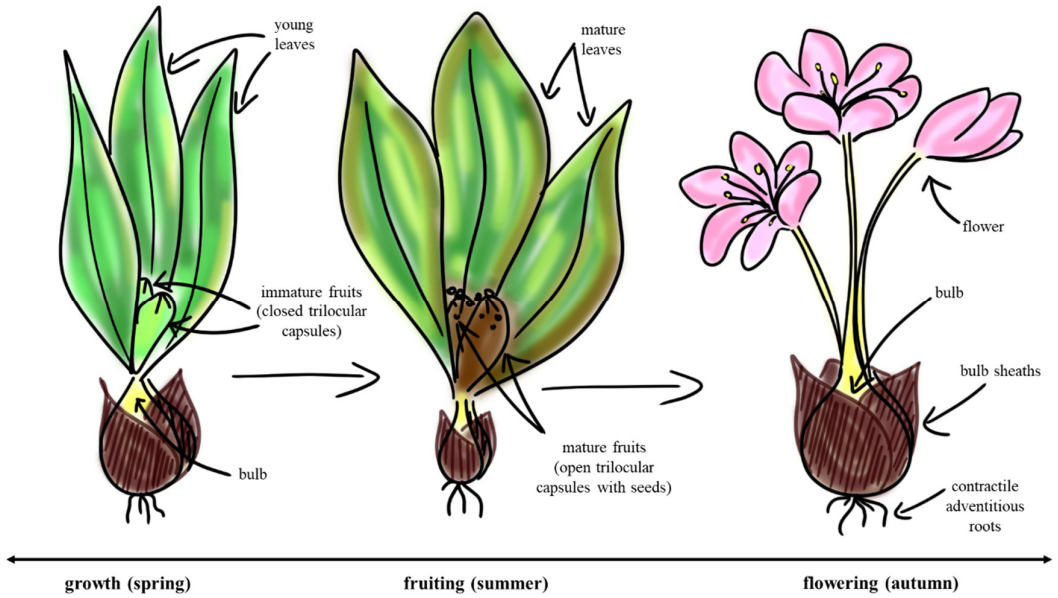

Figure 1. Schematic representation of *Colchicum autumnale* plants' ontogenetic cycle

Research on *C. autumnale* spans pharmacology, ecology, and agriculture; however, several important gaps remain. Recent studies have highlighted organ-specific and environment-dependent variations in metabolite content (Boboev *et al.*, 2023; Dincheva *et al.*, 2025). However, systematic studies on how environmental factors, developmental stages, and plant parts influence the phytochemical spectrum are lacking. Although optimized methods for colchicine extraction exist, there is limited research on efficient and standardized extraction and quantification protocols for phytochemicals in *C. autumnale* (Çankaya *et al.*, 2018).

According to Burzo *et al.* (2005), aboveground organs contain starch, sucrose, lipids, phytosterols, benzoic acid, salicylic acid, alkaloids, and tannins. The alkaloids present in underground organs are colchicine, 2-demethylcolchicine, colchicoside, 2-desmethyldeacetylcolchicine, demecolcine, thiocolchicoside, 3-demethylcolchicine, 3-methylcolchicine, 3-demethyl-β-lumicolchicine, α-lumicolchicine, β-lumicolchicine, γ-lumicolchicine, colcamine, colchicene, colchicerine, N-methyldesacetylcolchicine, N-deacetyl-N-methylcolchicine, N-formyl-deacetylcolchicine, and O-demethyl-N-deacetylcolchicine. Aerial organs contain salicylic acid, chelidonic acid, and alkaloids. The alkaloids found in the above-ground organs of plants belonging to this species are 2-acetyl-2-demethylcolchicine, 2-acetyl-3-methylcolchicine, colchifoline, demecolceine, demecolcine, N-acetyl-demecolcine, and O-acetylcolchiceine and apigenin.

Colchicine ($C_{22}H_{25}O_6N$, N-[(7S)-1,2,3,10-tetramethoxy-9-oxo-6,7-dihydro-5H-benzo[a]heptalen-7-yl] acetamide), the primary bioactive component extracted from *C. autumnale*, is an alkaloid that inhibits mitosis by attaching to tubulin and preventing its polymerization, resulting in autopolyploidization. Utilizing the antimitotic property of colchicine, researchers have developed plants with enhanced traits (compared to their diploid counterparts) for commercial applications (Manzoor *et al*., 2019). Colchicine has been widely used in the medical field to address a range of ailments, such as gout, familial Mediterranean fever, skin vasculitis, and Paget's and Behçet's diseases (Nerlekar *et al*., 2014). Recently, colchicine has garnered attention in clinical trials for the treatment of pericarditis (Shah *et al*., 2016), cancer (Zhang *et al*., 2019), and COVID-19 (Lopes *et al*., 2021).

In addition to colchicine and its derivative compounds, *C. autumnale* contains other secondary metabolites that confer protection throughout its ontogenetic cycle. Polyphenols are important biochemical compounds in plants that are involved in the oxidative stability of different parts of the plant and in chemical defense mechanisms, such as allelopathy (Li *et al*., 2014; Singh *et al*., 2021; Zagoskina *et al*., 2023). They function by preventing or inhibiting the generation of reactive oxygen species (ROS) (Agati and Tattini, 2010; Singh *et al*., 2021; Dini and Grumetto, 2022; Zagoskina *et al*., 2023). Polyphenols contribute to the thickening of the secondary cell wall, conferring mechanical resistance and rigidity that prevent the destruction of healthy tissues in the vicinity of the affected tissues (Gunnaiah *et al*., 2012; Singh *et al*., 2021), and play a role in promoting tissue sclerification (Di Ferdinando *et al.*, 2014). Moreover, they modulate plant growth, development, and signaling pathways, influencing processes such as cell division and hormone activity (Dini and Grumetto, 2022; Zagoskina *et al*., 2023).

Flavonoids are polyphenols that play crucial roles in seed germination, plant growth, and development (Wang *et al*., 2022; Zhuang *et al*., 2023). These compounds protect plants against biotic and abiotic stress (Shomali *et al*., 2022; Zhuang *et al*., 2023), serve as significant signaling molecules (Mathesius, 2018; Shah and Smith, 2020; Wang *et al*., 2022; Kumar *et al*., 2024), and function as allelopathic compounds (Mathesius, 2018; Shah and Smith, 2020; Zhuang *et al*., 2023), phytoalexins (Mathesius, 2018; Wang *et al.*, 2022; Zhuang *et al*., 2023), detoxifying (Dias *et al*., 2021; Zhuang *et al*., 2023), and antimicrobial agents (Pollastri and Tattini, 2011; Agati *et al*., 2012; Shah and Smith, 2020; Wang *et al*., 2022). Flavonoids interact with membrane phospholipids, thereby protecting chloroplast membranes against photooxidation (Agati *et al*., 2013; Ferreyra *et al*., 2021; Laoué *et al*., 2022) and inhibiting singlet oxygen ($^1O_2$) (Agati *et al*., 2007). Furthermore, flavonoids can facilitate the morpho-anatomical adaptation of plants to stress conditions (Agati and Tattini, 2010; Agati *et al*., 2013; Buer *et al*., 2013; Shah and Smith, 2020; Shomali *et al*., 2022; Zhuang *et al*., 2023).

Studying and comparing alcoholic and aqueous extracts of *C. autumnale* organs at different ontogenetic (developmental) stages is important because the extraction method, plant organ, and developmental stage significantly influence the chemical composition and biological activity of the extracts. Alcoholic (ethanolic) and aqueous extracts yield different profiles of bioactive compounds. For example, ethanolic extracts of *C. autumnale* leaves showed much higher acaricidal activity than aqueous extracts, indicating that ethanol extracts contain more potent or different active compounds from the leaves than water (Norouzi *et al*., 2020). The concentration and types of alkaloids and other bioactive compounds in *C. autumnale* vary significantly depending on the plant developmental stage. Although studies have focused on the organ and solvent, it is well established in phytochemistry that the ontogenetic stage impacts secondary metabolite content, which in turn affects extract potency and pharmacological properties (Ellington *et al*., 2003).

This study aimed to perform a comparative analysis of the aqueous and alcoholic extracts of *C. autumnale*, focusing on their phytochemical composition and antioxidant potential. Specifically, the objectives were to quantify and compare the contents of colchicine, total polyphenols, and flavonoids in aqueous and alcoholic extracts; evaluate and compare the antioxidant activity of the extracts and examine its correlation with polyphenol and flavonoid levels; investigate the distribution of these bioactive compounds across different

plant organs; assess the influence of ontogenetic stage on colchicine accumulation, phenol content, and antioxidant potential; and identify the extraction method, plant organ, and developmental stage that maximizes the yield of pharmacologically relevant compounds, providing insight into potential applications in medicine and phytotherapy. An analysis of the biochemical composition of a plant species may also provide insights into the mechanisms utilized to adapt to stress induced by interspecific competition, herbivores, pathogens, pests, and adverse environmental conditions. By comparing extracts from different organs and developmental stages, researchers can identify the optimal combination for maximum yield of desired compounds. Understanding these differences will facilitate the development of more effective medicines and biopesticides.

Materials and Methods

Plant material collection

The plant material comprising bulbs, leaves, and immature fruits (Figures 2 and 4) of *C. autumnale* was collected on 15.05.2021 (with the voucher specimen deposited in the Herbarium of the Faculty of Biology of the "Alexandru Ioan Cuza" University in Iasi, I186.542), which corresponds to the active growth period in the ontogenetic cycle of this species. Leaves and mature fruits (Figure 3) were obtained during the fruiting period, on 8.07.2021 (I186.555), and bulbs and flowers (Figure 5) were collected on 5.11.2021, during the flowering period (I186.556) from a meadow in Voroneț, Suceava County (Figure 6A and 6B). Plant material from 32 individual plants was collected at each ontogenetic stage (96 plants in total). The coordinates were recorded using GPSEssentials: latitude 47.493889° longitude 25.885833° and imported into the NASA Worldview Map (*https://worldview.earthdata.nasa.gov*) (Figure 7).

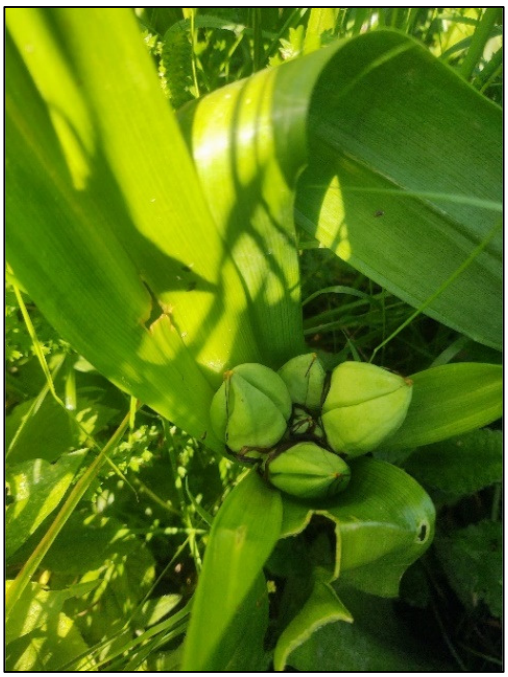

Figure 2. Immature *C. autumnale* fruits (closed trilocular capsule), photographed and collected on 15.05.2021

Figure 3. Mature *C. autumnale* fruits (open trilocular capsule with seeds), photographed and collected on 8.07.2021

Figure 4. Young *C. autumnale* plants with leaves, immature fruits, bulbs, and roots, collected on 15.05.2021

Figure 5. *C. autumnale* plants, showing only flowers, bulbs, and roots, collected during the flowering stage, on 5.11.2021

Soil samples were collected from the plant material harvest point, dried, and ground into fine powder. Five grams of soil powder was weighed and dissolved in 12.5 mL of distilled water (1:2.5 m/V). The solution thus obtained was analyzed using a pH meter, according to the method described by Blakemore *et al*. (1987), recommended by the Food and Drug Administration (FDA), and the results were confirmed by comparison with those obtained using pH indicator paper.

Data on the average temperature and relative humidity were obtained from the NASA POWER Daily API (*https://power.larc.nasa.gov*). The soil type was identified according to the World Reference Base (2006) Soil Groups (*https://soilgrids.org*) for the plant material collection point.

(A) **(B)**

Figure 6. The habitat from which the individuals of *C. autumnale* were collected on 15.05.2021 (A) and in 5.11.2021 (B)

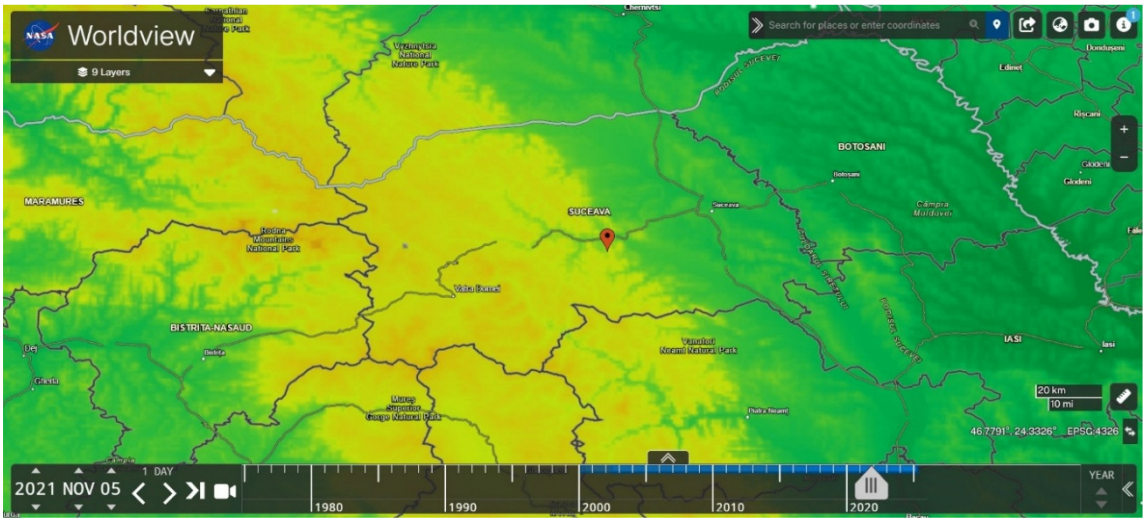

Figure 7. *C. autumnale* individuals harvest point (latitude 47.493889° longitude 25.885833°) (*https://worldview.earthdata.nasa.gov*)

Plant material preparation

The collected plant material was subjected to oven treatment at 65 °C for 12 h to inhibit enzymatic reactions, followed by desiccation in the dark at ambient temperature (23 ± 2 °C) for 7 days (Figure 8A and 8B).

(A) **(B)**

Figure 8. Dried plant parts of *C. autumnale*: leaves and fruits (A); bulb sheaths, bulb fragments, and flowers (B)

Extract preparation

Methanol and ethanol are the most effective solvents for extracting colchicine from *Colchicum autumnale*, with methanol generally yielding the highest extraction efficiency, followed closely by ethanol. In general, aqueous and ethanol extracts are also used for broader phytochemical and biological activity studies,

however, for maximum colchicine yield, methanol and ethanol are preferred (Çankaya *et al*., 2018; Norouzi *et al*., 2020)

Alcoholic extracts were prepared using a Soxhlet apparatus by extracting 5 g of plant material from each organ in absolute methanol, which was recirculated for 8-10 hours (method adapted from Rocchetti *et al*., (2019). The solvent was evaporated to dryness using a rotary evaporator (IKA RV3, Staufen, Germany), and the dry extract was re-dissolved in 50 mL of 70% ethanol.

Aqueous extracts were prepared by infusing 5 g of plant material from each collected organ in distilled water (Figure 9) for 24 h on a shaker at 25 °C, followed by filtration through filter paper. The solvent was evaporated to dryness using a lyophilizer (Christ Alpha 3-4, Osterode am Harz, Germany), and each dry extract was re-dissolved in 50 mL of distilled water.

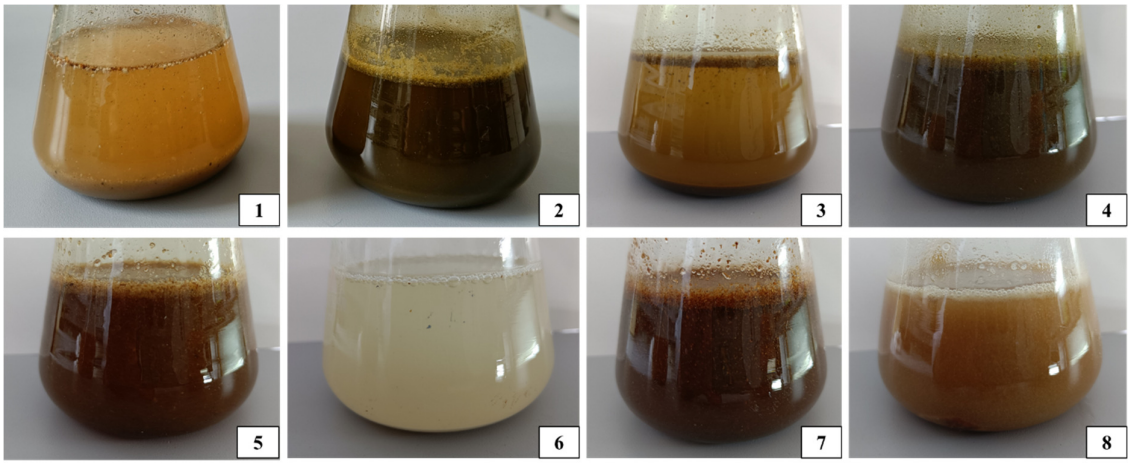

Figure 9. Aqueous extracts from *C. autumnale* organs before filtration
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract

Finally, eight alcoholic and eight aqueous extracts were obtained from the same mass of plant organ powder (5 g) and concentrated using 50 mL of solvent. The extracts were annotated as follows: 1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract.

Extract content analysis

The main bioactive compounds from the obtained extracts were identified and quantified through reversed-phase ultra-performance liquid chromatography (RP-UPLC), using a Shimadzu Prominence UPLC system (2 LC20AD pumps, SIL20AC autosampler, oven CT20AC, SPD M20A DAD detector, RF 20A XS florescence detector) coupled to a Zorbax Eclipse XDB - C18 column (length 250 mm, particle size 3 microns). Colchicine, colchicine, demecolcine, apigenin (purity ≥ 95%), and salicylic acid (purity ≥ 99%) were purchased from Sigma Aldrich (sigmaaldrich.com) and used for the construction of calibration curves in the range of 0.312-2.5 μg ml^{-1} for colchicine, 0.025 - 1.6 μg ml^{-1} for colchiceine, 0.05-0.5 μg ml^{-1} for demecolcine, 0.025 - 2 μg ml^{-1} for apigenin, and 0.04 - 4 μg ml^{-1} for salicylic acid.

For the detection of colchicine, colchicine, demecolcine, and apigenin, acetonitrile was used as mobile phase A, and 3% acetic acid was used as mobile phase B (Sigma Aldrich, Germany), according to the method used by Alali *et al*. (2004), which has also been used in previous studies (Moroșan *et al*., 2022). Elution was performed at a flow rate of 1 ml/min using the following program: 0-3 min 90% B isocratic, 3-11 min 90-40% B gradient, 11-12 min 40% B isocratic, 12-13 min gradient 40-90% B, 13-20 min 90% B isocratic.

For the detection of salicylic acid, the method of Toiu *et al.* (2011) was adapted, using 0.85% orthophosphoric acid as mobile phase A and acetonitrile as mobile phase B. Elution was performed at 1 ml/min using the program: 0-2 min 5% B isocratic, 2-5 min 5-80% B gradient, 5-10 min 80-100% B gradient, 10-12 min 100% B isocratic, 12-18 min 5% B isocratic.

Colchicine (Figure 10), colchiceine (Figure 11), and apigenin (Figure 12) were detected at 245, 254, and 350 nm, respectively, and eluted at 13.4 ± 0.08 min. Demecolcine was not detected in any of the extracts. Salicylic acid (Figure 13) was detected using a fluorescence detector at excitation and emission wavelengths of 310 and 450 nm, respectively. Chromatographic data were acquired using Shimadzu LC Software and interpreted manually by comparing the retention times and detection spectra of the standard compounds with those of the samples (extracts).

A volume of 20 µL was injected to detect compounds in standard solutions and extract samples for five technical replicates. The concentrations of the compounds in the extracts were quantified according to the area of the detected peaks and calculated according to the standard curves (Figure 10, 11, 12 and 13).

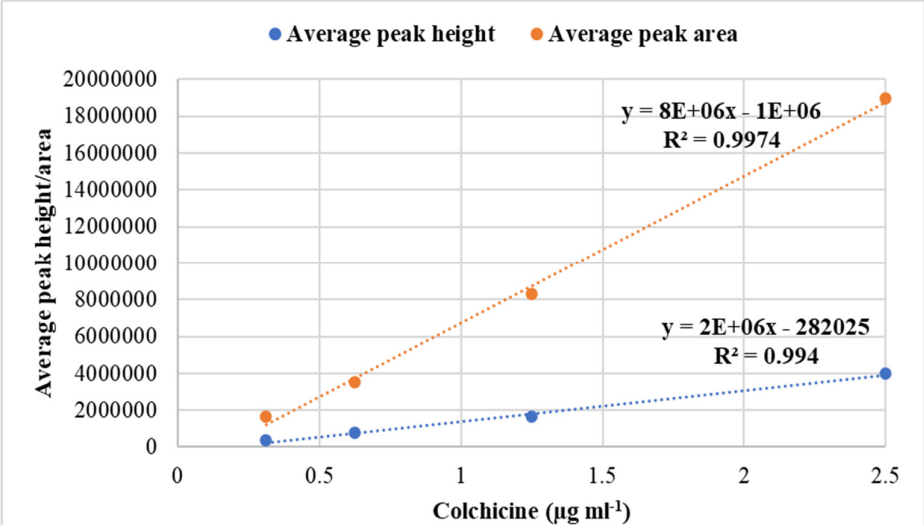

Figure 10. Standard curve used for the quantification of colchicine (µg ml^{-1}) content in *C. autumnale* extracts

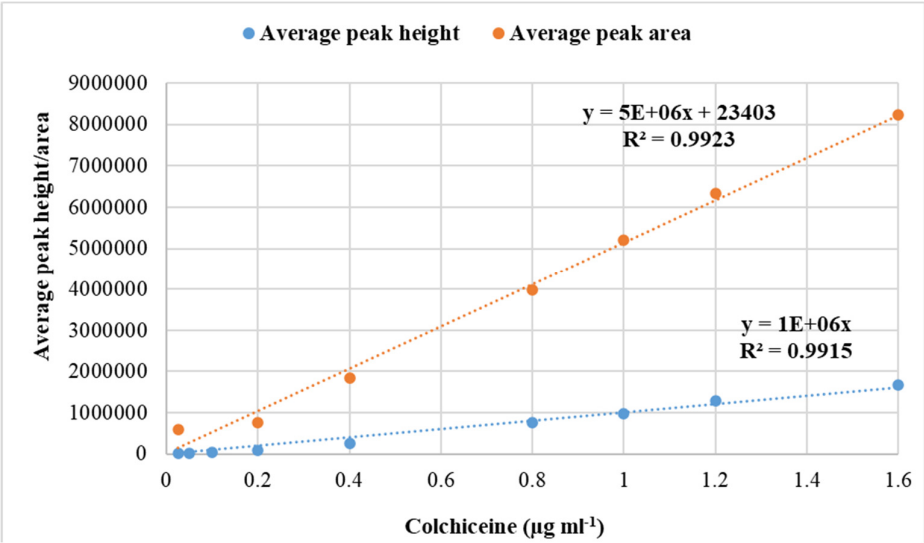

Figure 11. Standard curve used for the quantification of colchiceine (µg ml^{-1}) content in *C. autumnale* extracts

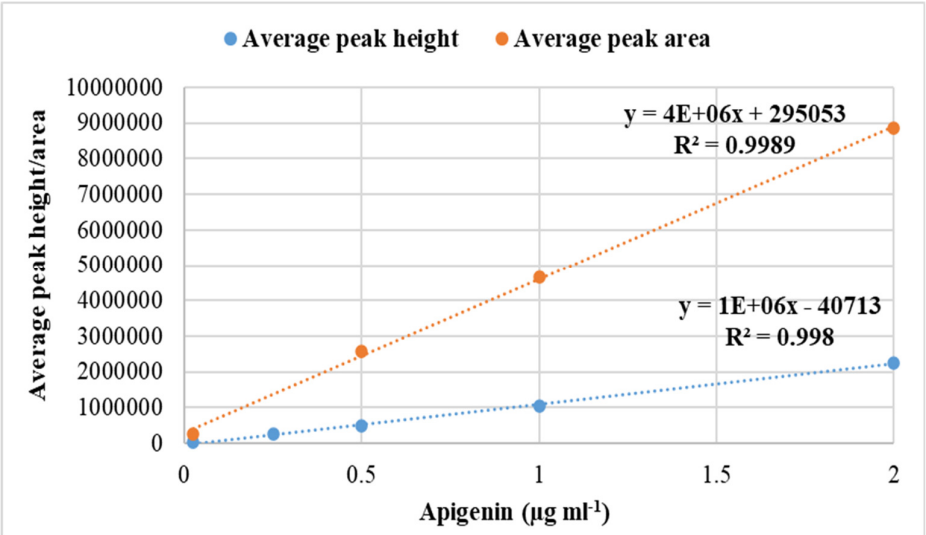

Figure 12. Standard curve used for the quantification of apigenin (µg ml^{-1}) content in *C. autumnale* extracts

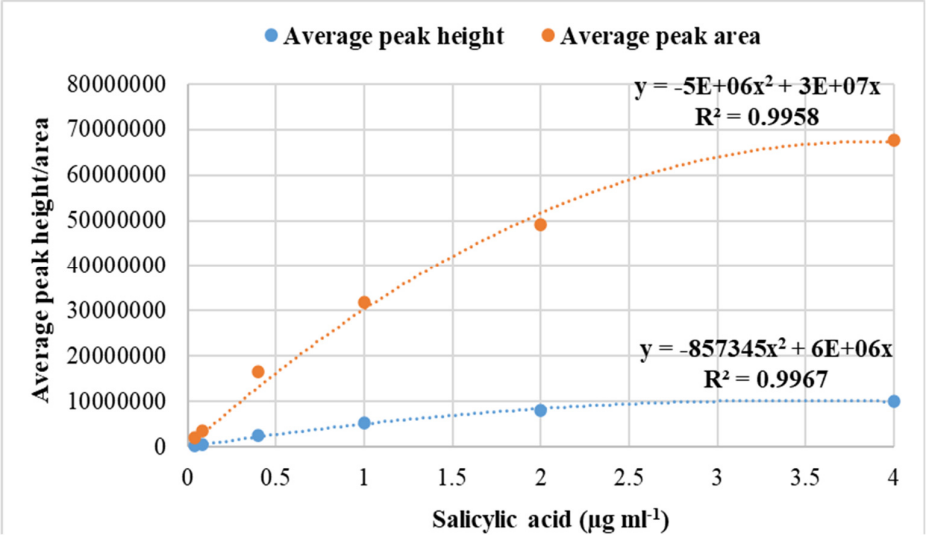

Figure 13. Standard curve used for the quantification of salicylic acid (µg ml^{-1}) content in *C. autumnale* extracts

The total polyphenol content was determined using the conventional method described by Singleton and Rossi (1965) in Herald *et al.* (2012). The standard curve was obtained by preparing standard solutions using gallic acid at concentrations of 0-400 µg ml^{-1}. The flavonoid content was determined using the conventional colorimetric method based on aluminum chloride, according to Herald *et al.* (2012), adapted from Zhishen *et al.* (1999). To determine the standard curve, standard solutions were prepared using quercetin at concentrations of 0-500 µg ml^{-1}. The concentrations of polyphenols and flavonoids in the extracts were calculated using the standard curve formula by calculating the mean values obtained by analyzing 3 biological replicates (n = 3) (Figure 14 and 15).

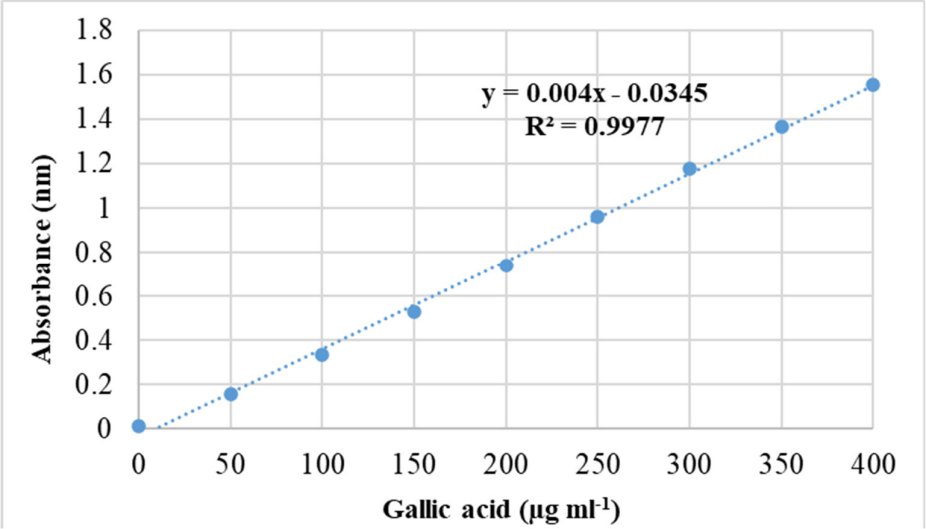

Figure 14. Standard curve used for the quantification of polyphenols (µg eq. gallic acid ml^{-1}) content in *C. autumnale* extracts

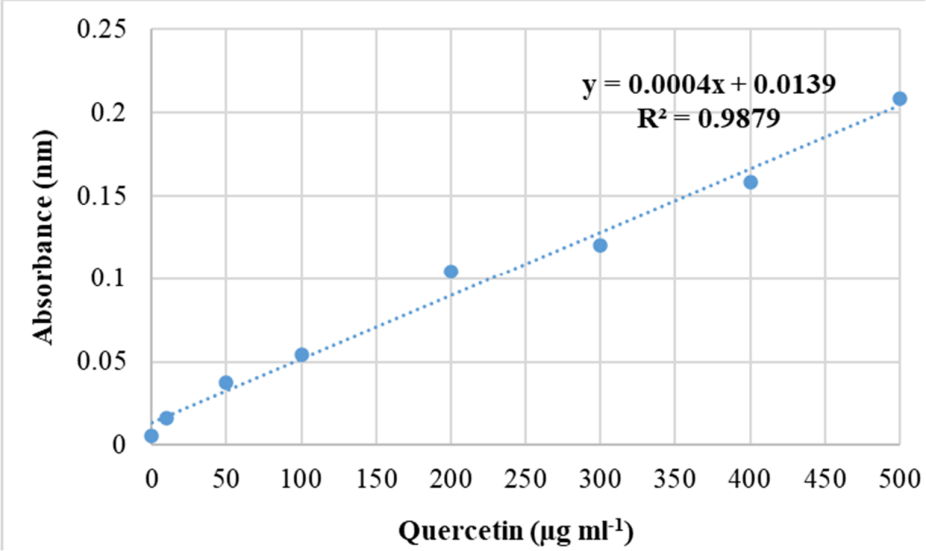

Figure 15. Standard curve used for the quantification of flavonoids (µg eq. quercetin ml^{-1}) content in *C. autumnale* extracts

The antioxidant activity of the *C. autumnale* extracts (n = 3) was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method described by Herald *et al.* (2012), Thaipong *et al.* (2006), and Brand-Williams *et al.* (1995). To obtain a standard curve, standard solutions were prepared using ascorbic acid at concentrations of 0-200 µg ml^{-1}. Antioxidant activity was expressed as equivalent mg ascorbic acid per gram of fresh plant material, calculated according to the standard curve formula (Figure 16).

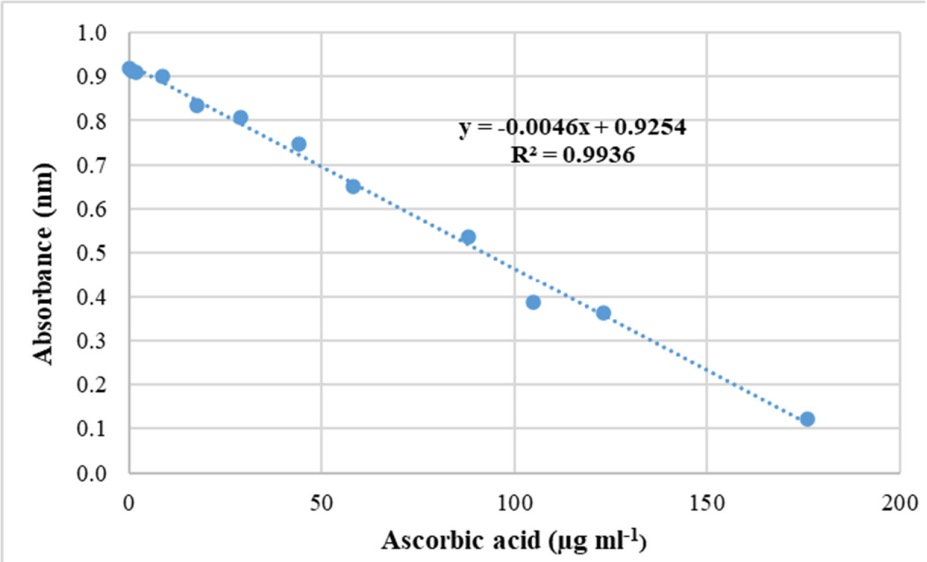

Figure 16. Standard curve used for the quantification of antioxidant activity (µg eq. ascorbic acid ml^{-1}) of *C. autumnale* extracts

Statistical analysis

For the interpretation of the results obtained from the biochemical analyses of the extracts, the two-way ANOVA statistical test, Tukey test for multiple comparisons (post-hoc), and Pearson's correlation coefficient were applied using GraphPad Prism 9.5.1 software. For UPLC analysis, the Relative Standard Deviation (%RSD), Limit of Detection (LOD), and Limit of Quantification (LOQ) are presented in Table 1.

Table 1. Relative Standard Deviation (%RSD), Limit of Detection (LOD), Limit of Quantification (LOQ) values for colchicine, colchiceine, apigenin and salicylic acid detected through UPLC

Parameters	Colchicine		Colchiceine		Apigenin		Salicylic acid	
	Peak height	Peak area	Peak height	Peak area	Peak height	Peak area	Peak height	Peak area
%RSD	1.047501	2.874768	1.406537	1.656845	2.449800	2.384739	1.546535	2.759667
LOD	0.000002	0.000000	0.000003	0.000001	0.000001	0.000000	0.000001	0.000000
LOQ	0.000006	0.000001	0.000009	0.000002	0.000003	0.000001	0.000004	0.000001

The values presented in the graphs and tables represent the mean ± standard error of the mean. Significant differences are marked in the graphs and tables with different letters ($p < 0.05$). Pearson's correlation coefficient was determined by comparing the biochemical analysis results of the alcoholic extracts with those of the aqueous extracts and by comparing the antioxidant activity of the extracts against the polyphenol and flavonoid content.

Results and Discussions

Influence of environmental conditions on secondary metabolites synthesis

Soil pH indirectly affects plants through nutrient availability, microbial activity, and metal solubility. Most plants thrive in slightly acidic to neutral soils (pH 6-7), where essential nutrients are most accessible and toxic metals are less soluble. At low pH, some nutrients become less available, whereas toxic metals can increase (Mróz, 2011).

According to the World Reference Base (2006) Soil Groups (*https://soilgrids.org*), the soil at the plant collection point is Cambisol, which usually contains moderate organic matter, sand, silt, and clay, is rich in essential nutrients, has good water retention, and has a slightly acidic to neutral pH (Twaij and Hasan, 2022).

Soil pH analysis at the collection site indicated that *C. autumnale* plants grow indicated an acidic pH of 5.8. *C. autumnale* grows ideally in light, nutritious, well-drained soils with a pH around 6. Poorly drained or highly acidic/alkaline soils can reduce plant size, flowering duration, and corm productivity, whereas neutral pH soils promote better vegetative growth, flowering, and seed production (Kaysarov and Akhmedov, 2021). Soil pH influences the uptake of nutrients in *C. autumnale* plants, which may explain the differences in plant performance and colchicine content in corms and other plant organs (Mróz, 2011).

The annual average temperature at the plant material collection point in 2021 was 6.3 °C and the annual relative humidity was 81.5%. The average temperature in spring was 4.24 °C, and the relative humidity was 82.73%; in summer, the temperature rose to an average of 17.54 °C, and the %RH (relative humidity) lowered to 75.73%; and during autumn, the average temperature was 6.77 °C, while %RH remained approximately constant at 75.52%. During winter (before the collection of plant material), the average temperature was -3.33 °C, with %RH of 90.9%. *C. autumnale* is adapted to temperate climates and can initiate growth at minimum positive air temperatures of 3-5 °C, with the growing season beginning shortly after snowmelt. High relative humidity (e.g., 81.5%) helps maintain soil moisture, which is beneficial for growth, improves plant vigor, and prolongs flowering duration. Seasonal and environmental factors, including temperature and humidity, influence the biosynthesis of secondary metabolites, with higher phenolic and flavonoid contents often linked to stress conditions (Davoodi *et al*., 2021).

Importance of choosing the right extraction methods and solvents

Soxhlet extraction is generally more effective than infusion and other conventional methods for extracting colchicine and other compounds from *C. autumnale* plants; however, advanced modern techniques may offer even higher yields and efficiency. In general, the optimal extraction method depends on the specific plant material, target compounds, and intended application (Wang and Weller, 2006; Danlami *et al*., 2014; Jibhkate *et al*., 2023). Conventional extraction methods include maceration, reflux, and Soxhlet extraction using solvents of varying polarities, such as methanol, ethanol, chloroform, and acetone. According to Abidin *et al*. (2015), Soxhlet extraction with methanol provides the highest colchicine yield (3.49% w/w from seeds), followed by reflux and maceration, with methanol being the most effective solvent.

Advanced extraction methods, such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and homogenizer-assisted extraction (HAE), generally achieve higher extraction yields and shorter processing times than conventional methods, such as Soxhlet and infusion (Chuo *et al*., 2020; Jha and Sit, 2021). For example, optimized UAE conditions (ultrasonication power: 602.4 W, time: 42 min, temperature: 64 °C) yielded 0.238% colchicine from bulbs (Çankaya *et al*., 2018), whereas SFE achieved >98% recovery in 110 min and matched the yield of conventional methods (Ellington *et al*., 2003). In addition, ultrasonication-based extraction (UBE) outperforms traditional extraction methods in terms of colchicine content and efficiency (Alqarni *et al*., 2022). The advantages of advanced extraction methods include shorter extraction times, lower temperatures, reduced solvent use, and eco-friendliness compared to conventional methods (Ellington *et al*., 2003; Çankaya *et al*., 2018; Alqarni *et al*., 2022).

However, these methods may not always be accessible in all settings. Soxhlet extraction can provide high yields of certain compounds, such as polyphenols, flavonoids, and antioxidants, and is sometimes superior to maceration or UAE for specific plant materials (Putra *et al*., 2022; Mokaizh *et al*., 2024). Infusion and decoction are simple, water-based methods suitable for extracting polar, water-soluble compounds; but they are less efficient for non-polar or less soluble bioactives and generally yield less extract than Soxhlet or advanced techniques (Abubakar and Haque, 2020).

The study of Abidin *et al*. (2015) that compares extraction methods for *Colchicum autumnale* seeds found that Soxhlet extraction produced the highest colchicine yield, especially when using methanol as the solvent, outperforming both the maceration and reflux methods. For related species (*Colchicum triphyllum*), both Soxhlet and infusion methods effectively extract alkaloids and polyphenols; however, Soxhlet extraction with methanol often yields higher concentrations of bioactive compounds, including colchicine derivatives. Infusion is effective for some phenolics and alkaloids, but generally yields less than Soxhlet extraction (Senizza *et al*., 2020).

Soxhlet extraction is a reliable and accessible method for extracting colchicine and polyphenols from *Colchicum autumnale*, offering high yields of these compounds, but it has both notable advantages and limitations. The positive aspects of Soxhlet extraction include high extraction efficiency, accessibility, and reproducibility. Soxhlet extraction yielded higher amounts of colchicine than maceration and was comparable to or slightly better than reflux extraction, especially when methanol was used as a solvent. This method is straightforward, does not require advanced equipment, and is suitable for settings lacking modern extraction technologies. Soxhlet extraction provides consistent results because of its continuous solvent cycling, ensuring the thorough extraction of target compounds. The disadvantages of Soxhlet extraction include high solvent and energy consumption, thermal degradation risks, and long extraction time (Abidin *et al*., 2015; Sridhar *et al*., 2021).

Recent research highlights that ultrasound-assisted extraction (UAE), especially when combined with deep eutectic solvents (DES), is one of the most effective and sustainable methods for extracting polyphenols from plant matrices. These approaches outperform conventional methods (such as maceration or Soxhlet extraction) in terms of yield, selectivity, reduced solvent use, and lower energy consumption (Liu *et al*., 2022; Wang *et al*., 2023; Aktaş and Kurek, 2024; Palos-Hernández *et al*., 2024; Szopa *et al*., 2024), yet Soxhlet extraction may provide a reliable extraction method when these advanced techniques are not available.

Biochemical content of C. autumnale in different ontogenetic stages

Regarding the biochemical content of meadow saffron plants, according to Wildman and Pursey (1960), all organs of *C. autumnale* accumulate colchicine, which is accompanied by several alkaloids in small amounts. Colchicine and other alkaloids are abundant in the endosperm, cotyledons, seeds, perisperm, testa, and fused pericarp (Wildman and Pursey, 1960). Preliminary phytochemical studies conducted by Davoodi *et al*. (2021) on *C. autumnale* reported the presence of alkaloids, phenolic compounds, tannins, flavonoids, coumarins, saponins, terpenoids, steroids, and glycosides in alcoholic extracts. The total tropolone alkaloid content was 9.8 ± 0.3 mg of colchicine per gram of plant material (bulbs), the polyphenol content was 5.6 ± 0.4 mg of gallic acid per gram of plant material, and the flavonoid content was 3.7 ± 0.4 g of quercetin per gram of plant material, using methanol and water at an 80:20 ratio as the extraction solvent. The total colchicine content was 4.4592 ± 0.0109 mg per gram of plant material represented by the bulbs (Davoodi *et al*., 2021).

In the present study, the colchicine content in bulbs was 1.328 ± 0.003 mg per gram of plant material collected during the growth period (spring) and 0.617 ± 0.002 mg per gram of bulbs collected during the flowering period (autumn), using a 70:30 ratio of ethanol to water. By comparing with the results obtained by Davoodi *et al*. (2021), methanol may be a more suitable solvent for colchicine, and this compound has a better solubility at higher methanol concentrations. When comparing the results of various studies, it is essential to consider several factors, including the environmental conditions experienced by *C. autumnale* plants, the neighboring plant species that compete with meadow saffron for nutritional resources, the availability of soil nutrients, and the extraction method employed. Overall, the content of bioactive compounds was higher in alcoholic extracts than in aqueous extracts prepared from the same plant material (using the same mass of powdered plant material in the preparation of the extracts, namely 5 g), except for the extract from bulbs collected during the flowering period (autumn).

UPLC analysis revealed that the highest concentration of colchicine, the primary bioactive compound, was found in the alcoholic and aqueous extracts obtained from flowers (8), whereas the lowest concentration was detected in the extracts from the bulb sheaths (7) (Table 2). Colchicine, like many other secondary metabolites, contributes to plant survival through its toxic effects on herbivores and pathogens (Ghosh and Jha, 2008). This may explain the higher colchicine content in the aboveground organs of meadow saffron compared to the underground organs. Pearson's correlation coefficient analysis indicated no correlation between the colchicine content of the alcoholic and aqueous extracts of *C. autumnale* ($p = 0.165$).

Table 1. Annotation of extracts, data on the plant material and compounds detected by RP-UPLC in alcoholic and aqueous extracts obtained from *C. autumnale* organs

Ontogenetic stage	Extract number	Collection date	Voucher specimen	Plant organ	Solvent	Content (mg ml^{-1})			
						Colchicine	Colchiceine	Apigenin	Salicylic acid
Growth	1	15.05.2021	I186.542	bulbs	EtOH 70%	**0.1328 ± 0.0003** ^{e}	0.0011 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.1204 ± 0.0003 ^{e}
					H_2O	**0.0885 ± 0.0015** ^{d}	0.0002 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
	2	15.05.2021	I186.542	leaves	EtOH 70%	**0.4461 ± 0.0004** ^{h}	0.0375 ± 0.0128 ^{d}	0.0184 ± 0.0001 ^{b}	0.1547 ± 0.0001 ^{f}
					H_2O	**0.1492 ± 0.0004** ^{e}	0.0184 ± 0.00 ^{b}	0.00 ± 0.00 ^{a}	0.0296 ± 0.0041 ^{b}
	3	15.05.2021	I186.542	fruits	EtOH 70%	**0.4308 ± 0.0008** ^{h}	0.0179 ± 0.0005 ^{b}	0.00 ± 0.00 ^{a}	0.0714 ± 0 ^{c}
					H_2O	**0.2624 ± 0.0046** ^{f}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
Fruiting	4	8.07.2021	I186.555	leaves	EtOH 70%	**0.204 ± 0.0006** ^{f}	0.0042 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.2026 ± 0.0007 ^{g}
					H_2O	**0.0559 ± 0.0003** ^{c}	0.00 ± 0.00 ^{a}	0.0004 ± 0.0001 ^{a}	0.0186 ± 0.0001 ^{b}
	5	8.07.2021	I186.555	fruits	EtOH 70%	**0.3993 ± 0.0005** ^{g}	0.0257 ± 0.0006 ^{c}	0.00 ± 0.00 ^{a}	0.1342 ± 0.0008 ^{e}
					H_2O	**0.0911 ± 0.0035** ^{d}	0.0005 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
Flowering	6	5.11.2021	I186.556	bulbs	EtOH 70%	**0.0617 ± 0.0002** ^{c}	0.0024 ± 0.0014 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
					H_2O	**0.0854 ± 0.0013** ^{d}	0.00003 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
	7	5.11.2021	I186.556	bulb sheats	EtOH 70%	**0.0305 ± 0.0001** ^{b}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.0915 ± 0 ^{d}
					H_2O	**0.0095 ± 0.0003** ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
	8	5.11.2021	I186.556	flowers	EtOH 70%	**0.7815 ± 0.003** ^{g}	0.0032 ± 0.0001 ^{a}	0.3765 ± 0.0005 ^{c}	0.327 ± 0.0142 ^{h}
					H_2O	**0.1227 ± 0.0001** ^{e}	0.0048 ± 0.0001 ^{a}	0.003 ± 0.0001 ^{a}	0.066 ± 0.00005 ^{c}

Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

Comparing the content of aqueous and alcoholic extracts, it was found that a lower amount of colchicine was obtained by extraction in water than by extraction in methanol performed with Soxhlet, except for the extract from bulbs collected in autumn (6), in which a higher concentration of colchicine was identified than in the alcoholic extract prepared from the same plant material. Wildman and Pursey (1960) reported that the colchicine content in bulbs was 3 times lower in the autumn period than in spring. However, in the present study, similar concentrations of colchicine were identified in the aqueous extracts of the bulbs (1 and 6 in Table

2). In contrast, by analyzing the alcoholic extracts, it was found that this compound was present in a quantity two times higher in bulbs during the growth period (spring) than in the flowering period (autumn). Compared to the results of previously conducted research (Moroșan *et al*., 2022), in which extracts were prepared from plant material (bulbs and flowers) collected from the same meadow (in October 2019), with colchicine concentrations detected by the same method (0.119 ± 0.007 mg ml^{-1} in the bulb extract and 0.286 ± 0.015 mg ml^{-1} in the flower extract), the colchicine concentration in the extracts obtained from the material collected in 2021 was two times lower in the bulbs and approximately three times higher in the flower extract. These differences may be due to environmental conditions, time of collection, total biomass of the harvested plant material, solvent used for extraction, and extraction time.

In the present study, colchiceine (Table 2) was identified at low concentrations in extracts from immature leaves (2) and in alcoholic extracts prepared from fruits (3 and 5), and at very low concentrations in extracts from bulbs (1 and 6), mature leaves (4), and flowers (8). This compound was not detected in the aqueous or ethanolic extracts of bulb sheaths (7). Colchiceine is considered the main degradation product of colchicine (in addition to lumicolchicines) (Kurek and Barczyński, 2016); thus, it is used for quality control of plant extracts. Pearson's correlation coefficient analysis indicated no correlation between the colchiceine content of the alcoholic and aqueous extracts ($p = 0.051$).

Although the presence of demecolcine has been reported in several studies on the biochemical composition of *C. autumnale* species (Malichová *et al*., 1979; Yoneda *et al*., 1984; Herbert *et al*., 1990; Davoodi *et al*., 2021), it was not detected in the extracts prepared in this study. This may indicate that the substrate in the area from which the plant material was collected, as well as the environmental or stress conditions experienced by *C. autumnale* individuals before collection, influenced the chemical composition of these plants, as revealed by the analysis of the alcoholic and aqueous extracts (Table 2).

Apigenin (Table 2) has been identified in the aqueous extracts of flowers (8) and mature leaves (4). Apigenin is a flavone with antioxidant properties that protects plant cells from oxidative stress, which is crucial for preventing damage caused by reactive oxygen species (Madunić *et al*., 2018; Azeem *et al*., 2024) and UV-B rays (Righini *et al*., 2018). As a secondary metabolite, apigenin contributes to plant defense against pathogens and environmental stressors (Mushtaq *et al*., 2023; Azeem *et al*., 2024). Comparing these results with the data from the literature (Burzo *et al*., 2006), it was confirmed that apigenin is present in the flowers of *C. autumnale*. Apigenin has also been identified in very small amounts in ethanolic extracts prepared from young leaves (2) and in aqueous extracts from mature leaves (4). Pearson's correlation coefficient analysis indicated a correlation between the apigenin content in alcoholic and aqueous extracts ($p = 0.000003$), indicating that both water and alcohol efficiently extract this compound.

Salicylic acid (Table 2) was identified at the highest concentration in both alcoholic and aqueous extracts of flowers (8) and leaves (2 and 4); however, the use of Soxhlet and methanol as extraction solvent made it possible to extract this compound from other organs, such as bulbs collected in spring (1), fruits (3 and 5), and bulb sheaths (7). Pearson's correlation coefficient analysis indicated a correlation in the salicylic acid content between alcoholic and aqueous extracts ($p = 0.004$), a compound that can be successfully extracted using either alcohol or water as solvents. Salicylic acid is involved in the flowering process and plays an important role in protecting plants against biotic and abiotic stress (Wani *et al*., 2017). It is also involved in the regulation of physiological and biochemical processes throughout the plant life cycle, thereby influencing its growth and development (Vicente and Plasencia, 2011; Koo *et al*., 2020).

According to a study by Jung *et al*. (2011), *C. autumnale* has a life cycle that begins with the flowering stage, which falls in the autumn period, in which the flowers appear above the ground, unaccompanied by leaves, with the gynoecium buried in the soil and protected by the bulb, the anthers are exposed in the above-ground area, and adventitious roots begin to appear on the young bulb disc (developed during summer). Flowering ends with a short period in which the fruits and leaves begin to develop underground, consuming part of the nutrient reserves in the bulb. This period is followed by dormancy during winter, during which the

fruits and leaf primordia are withdrawn into the soil by contractile adventitious roots, thus being protected from low temperatures. In spring, the fruits begin to appear above the ground surrounded by leaves, with a light green color and white developing seeds, whereas starch reserves in the old bulb are progressively depleted. As the old bulb shrinks, the new bulb begins to grow and accumulate starch. During summer, the fruits mature and open, with a brownish color, releasing blackish-brown seeds, and the leaves approach senescence. The plant has a small bulb (the old bulb), which enters the maceration process simultaneously with the development of the new bulb, while the aboveground organs begin to dry out. At that time, the plants consisted only of underground bulbs surrounded by sheaths that represented the leaves that had dried up, surrounded the bulb, and appeared as a brown tunic. The period of inactivity follows, and flowering resumes (Figure 1). Thus, we chose a different approach to study *C. autumnale*'s ontogenetic stages by starting with the growth period (in spring) because it is the first true stage of new metabolic activity within the plant, and the flowering period (in autumn) relies on reserves from the previous cycle.

During the growth period, which continued into the fruiting period, the colchicine and salicylic acid content in the leaves remained relatively constant (then decreased towards senescence in the bulb sheaths), whereas in the fruits, it increased during maturation. The colchicine content in the underground organs (bulbs) during the growth period was twice as high as that during the flowering period, which can be explained by the progressive reduction in bulb biomass.

Polyphenols are compounds with multiple and varied functions that contribute to the resistance and adaptation of plants to the environment, being essential in protection against stress, defense against herbivores, antioxidant activity, and regulation of growth and development processes (Sharma *et al.*, 2019; Singh *et al.*, 2021; Šamec *et al.*, 2021; Pinto *et al.*, 2021; Zagoskina *et al.*, 2023).

In the analyses of the available plant material, it was found that during the vegetative stage, the highest polyphenol content was found in the aboveground organs, especially in the leaves (2, 4) and flowers (8), and the polyphenol content determined was higher in the bulbs collected during the spring (1) than in the bulbs collected in the fall (6). In contrast, the analysis of the alcoholic extracts suggested that the accumulation of polyphenols in the fruits (3 and 5) would occur during maturation; however, in the aqueous extracts, this content remained approximately constant (Figure 17). Pearson's correlation test revealed a correlation between the polyphenol content of alcoholic and aqueous extracts ($p = 0.007$). This suggests that both ethanol/methanol and water can extract these compounds in a comparable manner, yet alcoholic solvents yield better results.

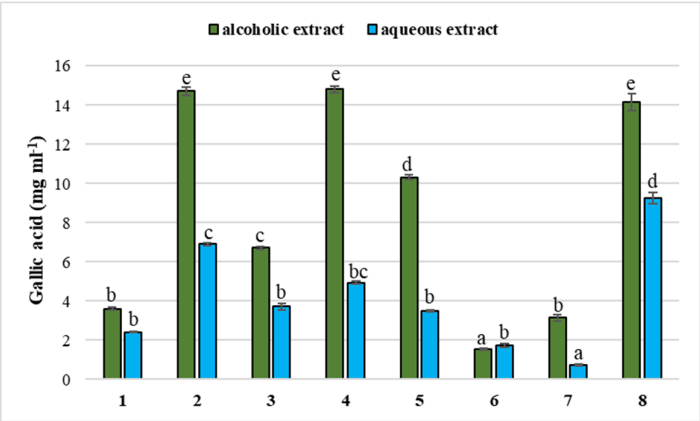

Figure 17. Polyphenols (mg eq. gallic acid ml^{-1}) content in alcoholic and aqueous *C. autumnale* extracts
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract
Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

Similarly to the analysis of the polyphenol content in alcoholic and aqueous extracts from plant organs of *C. autumnale*, it was found that during the growth stage (spring period) the highest flavonoids content is found in the aboveground organs, especially in leaves (2, 4), flowers (8) and fruits (3, 5). Flavonoids are phenolic compounds that regulate plant development and contribute to the pigmentation of flowers and fruits, which are essential characteristics for attracting pollinators (Mierziak *et al*., 2014; Mathesius, 2018), which explains their abundant presence in these organs. Moreover, these compounds accumulate in the epidermis and protect plants from harmful solar radiation (Shah and Smith, 2020; Ferreyra *et al*., 2021). Flavonoids exhibit strong antioxidant properties, helping to eliminate reactive oxygen species (ROS), and thus play a significant role in plant stress tolerance (Khalid *et al*., 2019; Dias *et al*., 2021; Shomali *et al*., 2022), as well as against pathogens and herbivores, increasing their resistance to biotic stress (Mathesius, 2018; Alseekh *et al*., 2020; Shah and Smith, 2020). Moreover, flavonoids are involved in cellular signaling processes, such as root-rhizosphere interactions, an interaction in which they can stimulate or inhibit microbial activity, affect nutrient uptake, and mediate, influence, or even determine allelopathic interactions (Hassan and Mathesius, 2012; Shah and Smith, 2020), which explains their presence, even in lower quantities, in underground organs.

The flavonoid content determined in the present study was higher in the alcoholic extract from mature fruits (5) than that prepared from immature fruits (3), whereas in the homologous aqueous extracts, it was similar in both extracts. Simultaneously, the flavonoid content in the leaves was similar in the alcoholic extracts (2 and 4), but higher in the aqueous extract prepared from leaves collected during the growth period (2) than in the aqueous extract from mature leaves (4). In addition, the flavonoid content was much higher in bulbs collected during spring (1) than in bulbs collected in autumn (6), an observation resulting from the analysis of both types of extracts (aqueous and alcoholic) (Figure 18). Pearson's test revealed a correlation between the flavonoid content of the alcoholic and aqueous extracts ($p = 0.00002$).

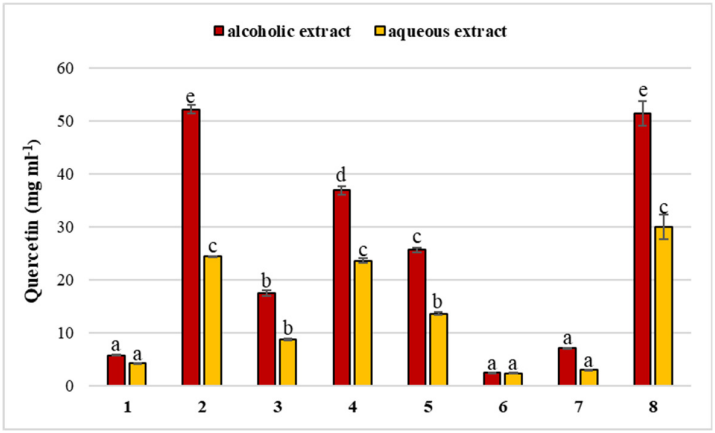

Figure 18. Flavonoids (mg eq. quercetin ml^{-1}) content in alcoholic and aqueous *C. autumnale* extracts
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract; Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

The polyphenol and flavonoid contents remain relatively constant in the leaves during the growth period towards fruiting and are also the most abundant in the leaves and flowers compared to the fruits, bulb sheaths, and bulbs. In the bulbs, this content decreases or remains relatively constant, and in the fruits, it increases significantly during maturation, providing additional protection against stress, pests, and herbivores.

The antioxidant activity (expressed as mg of ascorbic acid per gram of dry plant material) was considerably higher in alcoholic extracts prepared from the vegetative and reproductive organs of *C. autumnale* plants than in aqueous extracts (Figures 17 and 18). All meadow saffron organs exhibited high antioxidant

activity, except for the extract from bulbs collected in spring. In the case of aqueous extracts, the detected antioxidant activity was weak, and no significant variations were observed between the extracts. Pearson's correlation coefficient analysis indicated no correlation between the antioxidant activities of the alcoholic and aqueous extracts ($p = 0.162$).

The highest antioxidant activity was detected in alcoholic extracts from fruits (3 and 5), bulbs collected in autumn (6), flowers (8E), and leaves (2, 4, and 7), without significant variations (Figure 17). However, polyphenols and flavonoids were detected in extracts from flowers (8), leaves (2 and 4, but not 7), and fruits (3 and 5). Flavonoids may be the main compounds contributing to the antioxidant activity in the aboveground organs of meadow saffron, although there are significant differences in flavonoid content between the alcoholic extracts prepared from these organs (Figure 19 and 20).

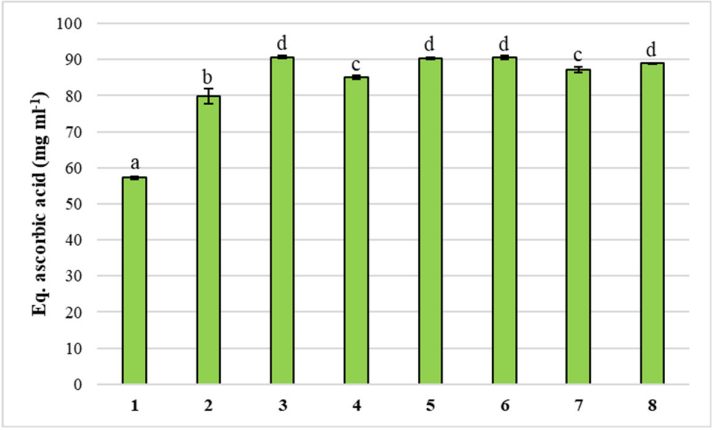

Figure 19. Antioxidant activity (mg eq. ascorbic acid ml^{-1}) of alcoholic extracts
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract; Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

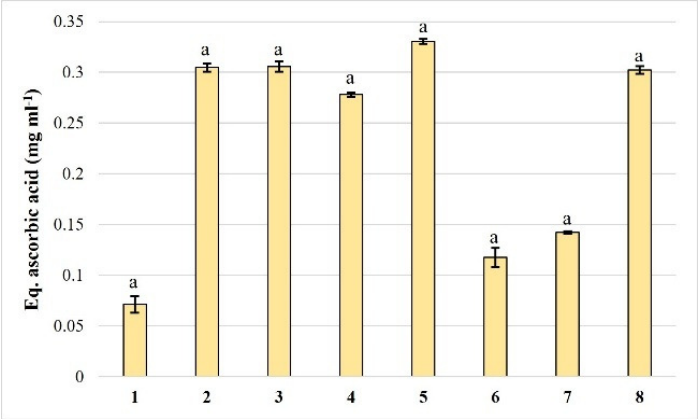

Figure 20. Antioxidant activity (mg eq. ascorbic acid ml^{-1}) of aqueous extracts
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract; Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

Pearson's correlation test also revealed a strong correlation between polyphenol and flavonoid content (as expected) in both extract types (alcoholic: $p = 0.0007$ and aqueous: $p = 0.0002$). When the polyphenol and

flavonoid contents of the alcoholic extracts were compared to the antioxidant activity of the extracts, no significant correlations were observed (polyphenols vs. DPPH: $p = 0.342$; flavonoids vs. DPPH: $p = 0.332$). Only the polyphenols and flavonoids from the aqueous *C. autumnale* extracts displayed a strong correlation with the antioxidant activity of the extracts (polyphenols vs. DPPH: $p = 0.034$; flavonoids vs. DPPH: $p = 0.019$), although it was weak, and there were no significant differences identified between the antioxidant activities of the aqueous extracts (Figure 20). The low antioxidant activity of the aqueous extracts may indicate the presence of compounds with higher solubility in alcohol or compounds that are either insoluble or less soluble in water. In addition, the polyphenol and flavonoid content detected in alcoholic extracts from bulbs collected in autumn (6) was very low, yet its antioxidant activity increased (Figure 19). This may provide clues regarding the presence of other compounds in this extract that contribute to the increased antioxidant activity.

The findings of this study confirm that all plant organs of *C. autumnale* accumulate colchicine and suggest that the time of collection, extraction method, and solvents significantly affect the yield of key bioactive compounds in *C. autumnale*. The alcoholic extracts were richer in secondary metabolites than their homologous aqueous extracts (except for the autumn bulb extracts, where the aqueous extract had a higher concentration). Apigenin and salicylic acid were detected at their highest concentrations in the flower and leaf extracts, respectively, indicating their roles in flowering and plant defence. Overall, the highest concentrations of polyphenols and flavonoids were observed in the aboveground organs during the growth stage, particularly in the leaves and flowers. Moreover, the antioxidant activity of the alcoholic extracts was significantly higher than that of the aqueous extracts, particularly in the extracts from the leaves, fruits, and flowers. This underscores the need for carefully planned and executed collection and processing protocols to maximize the extraction of desired substances from *C. autumnale*.

Conclusions

Soxhlet extraction is a reliable and accessible method for extracting colchicine and polyphenols from *C. autumnale* plants, with high efficiency, accessibility, and reproducibility. It provides consistent results owing to continuous solvent cycling, ensuring thorough extraction of target compounds when modern extraction techniques are unavailable. Usually, in extract preparation, the evaporation of solvents using a rotary evaporator is ideal for obtaining a more concentrated alcoholic extract, whereas lyophilization is better for aqueous extracts because it preserves the extract's integrity, given that many bioactive secondary metabolites (such as polyphenols and alkaloids) are thermolabile, thus avoiding thermal degradation of compounds. This is especially applicable to colchicine, which is a thermolabile compound in aqueous solutions but is more stable in ethanol.

This study revealed how colchicine and phenol concentrations vary across different developmental stages (growth, flowering, and fruiting) in various plant organs, providing new insights into the dynamic accumulation patterns throughout the plant's ontogenetic cycle. Flowers contain the highest colchicine concentrations, whereas bulbs and other organs exhibit varying levels depending on the season, emphasizing the influence of both plant organ and harvest time on bioactive compound content. Alcoholic extracts generally yield higher amounts of colchicine and phenols than water extracts, except in certain cases, such as bulbs during flowering, highlighting the importance of solvent choice in maximizing bioactive compound extraction. By analyzing environmental parameters, ontogenetic factors, and extraction methods, this study provides a comprehensive understanding that can inform optimized harvesting and processing strategies for medicinal and phytotherapeutic applications. The application of UPLC and spectrophotometry can offer precise quantification of secondary metabolites, setting a methodological precedent for future phytochemical studies in this species and potential applications in other plants with similar properties.

The biochemical diversity in *C. autumnale*, especially its high alkaloid content (notably colchicine), plays a key role in plant defense against herbivores and pathogens and supports survival during dormancy. Metabolite distribution, such as the abundance of colchicine and polyphenols in flowers, fruits, and leaves, reflects adaptation to ecological pressures and reproductive strategies. These compounds also contribute to stress tolerance and energy storage, influencing the ecological interactions and fitness of plants. Variations in the secondary metabolite content between plant parts are critical for pharmacognostic identification and safety, as colchicine has a narrow therapeutic index.

Authors' Contributions

Conceptualization: ICM, MMZ; Data curation: ICM, MM; Formal analysis: ICM, MM; Funding acquisition: MM, MMZ; Investigation: ICM, MM; Methodology: ICM, MM; Project administration: MMZ; Resources: MMZ, MM; Software: ICM, MM; Supervision: MMZ, MM; Validation: MMZ, MM; Visualization: ICM, MM, MMZ; Writing - original draft: ICM; Writing - review and editing: ICM, MM, MMZ.

All authors read and approved the final manuscript

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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An insight into the biochemical content of *Colchicum autumnale* L. throughout its ontogenetic cycle

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Abstract

Meadow saffron (*Colchicum autumnale* L.) is a poisonous perennial species with an unusual ontogenetic cycle. This study investigated its biochemical composition during three key developmental phases: growth, fruiting, and flowering. Aqueous and alcoholic extracts from different plant organs were analyzed using UPLC and spectrophotometry to evaluate the influence of developmental stage, extraction method, and solvent on secondary metabolite accumulation, with implications for extract potency and pharmacological properties. Colchicine and phenolic compounds were detected in all organs, though their concentrations varied with extraction technique and harvest time. Flowers contained the highest colchicine levels (0.7815 mg ml^{-1} in ethanol, 0.1227 mg ml^{-1} in water), while bulbs accumulated lower amounts that decreased from spring to autumn. In general, alcoholic extracts yielded more bioactive compounds than aqueous ones. Flowers and leaves also contained significant levels of polyphenols, with ethanol extracts showing markedly higher concentrations than water extracts. Antioxidant assays revealed strong activity in all alcoholic extracts (79.8 - 90.7 mg ml^{-1} eq. ascorbic acid), except for spring bulbs (57.2 mg ml^{-1}), whereas aqueous extracts exhibited negligible antioxidant capacity (0.07 - 0.3 mg ml^{-1}). These results highlight the dynamic phytochemistry of *C. autumnale*, demonstrating that the concentration of bioactive compounds is strongly influenced by plant organ, developmental stage, extraction method, and solvent. Such variability should be considered in future research and practical applications, including ecological studies, weed management, and standardization of extracts for medicinal use.

Keywords: antioxidant activity; colchicine; flavonoids; polyphenols; Soxhlet

Introduction

Colchicum autumnale, a member of the Colchicaceae family, is a toxic bulbous perennial plant typically found in mountain grasslands (Pop *et al*., 1983). This species thrives in various damp grassland environments, from hilly to mountainous regions, and it is widely distributed, particularly in Europe, being considered abundant (Chadburn, 2014).

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The plant features a dense tuber that produces an axillary bud, which gives rise to one to three violet flowers in autumn (Ștefan and Ivănescu, 2002). The leaves are basal and originate from the primordia of the underground bulb disc. The flower consists of a perigone with six pinkish-purple tepals, six stamens, and an extended perigone tube that forms on the bulb disc alongside the ovary, shielded by bulb cataphylls (Pop *et al.*, 1983). In spring, the fruit emerges aboveground and is encircled by 3-4 oblong-lanceolate leaves (Ștefan and Ivănescu, 2002). The fruit is a trilocular capsule containing colchicine-rich seeds (Pop *et al.*, 1983).

The plant's distinctive ontogenetic cycle begins with autumn flowering, followed by a dormant winter period during which the fruit remains underground. Spring marks the peak of photosynthetic activity, with fruits and leaves appearing aboveground. Plants enter a period of inactivity after fruit ripening (Jung *et al.*, 2011) (Figure 1).

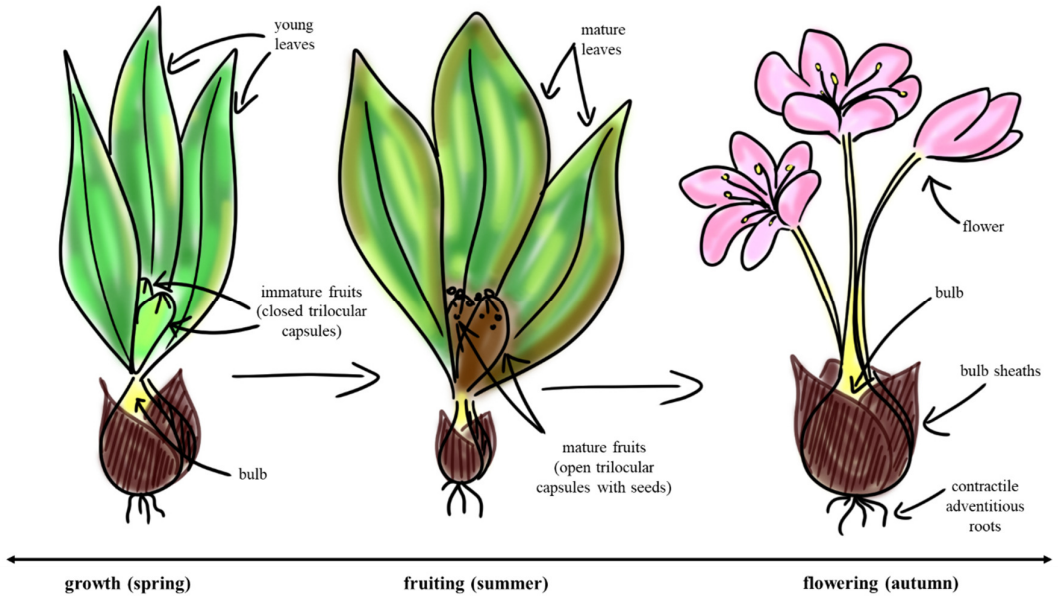

Figure 1. Schematic representation of *Colchicum autumnale* plants' ontogenetic cycle

Research on *C. autumnale* spans pharmacology, ecology, and agriculture; however, several important gaps remain. Recent studies have highlighted organ-specific and environment-dependent variations in metabolite content (Boboev *et al.*, 2023; Dincheva *et al.*, 2025). However, systematic studies on how environmental factors, developmental stages, and plant parts influence the phytochemical spectrum are lacking. Although optimized methods for colchicine extraction exist, there is limited research on efficient and standardized extraction and quantification protocols for phytochemicals in *C. autumnale* (Çankaya *et al.*, 2018).

According to Burzo *et al.* (2005), aboveground organs contain starch, sucrose, lipids, phytosterols, benzoic acid, salicylic acid, alkaloids, and tannins. The alkaloids present in underground organs are colchicine, 2-demethylcolchicine, colchicoside, 2-desmethyldeacetylcolchicine, demecolcine, thiocolchicoside, 3-demethylcolchicine, 3-methylcolchicine, 3-demethyl-β-lumicolchicine, α-lumicolchicine, β-lumicolchicine, γ-lumicolchicine, colcamine, colchicene, colchicerine, N-methyldesacetylcolchicine, N-deacetyl-N-methylcolchicine, N-formyl-deacetylcolchicine, and O-demethyl-N-deacetylcolchicine. Aerial organs contain salicylic acid, chelidonic acid, and alkaloids. The alkaloids found in the above-ground organs of plants belonging to this species are 2-acetyl-2-demethylcolchicine, 2-acetyl-3-methylcolchicine, colchifoline, demecolceine, demecolcine, N-acetyl-demecolcine, and O-acetylcolchiceine and apigenin.

Colchicine ($C_{22}H_{25}O_6N$, N-[(7S)-1,2,3,10-tetramethoxy-9-oxo-6,7-dihydro-5H-benzo[a]heptalen-7-yl] acetamide), the primary bioactive component extracted from *C. autumnale*, is an alkaloid that inhibits mitosis by attaching to tubulin and preventing its polymerization, resulting in autopolyploidization. Utilizing the antimitotic property of colchicine, researchers have developed plants with enhanced traits (compared to their diploid counterparts) for commercial applications (Manzoor *et al*., 2019). Colchicine has been widely used in the medical field to address a range of ailments, such as gout, familial Mediterranean fever, skin vasculitis, and Paget's and Behçet's diseases (Nerlekar *et al*., 2014). Recently, colchicine has garnered attention in clinical trials for the treatment of pericarditis (Shah *et al*., 2016), cancer (Zhang *et al*., 2019), and COVID-19 (Lopes *et al*., 2021).

In addition to colchicine and its derivative compounds, *C. autumnale* contains other secondary metabolites that confer protection throughout its ontogenetic cycle. Polyphenols are important biochemical compounds in plants that are involved in the oxidative stability of different parts of the plant and in chemical defense mechanisms, such as allelopathy (Li *et al*., 2014; Singh *et al*., 2021; Zagoskina *et al*., 2023). They function by preventing or inhibiting the generation of reactive oxygen species (ROS) (Agati and Tattini, 2010; Singh *et al*., 2021; Dini and Grumetto, 2022; Zagoskina *et al*., 2023). Polyphenols contribute to the thickening of the secondary cell wall, conferring mechanical resistance and rigidity that prevent the destruction of healthy tissues in the vicinity of the affected tissues (Gunnaiah *et al*., 2012; Singh *et al*., 2021), and play a role in promoting tissue sclerification (Di Ferdinando *et al.*, 2014). Moreover, they modulate plant growth, development, and signaling pathways, influencing processes such as cell division and hormone activity (Dini and Grumetto, 2022; Zagoskina *et al*., 2023).

Flavonoids are polyphenols that play crucial roles in seed germination, plant growth, and development (Wang *et al*., 2022; Zhuang *et al*., 2023). These compounds protect plants against biotic and abiotic stress (Shomali *et al*., 2022; Zhuang *et al*., 2023), serve as significant signaling molecules (Mathesius, 2018; Shah and Smith, 2020; Wang *et al*., 2022; Kumar *et al*., 2024), and function as allelopathic compounds (Mathesius, 2018; Shah and Smith, 2020; Zhuang *et al*., 2023), phytoalexins (Mathesius, 2018; Wang *et al.*, 2022; Zhuang *et al*., 2023), detoxifying (Dias *et al*., 2021; Zhuang *et al*., 2023), and antimicrobial agents (Pollastri and Tattini, 2011; Agati *et al*., 2012; Shah and Smith, 2020; Wang *et al*., 2022). Flavonoids interact with membrane phospholipids, thereby protecting chloroplast membranes against photooxidation (Agati *et al*., 2013; Ferreyra *et al*., 2021; Laoué *et al*., 2022) and inhibiting singlet oxygen ($^1O_2$) (Agati *et al*., 2007). Furthermore, flavonoids can facilitate the morpho-anatomical adaptation of plants to stress conditions (Agati and Tattini, 2010; Agati *et al*., 2013; Buer *et al*., 2013; Shah and Smith, 2020; Shomali *et al*., 2022; Zhuang *et al*., 2023).

Studying and comparing alcoholic and aqueous extracts of *C. autumnale* organs at different ontogenetic (developmental) stages is important because the extraction method, plant organ, and developmental stage significantly influence the chemical composition and biological activity of the extracts. Alcoholic (ethanolic) and aqueous extracts yield different profiles of bioactive compounds. For example, ethanolic extracts of *C. autumnale* leaves showed much higher acaricidal activity than aqueous extracts, indicating that ethanol extracts contain more potent or different active compounds from the leaves than water (Norouzi *et al*., 2020). The concentration and types of alkaloids and other bioactive compounds in *C. autumnale* vary significantly depending on the plant developmental stage. Although studies have focused on the organ and solvent, it is well established in phytochemistry that the ontogenetic stage impacts secondary metabolite content, which in turn affects extract potency and pharmacological properties (Ellington *et al*., 2003).

This study aimed to perform a comparative analysis of the aqueous and alcoholic extracts of *C. autumnale*, focusing on their phytochemical composition and antioxidant potential. Specifically, the objectives were to quantify and compare the contents of colchicine, total polyphenols, and flavonoids in aqueous and alcoholic extracts; evaluate and compare the antioxidant activity of the extracts and examine its correlation with polyphenol and flavonoid levels; investigate the distribution of these bioactive compounds across different

plant organs; assess the influence of ontogenetic stage on colchicine accumulation, phenol content, and antioxidant potential; and identify the extraction method, plant organ, and developmental stage that maximizes the yield of pharmacologically relevant compounds, providing insight into potential applications in medicine and phytotherapy. An analysis of the biochemical composition of a plant species may also provide insights into the mechanisms utilized to adapt to stress induced by interspecific competition, herbivores, pathogens, pests, and adverse environmental conditions. By comparing extracts from different organs and developmental stages, researchers can identify the optimal combination for maximum yield of desired compounds. Understanding these differences will facilitate the development of more effective medicines and biopesticides.

Materials and Methods

Plant material collection

The plant material comprising bulbs, leaves, and immature fruits (Figures 2 and 4) of *C. autumnale* was collected on 15.05.2021 (with the voucher specimen deposited in the Herbarium of the Faculty of Biology of the "Alexandru Ioan Cuza" University in Iasi, I186.542), which corresponds to the active growth period in the ontogenetic cycle of this species. Leaves and mature fruits (Figure 3) were obtained during the fruiting period, on 8.07.2021 (I186.555), and bulbs and flowers (Figure 5) were collected on 5.11.2021, during the flowering period (I186.556) from a meadow in Voroneț, Suceava County (Figure 6A and 6B). Plant material from 32 individual plants was collected at each ontogenetic stage (96 plants in total). The coordinates were recorded using GPSEssentials: latitude 47.493889° longitude 25.885833° and imported into the NASA Worldview Map (*https://worldview.earthdata.nasa.gov*) (Figure 7).

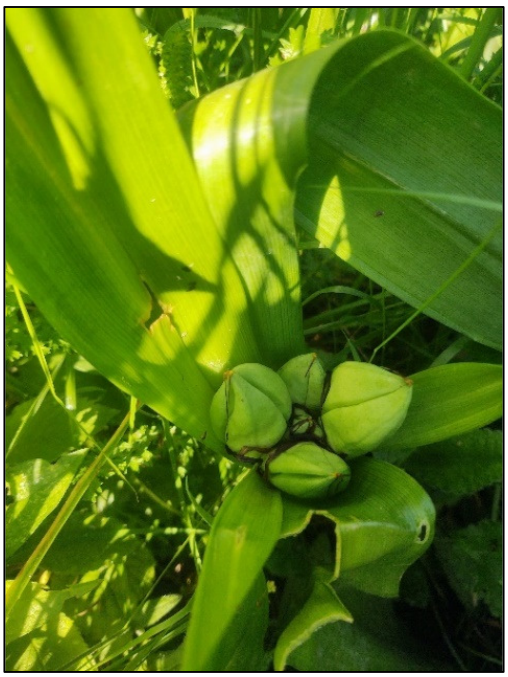

Figure 2. Immature *C. autumnale* fruits (closed trilocular capsule), photographed and collected on 15.05.2021

Figure 3. Mature *C. autumnale* fruits (open trilocular capsule with seeds), photographed and collected on 8.07.2021

Figure 4. Young *C. autumnale* plants with leaves, immature fruits, bulbs, and roots, collected on 15.05.2021

Figure 5. *C. autumnale* plants, showing only flowers, bulbs, and roots, collected during the flowering stage, on 5.11.2021

Soil samples were collected from the plant material harvest point, dried, and ground into fine powder. Five grams of soil powder was weighed and dissolved in 12.5 mL of distilled water (1:2.5 m/V). The solution thus obtained was analyzed using a pH meter, according to the method described by Blakemore *et al*. (1987), recommended by the Food and Drug Administration (FDA), and the results were confirmed by comparison with those obtained using pH indicator paper.

Data on the average temperature and relative humidity were obtained from the NASA POWER Daily API (*https://power.larc.nasa.gov*). The soil type was identified according to the World Reference Base (2006) Soil Groups (*https://soilgrids.org*) for the plant material collection point.

(A) **(B)**

Figure 6. The habitat from which the individuals of *C. autumnale* were collected on 15.05.2021 (A) and in 5.11.2021 (B)

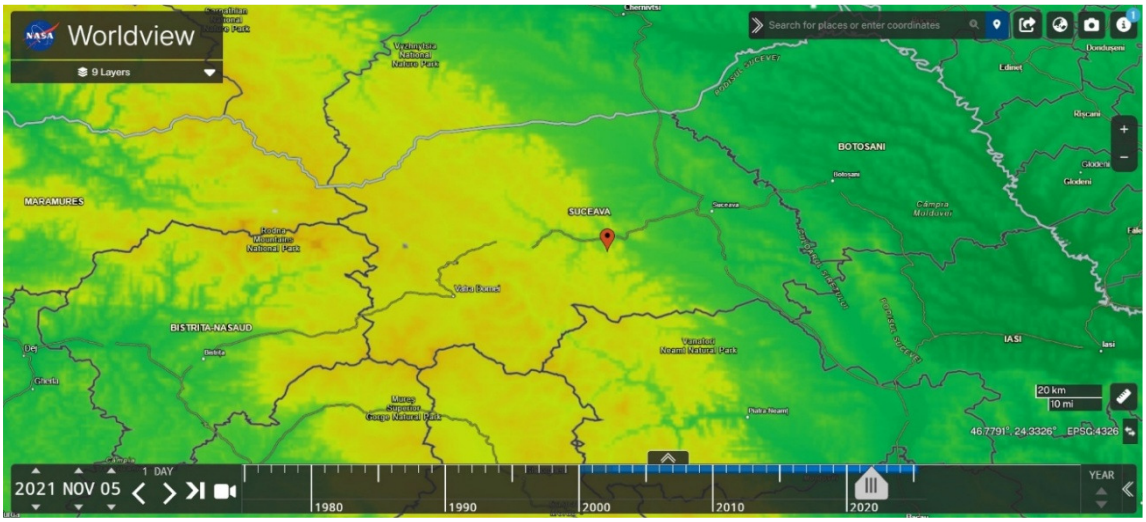

Figure 7. *C. autumnale* individuals harvest point (latitude 47.493889° longitude 25.885833°) (*https://worldview.earthdata.nasa.gov*)

Plant material preparation

The collected plant material was subjected to oven treatment at 65 °C for 12 h to inhibit enzymatic reactions, followed by desiccation in the dark at ambient temperature (23 ± 2 °C) for 7 days (Figure 8A and 8B).

(A) **(B)**

Figure 8. Dried plant parts of *C. autumnale*: leaves and fruits (A); bulb sheaths, bulb fragments, and flowers (B)

Extract preparation

Methanol and ethanol are the most effective solvents for extracting colchicine from *Colchicum autumnale*, with methanol generally yielding the highest extraction efficiency, followed closely by ethanol. In general, aqueous and ethanol extracts are also used for broader phytochemical and biological activity studies,

however, for maximum colchicine yield, methanol and ethanol are preferred (Çankaya *et al*., 2018; Norouzi *et al*., 2020)

Alcoholic extracts were prepared using a Soxhlet apparatus by extracting 5 g of plant material from each organ in absolute methanol, which was recirculated for 8-10 hours (method adapted from Rocchetti *et al*., (2019). The solvent was evaporated to dryness using a rotary evaporator (IKA RV3, Staufen, Germany), and the dry extract was re-dissolved in 50 mL of 70% ethanol.

Aqueous extracts were prepared by infusing 5 g of plant material from each collected organ in distilled water (Figure 9) for 24 h on a shaker at 25 °C, followed by filtration through filter paper. The solvent was evaporated to dryness using a lyophilizer (Christ Alpha 3-4, Osterode am Harz, Germany), and each dry extract was re-dissolved in 50 mL of distilled water.

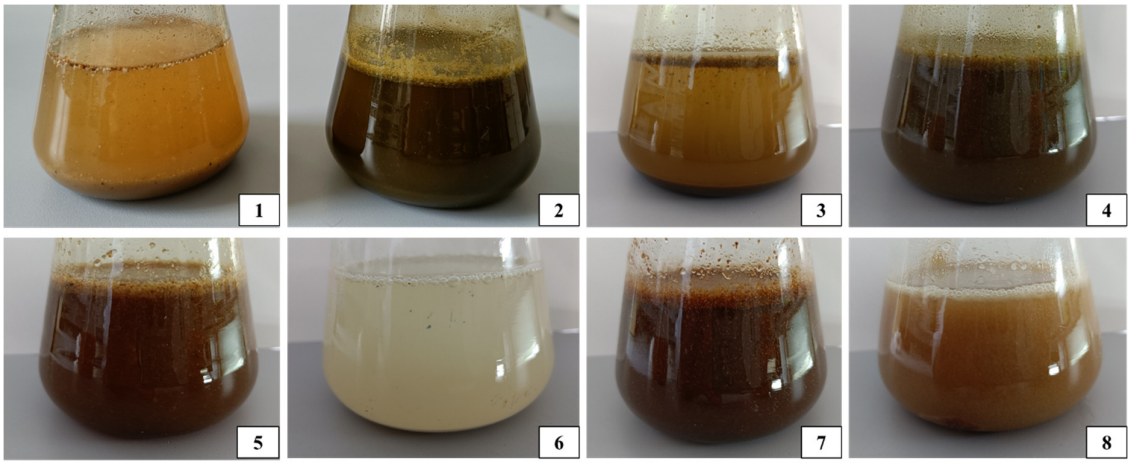

Figure 9. Aqueous extracts from *C. autumnale* organs before filtration
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract

Finally, eight alcoholic and eight aqueous extracts were obtained from the same mass of plant organ powder (5 g) and concentrated using 50 mL of solvent. The extracts were annotated as follows: 1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract.

Extract content analysis

The main bioactive compounds from the obtained extracts were identified and quantified through reversed-phase ultra-performance liquid chromatography (RP-UPLC), using a Shimadzu Prominence UPLC system (2 LC20AD pumps, SIL20AC autosampler, oven CT20AC, SPD M20A DAD detector, RF 20A XS florescence detector) coupled to a Zorbax Eclipse XDB - C18 column (length 250 mm, particle size 3 microns). Colchicine, colchicine, demecolcine, apigenin (purity ≥ 95%), and salicylic acid (purity ≥ 99%) were purchased from Sigma Aldrich (sigmaaldrich.com) and used for the construction of calibration curves in the range of 0.312-2.5 μg ml^{-1} for colchicine, 0.025 - 1.6 μg ml^{-1} for colchiceine, 0.05-0.5 μg ml^{-1} for demecolcine, 0.025 - 2 μg ml^{-1} for apigenin, and 0.04 - 4 μg ml^{-1} for salicylic acid.

For the detection of colchicine, colchicine, demecolcine, and apigenin, acetonitrile was used as mobile phase A, and 3% acetic acid was used as mobile phase B (Sigma Aldrich, Germany), according to the method used by Alali *et al*. (2004), which has also been used in previous studies (Moroșan *et al*., 2022). Elution was performed at a flow rate of 1 ml/min using the following program: 0-3 min 90% B isocratic, 3-11 min 90-40% B gradient, 11-12 min 40% B isocratic, 12-13 min gradient 40-90% B, 13-20 min 90% B isocratic.

For the detection of salicylic acid, the method of Toiu *et al.* (2011) was adapted, using 0.85% orthophosphoric acid as mobile phase A and acetonitrile as mobile phase B. Elution was performed at 1 ml/min using the program: 0-2 min 5% B isocratic, 2-5 min 5-80% B gradient, 5-10 min 80-100% B gradient, 10-12 min 100% B isocratic, 12-18 min 5% B isocratic.

Colchicine (Figure 10), colchiceine (Figure 11), and apigenin (Figure 12) were detected at 245, 254, and 350 nm, respectively, and eluted at 13.4 ± 0.08 min. Demecolcine was not detected in any of the extracts. Salicylic acid (Figure 13) was detected using a fluorescence detector at excitation and emission wavelengths of 310 and 450 nm, respectively. Chromatographic data were acquired using Shimadzu LC Software and interpreted manually by comparing the retention times and detection spectra of the standard compounds with those of the samples (extracts).

A volume of 20 µL was injected to detect compounds in standard solutions and extract samples for five technical replicates. The concentrations of the compounds in the extracts were quantified according to the area of the detected peaks and calculated according to the standard curves (Figure 10, 11, 12 and 13).

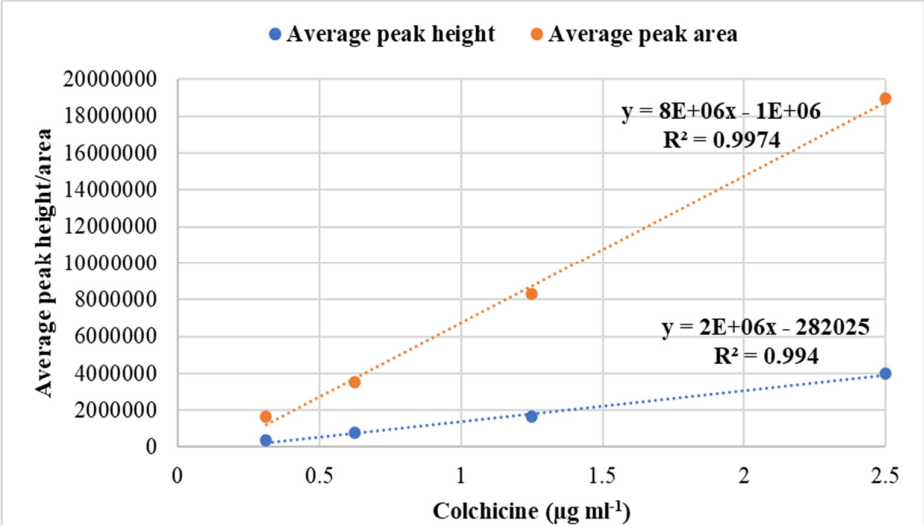

Figure 10. Standard curve used for the quantification of colchicine (µg ml^{-1}) content in *C. autumnale* extracts

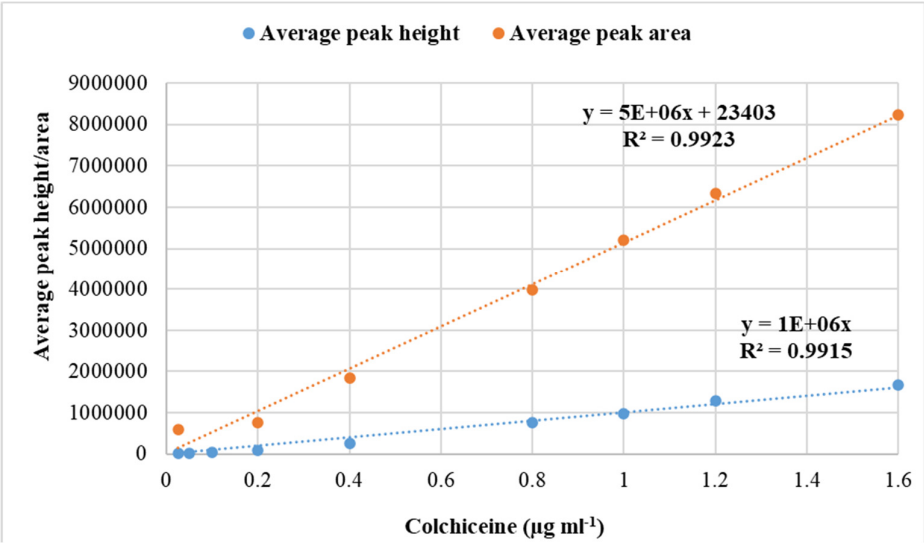

Figure 11. Standard curve used for the quantification of colchiceine (µg ml^{-1}) content in *C. autumnale* extracts

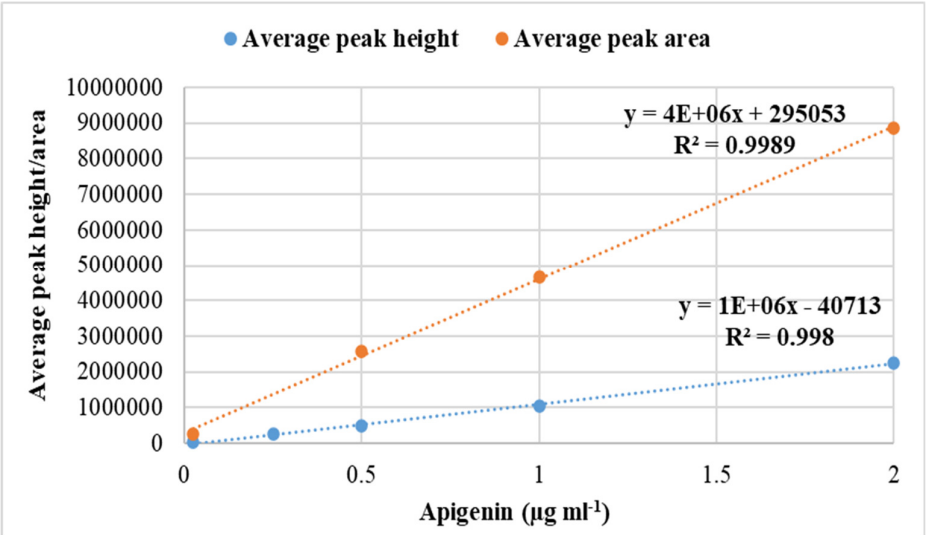

Figure 12. Standard curve used for the quantification of apigenin (µg ml^{-1}) content in *C. autumnale* extracts

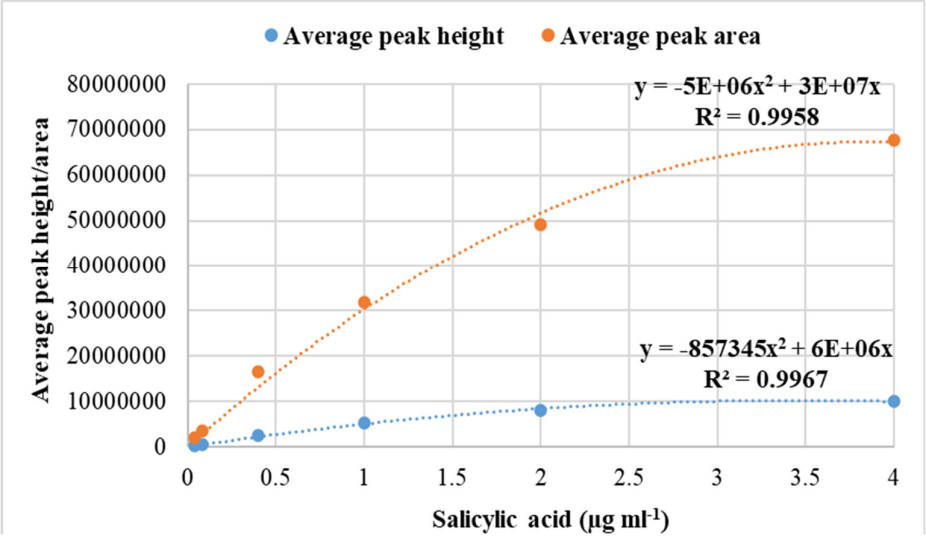

Figure 13. Standard curve used for the quantification of salicylic acid (µg ml^{-1}) content in *C. autumnale* extracts

The total polyphenol content was determined using the conventional method described by Singleton and Rossi (1965) in Herald *et al.* (2012). The standard curve was obtained by preparing standard solutions using gallic acid at concentrations of 0-400 µg ml^{-1}. The flavonoid content was determined using the conventional colorimetric method based on aluminum chloride, according to Herald *et al.* (2012), adapted from Zhishen *et al.* (1999). To determine the standard curve, standard solutions were prepared using quercetin at concentrations of 0-500 µg ml^{-1}. The concentrations of polyphenols and flavonoids in the extracts were calculated using the standard curve formula by calculating the mean values obtained by analyzing 3 biological replicates (n = 3) (Figure 14 and 15).

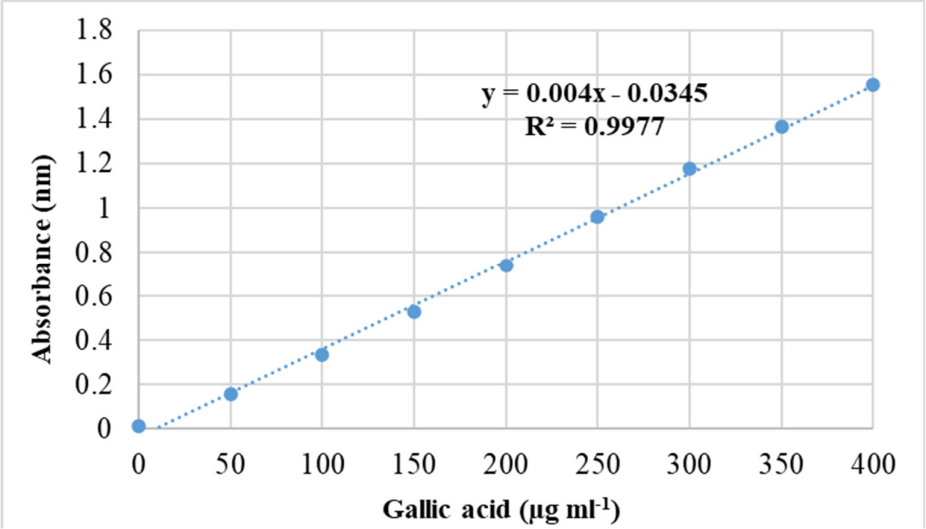

Figure 14. Standard curve used for the quantification of polyphenols (µg eq. gallic acid ml^{-1}) content in *C. autumnale* extracts

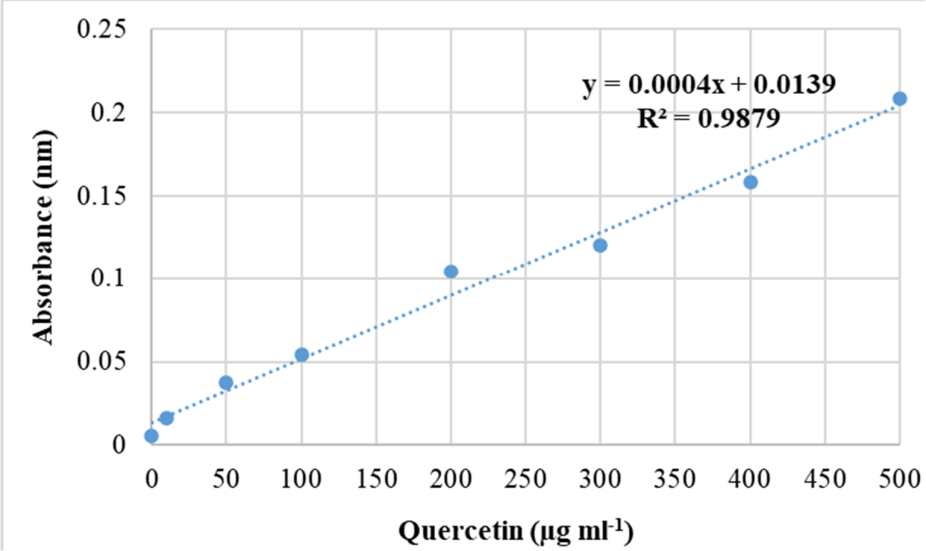

Figure 15. Standard curve used for the quantification of flavonoids (µg eq. quercetin ml^{-1}) content in *C. autumnale* extracts

The antioxidant activity of the *C. autumnale* extracts (n = 3) was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method described by Herald *et al.* (2012), Thaipong *et al.* (2006), and Brand-Williams *et al.* (1995). To obtain a standard curve, standard solutions were prepared using ascorbic acid at concentrations of 0-200 µg ml^{-1}. Antioxidant activity was expressed as equivalent mg ascorbic acid per gram of fresh plant material, calculated according to the standard curve formula (Figure 16).

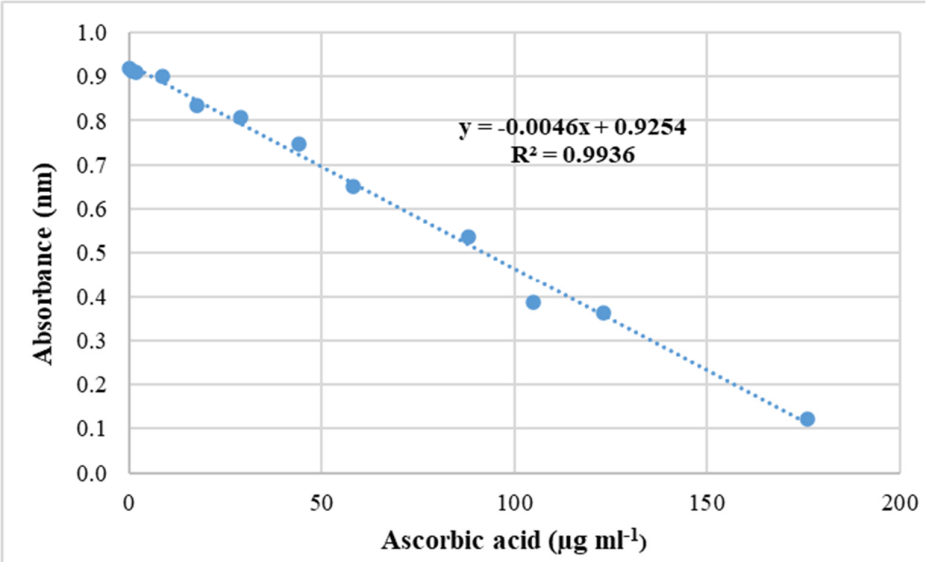

Figure 16. Standard curve used for the quantification of antioxidant activity (µg eq. ascorbic acid ml^{-1}) of *C. autumnale* extracts

Statistical analysis

For the interpretation of the results obtained from the biochemical analyses of the extracts, the two-way ANOVA statistical test, Tukey test for multiple comparisons (post-hoc), and Pearson's correlation coefficient were applied using GraphPad Prism 9.5.1 software. For UPLC analysis, the Relative Standard Deviation (%RSD), Limit of Detection (LOD), and Limit of Quantification (LOQ) are presented in Table 1.

Table 1. Relative Standard Deviation (%RSD), Limit of Detection (LOD), Limit of Quantification (LOQ) values for colchicine, colchiceine, apigenin and salicylic acid detected through UPLC

Parameters	Colchicine		Colchiceine		Apigenin		Salicylic acid	
	Peak height	Peak area	Peak height	Peak area	Peak height	Peak area	Peak height	Peak area
%RSD	1.047501	2.874768	1.406537	1.656845	2.449800	2.384739	1.546535	2.759667
LOD	0.000002	0.000000	0.000003	0.000001	0.000001	0.000000	0.000001	0.000000
LOQ	0.000006	0.000001	0.000009	0.000002	0.000003	0.000001	0.000004	0.000001

The values presented in the graphs and tables represent the mean ± standard error of the mean. Significant differences are marked in the graphs and tables with different letters ($p < 0.05$). Pearson's correlation coefficient was determined by comparing the biochemical analysis results of the alcoholic extracts with those of the aqueous extracts and by comparing the antioxidant activity of the extracts against the polyphenol and flavonoid content.

Results and Discussions

Influence of environmental conditions on secondary metabolites synthesis

Soil pH indirectly affects plants through nutrient availability, microbial activity, and metal solubility. Most plants thrive in slightly acidic to neutral soils (pH 6-7), where essential nutrients are most accessible and toxic metals are less soluble. At low pH, some nutrients become less available, whereas toxic metals can increase (Mróz, 2011).

According to the World Reference Base (2006) Soil Groups (*https://soilgrids.org*), the soil at the plant collection point is Cambisol, which usually contains moderate organic matter, sand, silt, and clay, is rich in essential nutrients, has good water retention, and has a slightly acidic to neutral pH (Twaij and Hasan, 2022).

Soil pH analysis at the collection site indicated that *C. autumnale* plants grow indicated an acidic pH of 5.8. *C. autumnale* grows ideally in light, nutritious, well-drained soils with a pH around 6. Poorly drained or highly acidic/alkaline soils can reduce plant size, flowering duration, and corm productivity, whereas neutral pH soils promote better vegetative growth, flowering, and seed production (Kaysarov and Akhmedov, 2021). Soil pH influences the uptake of nutrients in *C. autumnale* plants, which may explain the differences in plant performance and colchicine content in corms and other plant organs (Mróz, 2011).

The annual average temperature at the plant material collection point in 2021 was 6.3 °C and the annual relative humidity was 81.5%. The average temperature in spring was 4.24 °C, and the relative humidity was 82.73%; in summer, the temperature rose to an average of 17.54 °C, and the %RH (relative humidity) lowered to 75.73%; and during autumn, the average temperature was 6.77 °C, while %RH remained approximately constant at 75.52%. During winter (before the collection of plant material), the average temperature was -3.33 °C, with %RH of 90.9%. *C. autumnale* is adapted to temperate climates and can initiate growth at minimum positive air temperatures of 3-5 °C, with the growing season beginning shortly after snowmelt. High relative humidity (e.g., 81.5%) helps maintain soil moisture, which is beneficial for growth, improves plant vigor, and prolongs flowering duration. Seasonal and environmental factors, including temperature and humidity, influence the biosynthesis of secondary metabolites, with higher phenolic and flavonoid contents often linked to stress conditions (Davoodi *et al*., 2021).

Importance of choosing the right extraction methods and solvents

Soxhlet extraction is generally more effective than infusion and other conventional methods for extracting colchicine and other compounds from *C. autumnale* plants; however, advanced modern techniques may offer even higher yields and efficiency. In general, the optimal extraction method depends on the specific plant material, target compounds, and intended application (Wang and Weller, 2006; Danlami *et al*., 2014; Jibhkate *et al*., 2023). Conventional extraction methods include maceration, reflux, and Soxhlet extraction using solvents of varying polarities, such as methanol, ethanol, chloroform, and acetone. According to Abidin *et al*. (2015), Soxhlet extraction with methanol provides the highest colchicine yield (3.49% w/w from seeds), followed by reflux and maceration, with methanol being the most effective solvent.

Advanced extraction methods, such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and homogenizer-assisted extraction (HAE), generally achieve higher extraction yields and shorter processing times than conventional methods, such as Soxhlet and infusion (Chuo *et al*., 2020; Jha and Sit, 2021). For example, optimized UAE conditions (ultrasonication power: 602.4 W, time: 42 min, temperature: 64 °C) yielded 0.238% colchicine from bulbs (Çankaya *et al*., 2018), whereas SFE achieved >98% recovery in 110 min and matched the yield of conventional methods (Ellington *et al*., 2003). In addition, ultrasonication-based extraction (UBE) outperforms traditional extraction methods in terms of colchicine content and efficiency (Alqarni *et al*., 2022). The advantages of advanced extraction methods include shorter extraction times, lower temperatures, reduced solvent use, and eco-friendliness compared to conventional methods (Ellington *et al*., 2003; Çankaya *et al*., 2018; Alqarni *et al*., 2022).

However, these methods may not always be accessible in all settings. Soxhlet extraction can provide high yields of certain compounds, such as polyphenols, flavonoids, and antioxidants, and is sometimes superior to maceration or UAE for specific plant materials (Putra *et al*., 2022; Mokaizh *et al*., 2024). Infusion and decoction are simple, water-based methods suitable for extracting polar, water-soluble compounds; but they are less efficient for non-polar or less soluble bioactives and generally yield less extract than Soxhlet or advanced techniques (Abubakar and Haque, 2020).

The study of Abidin *et al*. (2015) that compares extraction methods for *Colchicum autumnale* seeds found that Soxhlet extraction produced the highest colchicine yield, especially when using methanol as the solvent, outperforming both the maceration and reflux methods. For related species (*Colchicum triphyllum*), both Soxhlet and infusion methods effectively extract alkaloids and polyphenols; however, Soxhlet extraction with methanol often yields higher concentrations of bioactive compounds, including colchicine derivatives. Infusion is effective for some phenolics and alkaloids, but generally yields less than Soxhlet extraction (Senizza *et al*., 2020).

Soxhlet extraction is a reliable and accessible method for extracting colchicine and polyphenols from *Colchicum autumnale*, offering high yields of these compounds, but it has both notable advantages and limitations. The positive aspects of Soxhlet extraction include high extraction efficiency, accessibility, and reproducibility. Soxhlet extraction yielded higher amounts of colchicine than maceration and was comparable to or slightly better than reflux extraction, especially when methanol was used as a solvent. This method is straightforward, does not require advanced equipment, and is suitable for settings lacking modern extraction technologies. Soxhlet extraction provides consistent results because of its continuous solvent cycling, ensuring the thorough extraction of target compounds. The disadvantages of Soxhlet extraction include high solvent and energy consumption, thermal degradation risks, and long extraction time (Abidin *et al*., 2015; Sridhar *et al*., 2021).

Recent research highlights that ultrasound-assisted extraction (UAE), especially when combined with deep eutectic solvents (DES), is one of the most effective and sustainable methods for extracting polyphenols from plant matrices. These approaches outperform conventional methods (such as maceration or Soxhlet extraction) in terms of yield, selectivity, reduced solvent use, and lower energy consumption (Liu *et al*., 2022; Wang *et al*., 2023; Aktaş and Kurek, 2024; Palos-Hernández *et al*., 2024; Szopa *et al*., 2024), yet Soxhlet extraction may provide a reliable extraction method when these advanced techniques are not available.

Biochemical content of C. autumnale in different ontogenetic stages

Regarding the biochemical content of meadow saffron plants, according to Wildman and Pursey (1960), all organs of *C. autumnale* accumulate colchicine, which is accompanied by several alkaloids in small amounts. Colchicine and other alkaloids are abundant in the endosperm, cotyledons, seeds, perisperm, testa, and fused pericarp (Wildman and Pursey, 1960). Preliminary phytochemical studies conducted by Davoodi *et al*. (2021) on *C. autumnale* reported the presence of alkaloids, phenolic compounds, tannins, flavonoids, coumarins, saponins, terpenoids, steroids, and glycosides in alcoholic extracts. The total tropolone alkaloid content was 9.8 ± 0.3 mg of colchicine per gram of plant material (bulbs), the polyphenol content was 5.6 ± 0.4 mg of gallic acid per gram of plant material, and the flavonoid content was 3.7 ± 0.4 g of quercetin per gram of plant material, using methanol and water at an 80:20 ratio as the extraction solvent. The total colchicine content was 4.4592 ± 0.0109 mg per gram of plant material represented by the bulbs (Davoodi *et al*., 2021).

In the present study, the colchicine content in bulbs was 1.328 ± 0.003 mg per gram of plant material collected during the growth period (spring) and 0.617 ± 0.002 mg per gram of bulbs collected during the flowering period (autumn), using a 70:30 ratio of ethanol to water. By comparing with the results obtained by Davoodi *et al*. (2021), methanol may be a more suitable solvent for colchicine, and this compound has a better solubility at higher methanol concentrations. When comparing the results of various studies, it is essential to consider several factors, including the environmental conditions experienced by *C. autumnale* plants, the neighboring plant species that compete with meadow saffron for nutritional resources, the availability of soil nutrients, and the extraction method employed. Overall, the content of bioactive compounds was higher in alcoholic extracts than in aqueous extracts prepared from the same plant material (using the same mass of powdered plant material in the preparation of the extracts, namely 5 g), except for the extract from bulbs collected during the flowering period (autumn).

UPLC analysis revealed that the highest concentration of colchicine, the primary bioactive compound, was found in the alcoholic and aqueous extracts obtained from flowers (8), whereas the lowest concentration was detected in the extracts from the bulb sheaths (7) (Table 2). Colchicine, like many other secondary metabolites, contributes to plant survival through its toxic effects on herbivores and pathogens (Ghosh and Jha, 2008). This may explain the higher colchicine content in the aboveground organs of meadow saffron compared to the underground organs. Pearson's correlation coefficient analysis indicated no correlation between the colchicine content of the alcoholic and aqueous extracts of *C. autumnale* ($p = 0.165$).

Table 1. Annotation of extracts, data on the plant material and compounds detected by RP-UPLC in alcoholic and aqueous extracts obtained from *C. autumnale* organs

Ontogenetic stage	Extract number	Collection date	Voucher specimen	Plant organ	Solvent	Content (mg ml^{-1})			
						Colchicine	Colchiceine	Apigenin	Salicylic acid
Growth	1	15.05.2021	I186.542	bulbs	EtOH 70%	**0.1328 ± 0.0003** ^{e}	0.0011 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.1204 ± 0.0003 ^{e}
					H_2O	**0.0885 ± 0.0015** ^{d}	0.0002 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
	2	15.05.2021	I186.542	leaves	EtOH 70%	**0.4461 ± 0.0004** ^{h}	0.0375 ± 0.0128 ^{d}	0.0184 ± 0.0001 ^{b}	0.1547 ± 0.0001 ^{f}
					H_2O	**0.1492 ± 0.0004** ^{e}	0.0184 ± 0.00 ^{b}	0.00 ± 0.00 ^{a}	0.0296 ± 0.0041 ^{b}
	3	15.05.2021	I186.542	fruits	EtOH 70%	**0.4308 ± 0.0008** ^{h}	0.0179 ± 0.0005 ^{b}	0.00 ± 0.00 ^{a}	0.0714 ± 0 ^{c}
					H_2O	**0.2624 ± 0.0046** ^{f}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
Fruiting	4	8.07.2021	I186.555	leaves	EtOH 70%	**0.204 ± 0.0006** ^{f}	0.0042 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.2026 ± 0.0007 ^{g}
					H_2O	**0.0559 ± 0.0003** ^{c}	0.00 ± 0.00 ^{a}	0.0004 ± 0.0001 ^{a}	0.0186 ± 0.0001 ^{b}
	5	8.07.2021	I186.555	fruits	EtOH 70%	**0.3993 ± 0.0005** ^{g}	0.0257 ± 0.0006 ^{c}	0.00 ± 0.00 ^{a}	0.1342 ± 0.0008 ^{e}
					H_2O	**0.0911 ± 0.0035** ^{d}	0.0005 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
Flowering	6	5.11.2021	I186.556	bulbs	EtOH 70%	**0.0617 ± 0.0002** ^{c}	0.0024 ± 0.0014 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
					H_2O	**0.0854 ± 0.0013** ^{d}	0.00003 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
	7	5.11.2021	I186.556	bulb sheats	EtOH 70%	**0.0305 ± 0.0001** ^{b}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.0915 ± 0 ^{d}
					H_2O	**0.0095 ± 0.0003** ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
	8	5.11.2021	I186.556	flowers	EtOH 70%	**0.7815 ± 0.003** ^{g}	0.0032 ± 0.0001 ^{a}	0.3765 ± 0.0005 ^{c}	0.327 ± 0.0142 ^{h}
					H_2O	**0.1227 ± 0.0001** ^{e}	0.0048 ± 0.0001 ^{a}	0.003 ± 0.0001 ^{a}	0.066 ± 0.00005 ^{c}

Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

Comparing the content of aqueous and alcoholic extracts, it was found that a lower amount of colchicine was obtained by extraction in water than by extraction in methanol performed with Soxhlet, except for the extract from bulbs collected in autumn (6), in which a higher concentration of colchicine was identified than in the alcoholic extract prepared from the same plant material. Wildman and Pursey (1960) reported that the colchicine content in bulbs was 3 times lower in the autumn period than in spring. However, in the present study, similar concentrations of colchicine were identified in the aqueous extracts of the bulbs (1 and 6 in Table

2). In contrast, by analyzing the alcoholic extracts, it was found that this compound was present in a quantity two times higher in bulbs during the growth period (spring) than in the flowering period (autumn). Compared to the results of previously conducted research (Moroșan *et al*., 2022), in which extracts were prepared from plant material (bulbs and flowers) collected from the same meadow (in October 2019), with colchicine concentrations detected by the same method (0.119 ± 0.007 mg ml^{-1} in the bulb extract and 0.286 ± 0.015 mg ml^{-1} in the flower extract), the colchicine concentration in the extracts obtained from the material collected in 2021 was two times lower in the bulbs and approximately three times higher in the flower extract. These differences may be due to environmental conditions, time of collection, total biomass of the harvested plant material, solvent used for extraction, and extraction time.

In the present study, colchiceine (Table 2) was identified at low concentrations in extracts from immature leaves (2) and in alcoholic extracts prepared from fruits (3 and 5), and at very low concentrations in extracts from bulbs (1 and 6), mature leaves (4), and flowers (8). This compound was not detected in the aqueous or ethanolic extracts of bulb sheaths (7). Colchiceine is considered the main degradation product of colchicine (in addition to lumicolchicines) (Kurek and Barczyński, 2016); thus, it is used for quality control of plant extracts. Pearson's correlation coefficient analysis indicated no correlation between the colchiceine content of the alcoholic and aqueous extracts ($p = 0.051$).

Although the presence of demecolcine has been reported in several studies on the biochemical composition of *C. autumnale* species (Malichová *et al*., 1979; Yoneda *et al*., 1984; Herbert *et al*., 1990; Davoodi *et al*., 2021), it was not detected in the extracts prepared in this study. This may indicate that the substrate in the area from which the plant material was collected, as well as the environmental or stress conditions experienced by *C. autumnale* individuals before collection, influenced the chemical composition of these plants, as revealed by the analysis of the alcoholic and aqueous extracts (Table 2).

Apigenin (Table 2) has been identified in the aqueous extracts of flowers (8) and mature leaves (4). Apigenin is a flavone with antioxidant properties that protects plant cells from oxidative stress, which is crucial for preventing damage caused by reactive oxygen species (Madunić *et al*., 2018; Azeem *et al*., 2024) and UV-B rays (Righini *et al*., 2018). As a secondary metabolite, apigenin contributes to plant defense against pathogens and environmental stressors (Mushtaq *et al*., 2023; Azeem *et al*., 2024). Comparing these results with the data from the literature (Burzo *et al*., 2006), it was confirmed that apigenin is present in the flowers of *C. autumnale*. Apigenin has also been identified in very small amounts in ethanolic extracts prepared from young leaves (2) and in aqueous extracts from mature leaves (4). Pearson's correlation coefficient analysis indicated a correlation between the apigenin content in alcoholic and aqueous extracts ($p = 0.000003$), indicating that both water and alcohol efficiently extract this compound.

Salicylic acid (Table 2) was identified at the highest concentration in both alcoholic and aqueous extracts of flowers (8) and leaves (2 and 4); however, the use of Soxhlet and methanol as extraction solvent made it possible to extract this compound from other organs, such as bulbs collected in spring (1), fruits (3 and 5), and bulb sheaths (7). Pearson's correlation coefficient analysis indicated a correlation in the salicylic acid content between alcoholic and aqueous extracts ($p = 0.004$), a compound that can be successfully extracted using either alcohol or water as solvents. Salicylic acid is involved in the flowering process and plays an important role in protecting plants against biotic and abiotic stress (Wani *et al*., 2017). It is also involved in the regulation of physiological and biochemical processes throughout the plant life cycle, thereby influencing its growth and development (Vicente and Plasencia, 2011; Koo *et al*., 2020).

According to a study by Jung *et al*. (2011), *C. autumnale* has a life cycle that begins with the flowering stage, which falls in the autumn period, in which the flowers appear above the ground, unaccompanied by leaves, with the gynoecium buried in the soil and protected by the bulb, the anthers are exposed in the above-ground area, and adventitious roots begin to appear on the young bulb disc (developed during summer). Flowering ends with a short period in which the fruits and leaves begin to develop underground, consuming part of the nutrient reserves in the bulb. This period is followed by dormancy during winter, during which the

fruits and leaf primordia are withdrawn into the soil by contractile adventitious roots, thus being protected from low temperatures. In spring, the fruits begin to appear above the ground surrounded by leaves, with a light green color and white developing seeds, whereas starch reserves in the old bulb are progressively depleted. As the old bulb shrinks, the new bulb begins to grow and accumulate starch. During summer, the fruits mature and open, with a brownish color, releasing blackish-brown seeds, and the leaves approach senescence. The plant has a small bulb (the old bulb), which enters the maceration process simultaneously with the development of the new bulb, while the aboveground organs begin to dry out. At that time, the plants consisted only of underground bulbs surrounded by sheaths that represented the leaves that had dried up, surrounded the bulb, and appeared as a brown tunic. The period of inactivity follows, and flowering resumes (Figure 1). Thus, we chose a different approach to study *C. autumnale*'s ontogenetic stages by starting with the growth period (in spring) because it is the first true stage of new metabolic activity within the plant, and the flowering period (in autumn) relies on reserves from the previous cycle.

During the growth period, which continued into the fruiting period, the colchicine and salicylic acid content in the leaves remained relatively constant (then decreased towards senescence in the bulb sheaths), whereas in the fruits, it increased during maturation. The colchicine content in the underground organs (bulbs) during the growth period was twice as high as that during the flowering period, which can be explained by the progressive reduction in bulb biomass.

Polyphenols are compounds with multiple and varied functions that contribute to the resistance and adaptation of plants to the environment, being essential in protection against stress, defense against herbivores, antioxidant activity, and regulation of growth and development processes (Sharma *et al.*, 2019; Singh *et al.*, 2021; Šamec *et al.*, 2021; Pinto *et al.*, 2021; Zagoskina *et al.*, 2023).

In the analyses of the available plant material, it was found that during the vegetative stage, the highest polyphenol content was found in the aboveground organs, especially in the leaves (2, 4) and flowers (8), and the polyphenol content determined was higher in the bulbs collected during the spring (1) than in the bulbs collected in the fall (6). In contrast, the analysis of the alcoholic extracts suggested that the accumulation of polyphenols in the fruits (3 and 5) would occur during maturation; however, in the aqueous extracts, this content remained approximately constant (Figure 17). Pearson's correlation test revealed a correlation between the polyphenol content of alcoholic and aqueous extracts ($p = 0.007$). This suggests that both ethanol/methanol and water can extract these compounds in a comparable manner, yet alcoholic solvents yield better results.

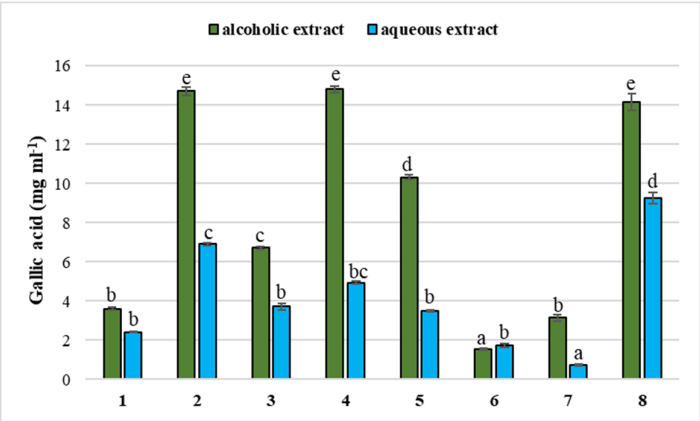

Figure 17. Polyphenols (mg eq. gallic acid ml^{-1}) content in alcoholic and aqueous *C. autumnale* extracts
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract
Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

Similarly to the analysis of the polyphenol content in alcoholic and aqueous extracts from plant organs of *C. autumnale*, it was found that during the growth stage (spring period) the highest flavonoids content is found in the aboveground organs, especially in leaves (2, 4), flowers (8) and fruits (3, 5). Flavonoids are phenolic compounds that regulate plant development and contribute to the pigmentation of flowers and fruits, which are essential characteristics for attracting pollinators (Mierziak *et al*., 2014; Mathesius, 2018), which explains their abundant presence in these organs. Moreover, these compounds accumulate in the epidermis and protect plants from harmful solar radiation (Shah and Smith, 2020; Ferreyra *et al*., 2021). Flavonoids exhibit strong antioxidant properties, helping to eliminate reactive oxygen species (ROS), and thus play a significant role in plant stress tolerance (Khalid *et al*., 2019; Dias *et al*., 2021; Shomali *et al*., 2022), as well as against pathogens and herbivores, increasing their resistance to biotic stress (Mathesius, 2018; Alseekh *et al*., 2020; Shah and Smith, 2020). Moreover, flavonoids are involved in cellular signaling processes, such as root-rhizosphere interactions, an interaction in which they can stimulate or inhibit microbial activity, affect nutrient uptake, and mediate, influence, or even determine allelopathic interactions (Hassan and Mathesius, 2012; Shah and Smith, 2020), which explains their presence, even in lower quantities, in underground organs.

The flavonoid content determined in the present study was higher in the alcoholic extract from mature fruits (5) than that prepared from immature fruits (3), whereas in the homologous aqueous extracts, it was similar in both extracts. Simultaneously, the flavonoid content in the leaves was similar in the alcoholic extracts (2 and 4), but higher in the aqueous extract prepared from leaves collected during the growth period (2) than in the aqueous extract from mature leaves (4). In addition, the flavonoid content was much higher in bulbs collected during spring (1) than in bulbs collected in autumn (6), an observation resulting from the analysis of both types of extracts (aqueous and alcoholic) (Figure 18). Pearson's test revealed a correlation between the flavonoid content of the alcoholic and aqueous extracts ($p = 0.00002$).

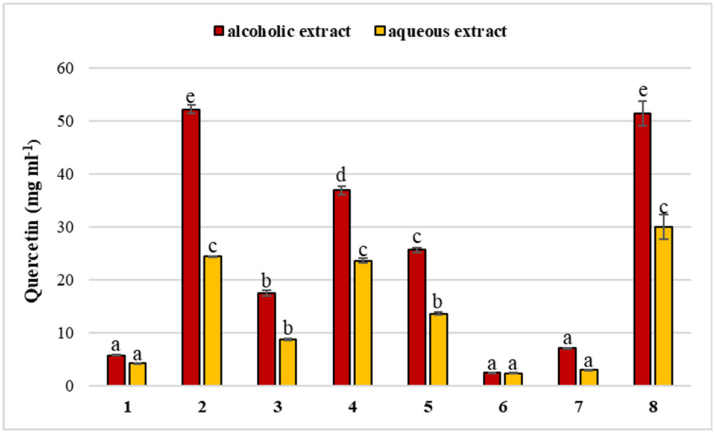

Figure 18. Flavonoids (mg eq. quercetin ml^{-1}) content in alcoholic and aqueous *C. autumnale* extracts
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract; Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

The polyphenol and flavonoid contents remain relatively constant in the leaves during the growth period towards fruiting and are also the most abundant in the leaves and flowers compared to the fruits, bulb sheaths, and bulbs. In the bulbs, this content decreases or remains relatively constant, and in the fruits, it increases significantly during maturation, providing additional protection against stress, pests, and herbivores.

The antioxidant activity (expressed as mg of ascorbic acid per gram of dry plant material) was considerably higher in alcoholic extracts prepared from the vegetative and reproductive organs of *C. autumnale* plants than in aqueous extracts (Figures 17 and 18). All meadow saffron organs exhibited high antioxidant

activity, except for the extract from bulbs collected in spring. In the case of aqueous extracts, the detected antioxidant activity was weak, and no significant variations were observed between the extracts. Pearson's correlation coefficient analysis indicated no correlation between the antioxidant activities of the alcoholic and aqueous extracts ($p = 0.162$).

The highest antioxidant activity was detected in alcoholic extracts from fruits (3 and 5), bulbs collected in autumn (6), flowers (8E), and leaves (2, 4, and 7), without significant variations (Figure 17). However, polyphenols and flavonoids were detected in extracts from flowers (8), leaves (2 and 4, but not 7), and fruits (3 and 5). Flavonoids may be the main compounds contributing to the antioxidant activity in the aboveground organs of meadow saffron, although there are significant differences in flavonoid content between the alcoholic extracts prepared from these organs (Figure 19 and 20).

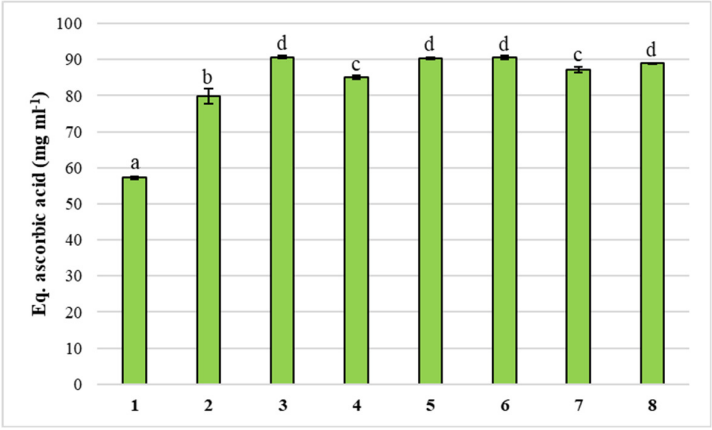

Figure 19. Antioxidant activity (mg eq. ascorbic acid ml^{-1}) of alcoholic extracts
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract; Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

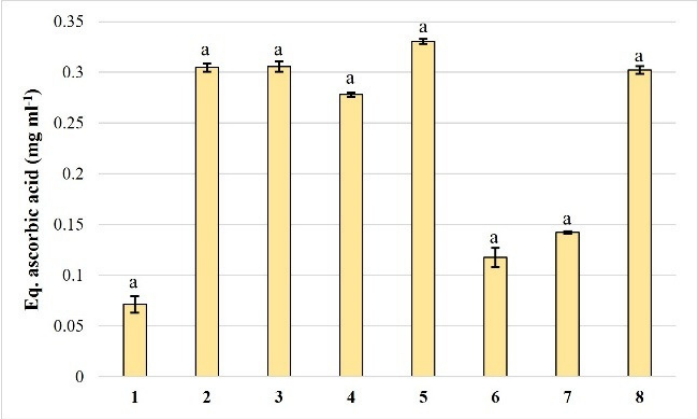

Figure 20. Antioxidant activity (mg eq. ascorbic acid ml^{-1}) of aqueous extracts
1 = spring bulb extract; 2 = young leaves extract; 3 = immature fruits extract; 4 = mature leaves extract; 5 = mature fruits extract; 6 = autumn bulb extract; 7 = bulb sheaths extract; 8 = flower extract; Data is presented as mean ± standard error of the mean; statistically significant differences determined through Tukey analysis are marked with different letters

Pearson's correlation test also revealed a strong correlation between polyphenol and flavonoid content (as expected) in both extract types (alcoholic: $p = 0.0007$ and aqueous: $p = 0.0002$). When the polyphenol and

flavonoid contents of the alcoholic extracts were compared to the antioxidant activity of the extracts, no significant correlations were observed (polyphenols vs. DPPH: $p = 0.342$; flavonoids vs. DPPH: $p = 0.332$). Only the polyphenols and flavonoids from the aqueous *C. autumnale* extracts displayed a strong correlation with the antioxidant activity of the extracts (polyphenols vs. DPPH: $p = 0.034$; flavonoids vs. DPPH: $p = 0.019$), although it was weak, and there were no significant differences identified between the antioxidant activities of the aqueous extracts (Figure 20). The low antioxidant activity of the aqueous extracts may indicate the presence of compounds with higher solubility in alcohol or compounds that are either insoluble or less soluble in water. In addition, the polyphenol and flavonoid content detected in alcoholic extracts from bulbs collected in autumn (6) was very low, yet its antioxidant activity increased (Figure 19). This may provide clues regarding the presence of other compounds in this extract that contribute to the increased antioxidant activity.

The findings of this study confirm that all plant organs of *C. autumnale* accumulate colchicine and suggest that the time of collection, extraction method, and solvents significantly affect the yield of key bioactive compounds in *C. autumnale*. The alcoholic extracts were richer in secondary metabolites than their homologous aqueous extracts (except for the autumn bulb extracts, where the aqueous extract had a higher concentration). Apigenin and salicylic acid were detected at their highest concentrations in the flower and leaf extracts, respectively, indicating their roles in flowering and plant defence. Overall, the highest concentrations of polyphenols and flavonoids were observed in the aboveground organs during the growth stage, particularly in the leaves and flowers. Moreover, the antioxidant activity of the alcoholic extracts was significantly higher than that of the aqueous extracts, particularly in the extracts from the leaves, fruits, and flowers. This underscores the need for carefully planned and executed collection and processing protocols to maximize the extraction of desired substances from *C. autumnale*.

Conclusions

Soxhlet extraction is a reliable and accessible method for extracting colchicine and polyphenols from *C. autumnale* plants, with high efficiency, accessibility, and reproducibility. It provides consistent results owing to continuous solvent cycling, ensuring thorough extraction of target compounds when modern extraction techniques are unavailable. Usually, in extract preparation, the evaporation of solvents using a rotary evaporator is ideal for obtaining a more concentrated alcoholic extract, whereas lyophilization is better for aqueous extracts because it preserves the extract's integrity, given that many bioactive secondary metabolites (such as polyphenols and alkaloids) are thermolabile, thus avoiding thermal degradation of compounds. This is especially applicable to colchicine, which is a thermolabile compound in aqueous solutions but is more stable in ethanol.

This study revealed how colchicine and phenol concentrations vary across different developmental stages (growth, flowering, and fruiting) in various plant organs, providing new insights into the dynamic accumulation patterns throughout the plant's ontogenetic cycle. Flowers contain the highest colchicine concentrations, whereas bulbs and other organs exhibit varying levels depending on the season, emphasizing the influence of both plant organ and harvest time on bioactive compound content. Alcoholic extracts generally yield higher amounts of colchicine and phenols than water extracts, except in certain cases, such as bulbs during flowering, highlighting the importance of solvent choice in maximizing bioactive compound extraction. By analyzing environmental parameters, ontogenetic factors, and extraction methods, this study provides a comprehensive understanding that can inform optimized harvesting and processing strategies for medicinal and phytotherapeutic applications. The application of UPLC and spectrophotometry can offer precise quantification of secondary metabolites, setting a methodological precedent for future phytochemical studies in this species and potential applications in other plants with similar properties.

The biochemical diversity in *C. autumnale*, especially its high alkaloid content (notably colchicine), plays a key role in plant defense against herbivores and pathogens and supports survival during dormancy. Metabolite distribution, such as the abundance of colchicine and polyphenols in flowers, fruits, and leaves, reflects adaptation to ecological pressures and reproductive strategies. These compounds also contribute to stress tolerance and energy storage, influencing the ecological interactions and fitness of plants. Variations in the secondary metabolite content between plant parts are critical for pharmacognostic identification and safety, as colchicine has a narrow therapeutic index.

Authors' Contributions

Conceptualization: ICM, MMZ; Data curation: ICM, MM; Formal analysis: ICM, MM; Funding acquisition: MM, MMZ; Investigation: ICM, MM; Methodology: ICM, MM; Project administration: MMZ; Resources: MMZ, MM; Software: ICM, MM; Supervision: MMZ, MM; Validation: MMZ, MM; Visualization: ICM, MM, MMZ; Writing - original draft: ICM; Writing - review and editing: ICM, MM, MMZ.

All authors read and approved the final manuscript

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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