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ECOSYSTEM SERVICES OF BLACKTHORN: EFFECTS OF HARVESTING TIME ON THE FUNCTIONAL EXTRACTION OF POLYPHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY

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ABSTRACT

The objective of this study was to examine the potential ecosystem services of Blackthorn (Prunus spinosa L.) in view of its traditional importance as a source of food and medicine. Fruits were sampled from a suburban population in the southwestern part of Belgrade at three time points: after ripening in late summer, in early winter, and in early spring. Using spectrophotometric methods and ethanol and distilled water as solvents, the contents of polyphenolic compounds and the antioxidant capacity were determined. To assess the influence of the harvesting period, the results were analysed using descriptive statistics, one-way ANOVA, Tukey's HSD and LS Means tests, Spearman's correlation coefficient, and confidence interval models. The biochemical composition of the fruits was shown to depend on the time of harvest, as confirmed by the statistically significant differences between the sampling periods for all the parameters except anthocyanins. In all the sampling periods, the ethanolic extracts exhibited higher values of polyphenolic compounds and antioxidant capacity compared to the aqueous extracts. Early spring and early winter were identified as the most suitable harvesting times, as the strongest antioxidant activity measured by the FRAP test was recorded in the third period, and by the DPPH and ABTS tests in the second period. This study makes a significant contribution by confirming that the harvest time does not affect the anthocyanin content, thus highlighting the commercial potential of Blackthorn fruits as natural colourants based on anthocyanins. The patterns of variability suggest optimal harvesting times for the efficient extraction of bioactive compounds and the use of Blackthorn fruits in the development of functional foods and pharmaceutical products. Furthermore, the findings serve as a basis for developing strategies aligned with climate change predictions, as the results confirm a positive influence of low air temperatures preceding the second and third sampling on the bioactive components of the fruits.

Key words:
Prunus spinosa L., fruit plants, targeted use, bioactivities, FRAP, ABTS+, DPPH

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INTRODUCTION

Woody plants bearing edible fruits represent valuable raw materials for meeting fundamental nutritional needs, as fruits serve diverse dietary functions. In addition to their basic nutritional value, they often contain bioactive compounds that may promote health and slow down the ageing process in humans (Orlandi et al., 2023). This is consistent with the increasing interest in fruit-bearing species and food products that not only serve utilitarian purposes but also enhance physical performance, support overall well-being, and reduce the risk of disease. Fruits rich in biologically active substances, particularly polyphenolic compounds, are considered the most beneficial (Moskalets et al., 2024). Polyphenols, a class of natural secondary metabolites that includes phenolics, tannins, flavonoids, anthocyanins, and others, contribute to the colour, aroma, and flavour of fruits, while also offering significant nutritional value and potential health-promoting effects (Araujo-Rodrigues et al., 2021). According to Zhang et al. (2023), polyphenolic compounds possess antimicrobial, antiviral, and anti-inflammatory properties, as well as strong antioxidant capacity. This antioxidant potential is influenced by factors such as plant taxonomy (Fernandes et al., 2023), the biochemical composition of the plant material (Angulo-Lopez et al., 2022), molecular interactions, and the degree of polymerisation (Moskalets et al., 2024).

In the current decade, there has been a rise in research on "underutilised fruit species" with biologically active compounds, including polyphenols, that grow spontaneously rather than through cultivation, requiring minimal maintenance and representing an environmentally safe source of raw material for consumption and processing (Beed et al., 2021). In such cases, the appropriate ecosystem service management and increased awareness of climate change are essential (Čukanović et al., 2024; Petrov et al., 2024; Ljubojević et al., 2025), alongside the efforts to promote the ecological and economic value of these fruit taxa (McLain et al., 2012; Petrov et al., 2025). Within this context, growing attention has been given to *Prunus spinosa* L., whose fresh fruits are rich in polyphenols, conferring anti-inflammatory and antioxidant effects on immune-related cells in humans (Magiera et al., 2022).

Prunus spinosa L. (Blackthorn) is a low to medium-sized tree or shrub native to Europe, western Asia, and northern Africa (Ocokoljić & Petrov, 2022), and has become naturalised in eastern parts of North America, as well as in New Zealand and Tasmania (Popescu & Caudullo, 2016). According to Brown et al. (2022), Blackthorn—like other "wild" fruit species—is particularly suitable for developing high-yielding fruit crop genotypes with aromatic fruits that are resistant to sub-zero temperatures and spring and summer drought conditions. They also highlight Blackthorn as a promising species for breeding programmes and hybridisation with plums and apricots. The fruits of Blackthorn are astringent drupes, dark bluish-purple in colour with a grey waxy coating (Ocokoljić & Petrov, 2022). They are used in the production of jams, beverages, and functional supplements, and can also be preserved (Negrean et al., 2023).

The ecosystem services of Blackthorn are considered promising due to the high content of phenolic compounds and the antioxidant activity of its fruits (Negrean et al., 2023). However, there is limited information regarding changes in the bioactive components of the fruits after ripening, i.e. their availability for processing from wild populations (Kolarov et al., 2021). Post-ripening conditions, primarily climatic, influence fruit characteristics and should be analysed to determine the optimal harvesting time without compromising bioactive compound content (Al-Maiman and Ahmad, 2002). Therefore, this study examines the characteristics of Blackthorn fruits in relation to different post-ripening harvesting periods. Particular attention is given to the content of polyphenolic compounds and the antioxidant capacity of fresh fruits harvested in late summer, early winter, and early spring, with the aim of identifying the most suitable solvent and optimal harvesting time. The study also highlights the bioactive potential of Blackthorn fruits for use in the food, cosmetic, and pharmaceutical industries, as well as in the development of functional products.

MATERIAL AND METHODS

Origin of samples

The fruit samples used for analysis were collected from a population of *Prunus spinosa* L. located in the Municipality of Čukarica, within the southwestern suburban zone of Belgrade. The site is oriented east-southeast (44º43'36.30"N, 20º19'36.60"E) to west-northwest (44º43'36.60"N, 20º19'36.12"E), at an elevation of 112 metres above sea level. The soil is classified as eutric brown soil (eutric cambisol) according to the WRB system (2015). Additional information regarding the sampling location is provided in Figure 1.

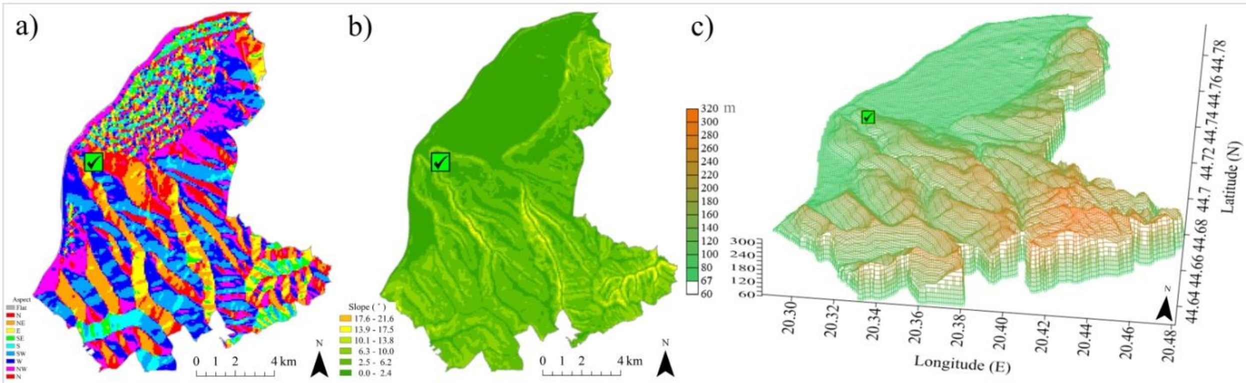

Figure 1. Study area: a) Aspect, b) Slope, and c) 3D surface plot of relative relief values across the territory of the Čukarica Municipality (Belgrade), with a marked location of the *Prunus spinosa* population from which fruit samples were collected

To investigate changes after ripening, fruit samples were collected from the southern parts of the crowns on three occasions (Figure 2): 1st sampling – at the time of ripeness (14 August, late climatological summer 2024), 2nd sampling – 135 days after ripening (27 December, early climatological and calendar winter 2024/2025), and 3rd sampling – 63 days after the second, or 198 days after the first sampling (1 March, early climatological spring 2025). The climatological seasons are defined as follows: winter from 1 December of the previous year to 28 February of the following year; spring from 1 March to 31 May; summer from 1 June to 31 August; and autumn from 1 September to 30 November (RHMZ, 2025).

Figure 2. Photographs of a part of the *Prunus spinosa* population at the fruit harvesting times: late summer (sampling 1), early winter (sampling 2), and early spring (sampling 3)

Determination of Polyphenolic Compounds and Antioxidant Capacity of Blackthorn Fruits

Based on the official analytical protocols and methods (AOAC, 1990), 5 g of mesocarp from fruits dried to constant weight were measured. Extraction was performed using 70% ethanol and distilled water (10 ml) in a 1:50 ratio. Samples were then left in a dark place for 24 hours, followed by centrifugation for 15 minutes at 10,000 rpm. The supernatant was transferred using a Pasteur pipette into new tubes and used for further analysis.

Total Phenolic Content

The total phenolic content in Blackthorn fruit extracts was determined according to Nagavani and Raghava Rao (2010) and Karakas et al. (2019) using the Folin–Ciocalteu method. To the reaction mixture, 3.4 ml of distilled water and 200 µl of Folin–Ciocalteu reagent were added, followed by 20 µl of the sample. After five minutes, 400 µl of Na_2CO_3 solution was added. A calibration curve was constructed using a series of standard quercetin dilutions in water. The absorbance was measured after 60 minutes at a wavelength of 730 nm using a spectrophotometer (Thermo Scientific Evolution 220). The total phenolic content in the tested extracts was expressed as mg quercetin equivalents per gram of fresh weight.

Total Tannins Content

The total tannin content was determined similarly to the total phenolics, following Nagavani and Raghava Rao (2010) and using the Folin–Ciocalteu method. The supernatant obtained by centrifugation (10,000 rpm; 10 min) was used in a reaction mixture consisting of 0.1 g polyvinylpolypyrrolidone (PVPP), 1 ml of water, and 1 ml of extract. After tannin precipitation, PVPP was employed to determine the content of non-tannin phenolics in the supernatant. The total tannin content was calculated as the difference between the total phenolic content and the non-tannin phenolic content in the sample. To the reaction mixture, 3.4 ml of distilled water and 200 µl of Folin–Ciocalteu reagent were added, followed by 40 µl of sample (40 µl of water in the blank). After 400 minutes, 400 µl of Na_2CO_3 solution was added. The absorbance was measured at 730 nm after 60 minutes using a spectrophotometer, as for the phenolics. The total tannin content was expressed as mg quercetin equivalents per gram of fresh weight.

Total Flavonoids Content

The total flavonoid content was determined according to Markham (1989) and Kotsou et al. (2023), based on the ability of flavonoids to form complexes with metal ions, with particular emphasis on the Al^{3+} complex. To the reaction mixture, 0.5 ml of water, 1.5 ml of $AlCl_3$ solution, and 200 µl of extract were added. For the blank, 100 µl of sample and 2 ml of water were added, with each sample having its own blank. After 15 minutes, the absorbance was measured at 430 nm using a spectrophotometer. The total flavonoid content in the tested extracts was expressed as mg quercetin equivalents per gram of fresh weight.

Total Anthocyanins Content

The total anthocyanin content was determined spectrophotometrically using two buffers at pH 1.0 and pH 4.5, each with a volume of 1 ml, to which 200 µl of sample was added. After 15 minutes, the absorbance was measured at wavelengths of 520 nm and 700 nm, and the results were expressed as mg cyanidin-3-glucoside equivalents per gram of fresh weight. The determination of total anthocyanins is based on their property of reversibly changing structure with pH, which affects their absorption spectrum. The content of monomeric anthocyanins was measured using the pH differential method, which relies on the characteristic that monomeric anthocyanins exist as red oxonium ions at pH 1.0, whereas at pH 4.5 they convert to colourless hemiketal forms (Kotsou et al., 2023).

Antioxidant Activities of Blackthorn Fruits Extracts

To assess the antioxidant activity of Blackthorn fruit extracts, three assays were employed: FRAP for measuring reducing power, and DPPH and ABTS assays for radical scavenging activity. The total reducing capacity (FRAP) and radical scavenging activities (DPPH and $ABTS^+$) were expressed as mg Trolox equivalents per gram of fresh weight.

FRAP Analysis

FRAP analyses were performed according to the method of Benzie and Strain (1996) with adaptations published by Kotsou et al. (2023). The absorbance was measured spectrophotometrically at λ=593 nm, 30 minutes after adding 2 ml of FRAP reagent and 10 µl of sample to the reaction mixture. The calibration curve was constructed using a series of FRAP dilutions in water.

DPPH Analysis

The radical scavenging ability of Blackthorn fruit extracts was determined spectrophotometrically by monitoring the "quenching" activity of the tested extracts, based on the reduction of the DPPH radical (2,2-diphenyl-1-picrylhydrazyl) to its reduced form (DPPH-H), as described by Przybylski et al. (1998). The DPPH radical is purple in colour and has an absorption maximum at 517 nm. Upon reaction with antioxidants, it accepts a hydrogen atom to pair its unpaired electron, converting into the yellow-colored DPPH-H. The procedure involves adding 3 ml of DPPH reagent and 10 µl of sample to the reaction mixture. After 30 minutes, the absorbance at 517 nm was measured using a spectrophotometer. The calibration curve was constructed using a series of Trolox dilutions in water.

ABTS+ Analysis
The $ABTS^+$ radical scavenging activity was measured following the method of Re et al. (1999), with minor modifications, to assess the ability of Blackthorn fruit extracts to neutralize the radical cation 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid). The procedure involved adding 3 ml of 70% $ABTS^{\cdot+}$ reagent and 20 µl of sample to the reaction mixture. After two hours, the absorbance was measured at 734 nm using a spectrophotometer. The calibration curve was constructed using a series of Trolox dilutions in water.

Statistical Analysis
All the experimental measurements were performed in six replicates per solvent used, for each of the three fruit sampling periods. The total number of experimental replicates was 252. The results obtained were presented using descriptive statistics, one-way ANOVA, Tukey's HSD, and Least Significant Means (LS means) tests to assess the impact of sampling time and solvent ($p < 0.05$) on the content of polyphenolic compounds and antioxidant capacity of Blackthorn fruits. Spearman's rank correlation coefficient (ρ) was used to evaluate the strength of linear correlations. The value and sign of ρ (ranging from -1 to 1) indicate the strength and direction of the relationship. The scale by Horvat and Mijoč (2012) was applied for interpreting correlation strength: 0 (no correlation), 0–0.24 (very weak), 0.25–0.49 (weak), 0.50–0.74 (moderate), 0.75–0.99 (strong to very strong), and 1 (perfect). Only significant correlations with $p < 0.05$ were analyzed. The confidence interval models were employed to quantify the effect of sampling time, for both solvents, on the content of polyphenolic compounds and antioxidant capacity. Statistical analyses were performed using XLSTAT 2022 and Past 4.11 software. Additionally, ArcGIS 10.8/ArcMap 10.8, Surfer 25, Google Earth Pro, and the authors' photographs were used in the study.

RESULTS AND DISCUSSION

Analysis of Air Temperature and Precipitation Between Harvesting Periods
The autumn of 2024 was generally warm and rainy in the study area, with September and October standing out as warm months according to percentile and tercile methods, whereas November was cold. September was classified as very rainy by the percentile method and rainy by the tercile method, while October and November fell within the normal range for precipitation by both methods compared to the reference period 1991–2020 (Tables 1 and 2).

Table 1. Average monthly air temperatures with corresponding percentiles and terciles, and their deviations for September, October, November, and December 2024, and January and February 2025, relative to the reference period 1991–2020

T_{mean} (°C)	1991-2020 Perc. Cat.*	1991-2020 T_{mean} (°C)	Deviation (°C)	1991-2020 33.-Perc.	1991-2020 50.-Perc.	1991-2020 66.-Perc.	Terciles** Cat.
September 2024							
19.9	W	18.0	1.9	17.1	17.9	18.8	1
October 2024							
14.4	W	12.8	1.6	12.2	12.8	13.8	1
November 2024							
5.7	C	7.4	-1.7	7.0	7.7	8.4	-1
December 2024							
3.2	N	2.2	1.0	1.6	2.3	3.4	0
January 2025							
4.0	VW	1.0	3.0	0.2	0.8	1.6	1
February 2025							
2.4	N	3.0	-0.6	2.3	3.4	4.7	0

Legend:
* EW - Extremely warm; VW - Very warm; W - Warm; N – Normal; C – Cold; VC - Very cold; EC - Extremely cold;
** 1 – Warm; 0 – Normal; -1 – Cold; categorization RHMZ (2025)

Deviations from the average monthly air temperature and precipitation norms for the reference period were +1.9°C and +30.8mm (September), +1.6°C and -6.7mm (October), and -1.7°C and +1.0mm (November). During autumn 2024, one heat wave was recorded in the study area — defined as a continuous period of five or more days with maximum daily air temperatures classified as very warm or extremely warm by the percentile method (RHMZ, 2025) — lasting eight days from 1 to 8 September (Figure 3). No cold waves — continuous periods of five or more days with maximum daily air temperatures classified as very cold or extremely cold (RHMZ, 2025) — were recorded;

however, cold spells occurred between 4 and 11 November with temperatures in the cold and very cold categories, on 17 and 18 November as very cold, and from 23 to 25 November as cold and very cold. Notably, November was cold preceding the second fruit harvest period. Winter 2024/2025 was warm and dry overall, with December and February falling within the normal range according to percentile and tercile methods, while January was very warm (percentiles) and warm (terciles). December was within the normal range for precipitation by both methods, whereas January and February were very dry (percentiles) and dry (terciles) compared to the reference period (Tables 1 and 2). Deviations from the average monthly air temperature and precipitation norms were +1.0°C and +14.9mm (December), +3.0°C and -26.0mm (January), and -0.6°C and -25.4mm (February). During winter 2024/2025, a single heat wave lasting five days was recorded from 27 to 31 January (Figure 3). No cold waves were observed, but a cold spell occurred from 19 to 21 February with temperatures classified as cold and very cold, preceding the third fruit harvest period.

Table 2. Monthly precipitation totals, corresponding percentiles and terciles, and their deviations for September, October, November, and December 2024, and January and February 2025, compared to the reference period 1991–2020

Sum (mm)	1991-2020		Deviation from	1991-2020			Terciles**
	Perc. Cat.*	Mean (mm)	the mean (mm)	33.-Perc.	50.-Perc.	66.-Perc.	Cat.
September 2024							
86.8	VW	56.0	30.8	32.2	51.6	65.7	1
October 2024							
44.0	N	50.7	-6.7	33.6	45.2	59.9	0
November 2024							
45.6	N	45.5	1.0	31.6	42.8	47.1	0
December 2024							
63.2	N	48.3	14.9	36.1	46.0	55.9	1
January 2025							
16.4	VD	42.4	-26.0	36.0	40.5	49.8	-1
February 2025							
8.6	VD	34.0	-25.4	23.5	33.9	46.1	-1

Legend:
* EW - Extremely wet; VW - Very wet; W – Wet; N – Normal, D – Dry; VD - Very dry; ED - Extremely dry;
** 1 – Wet; 0 – Normal; -1 – Dry; categorization RHMZ (2025)

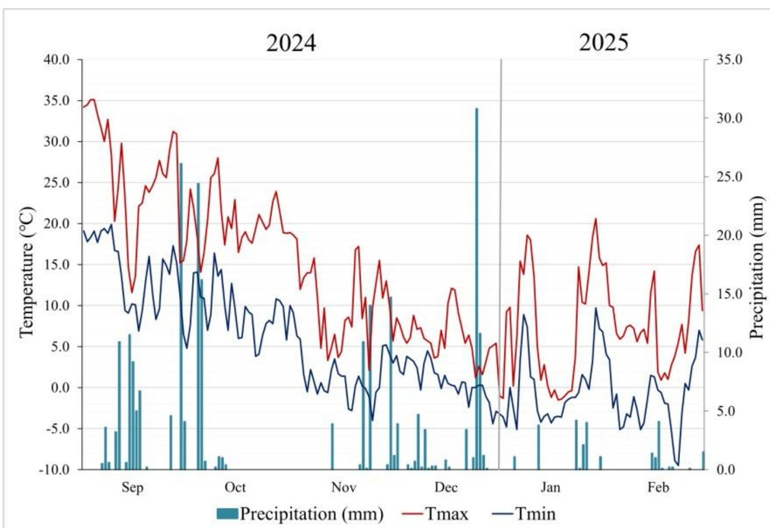

Figure 3. Daily maximum and minimum air temperature (°C) trends and precipitation amounts for the period from September 1, 2024, to February 28, 2025, based on the data from the Surčin Meteorological Station

Bioactive Components of Blackthorn Fruits in Late Summer, Early Winter, and Early Spring

Table 3 presents the descriptive statistics results for the bioactive components and antioxidant capacity of Blackthorn fruits for the three sampling periods and applied solvents, as well as ANOVA analysis between sampling periods conducted separately for each solvent at a significance level of $p<0.05$.

Table 3. Descriptive statistics for bioactive components and antioxidant capacity of Blackthorn fruits according to the sampling time, solvent, and one-way ANOVA results between the sampling times at significance level of p**<0.05**

Sampling time	**1***	**2***	**3***	**ANOVA**	
Traits	**Mean±Se**	**Mean±Se**	**Mean±Se**	**F**	**p**
Total Phenolics (mg quercetin equivalents/g fresh weight)					
Water	4.50±0.21	7.83±0.13	9.81±0.29	898.55	**0.0000**
Ethanol	9.99±0.48	12.24±0.15	13.59±1.29	31.140	**0.0000**
Total Tannins (mg quercetin equivalents/g fresh weight)					
Water	2.30±0.11	4.69±0.24	7.21±0.65	218.705	**0.0000**
Ethanol	4.94±0.50	7.40±0.24	9.71±0.96	83.739	**0.0000**
Total Flavonoids (mg quercetin equivalents/g fresh weight)					
Water	0.28±0.04	0.54±0.08	0.74±0.22	17.208	**0.0000**
Ethanol	2.44.6±0.24	2.48±0.34	4.15±1.08	12.789	**0.0000**
Total Anthocyanins (mg cyanidin-3-glucoside equivalents/g fresh weight)					
Water	0.13±0.02	0.21±0.7	0.18±0.04	3.889	**0.044**
Ethanol	2.76±0.51	2.41±0.44	2.56±0.95	0.413	0.669
FRAP (mg trolox equivalents/g fresh weight)					
Water	15.25±0.73	21.64±0.62	29.48±2.31	146.561	**0.0000**
Ethanol	31.18±0.52	35.00±0.75	39.66±0.79	222.463	**0.0000**
DPPH (mg trolox equivalents/g fresh weight)					
Water	10.54±0.42	15.73±0.79	12.91±0.63	101.824	**0.0000**
Ethanol	33.86±0.47	41.95±0.69	34.08±4.41	18.953	**0.0000**
ABTS (mg trolox equivalents/g fresh weight)					
Water	34.09±1.19	45.89±0.62	37.85±2.66	73.702	**0.0000**
Ethanol	72,47±1.38	79,36±0.93	61.08±2.51	169.480	**0.0000**

Legend: *1, 2, and 3 are the sampling dates; the bolded p-values are statistically significant

Differences between the sampling terms were observed, and statistical significance was confirmed by ANOVA for all the bioactive components and antioxidant capacity tests for both solvents, except for total anthocyanins in the ethanol solvent.

Considering the ANOVA results presented in Tables 4 and 5, the Tukey's HSD test results at a significance level of $p < 0.05$ are shown, indicating variations in the content of bioactive components and antioxidant capacity depending on the sampling term and solvent type. The lowest total phenolics content was recorded in the samples from the first sampling term extracted with water (4.502 mg/g), while the highest total phenolics content was found in the samples from the third term extracted with ethanol (13.592 mg/g). The Tukey test grouped the samples into three groups, with the water-extracted samples showing significantly lower phenolic content compared to those extracted with ethanol. The analysis of differences between the sampling terms, with a 95% confidence interval, was statistically significant between all the terms. The solvent has a key influence on phenolic extraction, and the samples from the third term, regardless of the solvent, showed higher total phenolic content, suggesting a possible impact of climatic factors on the phenolic content of Blackthorn fruits. The trends observed in the total phenolic and tannin contents analyses were similar, depending on the sampling term and solvent type. The lowest tannin content was recorded in the samples from the first sampling term extracted with water (2.302 mg/g), whereas the highest tannin content was found in samples from the third term extracted with ethanol (9.710 mg/g). The content of both types of bioactive compounds was higher in the ethanol-extracted samples compared to those extracted with water. The total phenolic content in all the samples was higher than the tannin content. The Tukey test for both parameters showed the same significant differences between the sampling terms (95% confidence interval), statistically significant for both solvents and among all the groups, further confirming the importance of solvent type and sampling term on the bioactive compound content in Blackthorn fruits. The results presented in Table 4 indicate significant differences in the total flavonoid and total anthocyanin contents depending on the sampling term and solvent type. Ethanol was a more efficient solvent for extracting both bioactive compounds, as the total flavonoid and total anthocyanin values were higher in the ethanol extracts compared to the water extracts. The lowest total flavonoid and total anthocyanin contents were observed in the samples from the first sampling term extracted with water (0.275 mg/g and 0.128 mg/g, respectively), while the highest total flavonoid content was found in the samples from the second term extracted with ethanol (2.482 mg/g), and the highest total anthocyanins content in the samples from the first term (2.760 mg/g).

Table 4. Results of Tukey's (HSD) test / Analysis of differences between the categories with a 95% confidence interval for the phenolic compounds determined in Blackthorn fruits at three sampling terms

Contrast	Difference	Standardized difference	Critical value	Pr > Diff	Significant	Lower bound (95%)	Upper bound (95%)	Lower bound (95%)	Upper bound (95%)
Total Phenolics (mg quercetin equivalents/g fresh weight) – solvent water									
3 vs 1	5.307	41.942	2.597	< 0.0001	Yes	4.978	5.635		
3 vs 2	1.978	15.636	2.597	< 0.0001	Yes	1.650	2.307		
2 vs 1	3.328	26.306	2.597	< 0.0001	Yes	3.000	3.657		
Total Phenolics (mg quercetin equivalents/g fresh weight) – solvent ethanol									
3 vs 1	3.598	7.812	2.597	< 0.0001	Yes	2.402	4.795		
3 vs 2	1.353	2.938	2.597	0.026	Yes	0.157	2.550		
2 vs 1	2.245	4.874	2.597	0.001	Yes	1.049	3.441		
Total Tannins (mg quercetin equivalents/g fresh weight) – solvent water									
3 vs 1	4.903	20.912	2.597	< 0.0001	Yes	4.294	5.512		
3 vs 2	2.512	10.712	2.597	< 0.0001	Yes	1.903	3.121		
2 vs 1	2.392	10.200	2.597	< 0.0001	Yes	1.783	3.001		
Total Tannins (mg quercetin equivalents/g fresh weight) – solvent ethanol									
3 vs 1	4.770	12.939	2.597	< 0.0001	Yes	3.812	5.728		
3 vs 2	2.313	6.275	2.597	< 0.0001	Yes	1.356	3.271		
2 vs 1	2.457	6.664	2.597	< 0.0001	Yes	1.499	3.414		
Total Flavonoids (mg quercetin equivalents/g fresh weight) – solvent water									
3 vs 1	0.460	5.840	2.597	< 0.0001	Yes	0.255	0.665		
3 vs 2	0.192	2.433	2.597	0.068	No	-0.013	0.396		
2 vs 1	0.268	3.406	2.597	0.010	Yes	0.064	0.473		
Total Flavonoids (mg quercetin equivalents/g fresh weight) – solvent ethanol									
3 vs 1	1.710	4.435	2.597	0.001	Yes	0.708	2.712		
3 vs 2	1.667	4.323	2.597	0.002	Yes	0.665	2.668		
2 vs 1	0.043	0.112	2.597	0.993	No	-0.958	1.045		
Total Anthocyanins (mg cyanidin-3-glucoside/g fresh weight) – solvent water									
2 vs 1	0.078	2.764	2.597	0.036	Yes	0.005	0.152		
2 vs 3	0.030	1.058	2.597	0.553	No	-0.044	0.104		
3 vs 1	0.048	1.705	2.597	0.236	No	-0.025	0.122		
Total Anthocyanins (mg cyanidin-3-glucoside/g fresh weight) – solvent ethanol									
1 vs 2	0.350	0.905	2.597	0.645	No	-0.654	1.354		
1 vs 3	0.200	0.517	2.597	0.864	No	-0.804	1.204		
3 vs 2	0.150	0.388	2.597	0.921	No	-0.854	1.154		

Legend: *1, 2, and 3 are the sampling dates

The Tukey test for these two parameters showed different grouping patterns that confirm the significance of both the solvent type and the sampling term, except for the total anthocyanins values extracted with ethanol, which belonged to the same group, indicating that the sampling term did not affect their content in Blackthorn fruits. Specifically, the analysis of differences between the sampling terms for total flavonoids, at a 95% confidence interval, was statistically significant for the water solvent between terms 3 and 1, as well as between terms 2 and 1, while no significant differences were observed between terms 3 and 2. For the ethanol solvent, statistically significant differences in the total flavonoids content were found between terms 3 and 1 and between terms 3 and 2, but not between terms 2 and 1. The analysis of differences between the sampling terms for anthocyanins, at a 95% confidence interval, was statistically significant only for the water solvent between terms 2 and 1, whereas no significant differences were observed between the sampling terms for ethanol extracts.

The results indicate that Blackthorn fruits contain a variety of bioactive polyphenolic compounds, including phenols, tannins, flavonoids, and anthocyanins, as confirmed by Radovanović et al. (2013) for Blackthorn fruits sampled during July-August 2011 in the Vlasina Nature Park in southeastern Serbia under typical mountain climate conditions. Using water as a solvent, the results for the three antioxidant assays showed low values in the first sampling period: FRAP (15.248 mg/g), DPPH (10.535 mg/g), and ABTS (34.087 mg/g). Using ethanol, the highest value was observed in the third sampling period for the FRAP test (36.660 mg/g), while for the other two antioxidant tests the highest values using ethanol were recorded in the second sampling period: DPPH (41.947 mg/g) and ABTS (79.357 mg/g). The Tukey test for FRAP and ABTS with both solvents showed different homogeneous group patterns, but the analysis of differences between sampling periods (95% confidence interval) was statistically significant for both solvents across all groups, further confirming the importance of the solvent type and sampling period (Table 5). For DPPH, the analysis of differences between sampling periods was statistically significant for water as the solvent across all groups, whereas for ethanol statistical significance was observed between periods 2 and 1, and between 2 and 3, while differences between periods 3 and 1 were not statistically significant.

Table 5. Results of Tukey's (HSD) test / Analysis of differences between the categories with a 95% confidence interval for antioxidant tests determined in Blackthorn fruits at three sampling periods

Contrast	**Difference**	**Standardized difference**	**Critical value**	**Pr > Diff**	**Significant**	**Lower bound (95%)**	**Upper bound (95%)**	**Lower bound (95%)**	**Upper bound (95%)**
FRAP (mg trolox equivalents/g fresh weight) – solvent water									
3 vs 1	14.233	17.091	2.597	< 0.0001	Yes	12.070	16.396		
3 vs 2	7.845	9.420	2.597	< 0.0001	Yes	5.682	10.008		
2 vs 1	6.388	7.671	2.597	< 0.0001	Yes	4.225	8.551		
FRAP (mg trolox equivalents/g fresh weight) – solvent ethanol									
3 vs 1	8.478	21.059	2.131	< 0.0001	Yes	7.620	9.336		
3 vs 2	4.660	11.575	2.131	< 0.0001	Yes	3.802	5.518		
2 vs 1	3.818	9.484	2.131	< 0.0001	Yes	2.960	4.676		
DPPH (mg trolox equivalents/g fresh weight) – solvent water									
2 vs 1	5.197	14.253	2.597	< 0.0001	Yes	4.250	6.144		
2 vs 3	2.822	7.739	2.597	< 0.0001	Yes	1.875	3.769		
3 vs 1	2.375	6.514	2.597	< 0.0001	Yes	1.428	3.322		
DPPH (mg trolox equivalents/g fresh weight) – solvent ethanol									
2 vs 1	8.083	5.403	2.597	0.000	Yes	4.198	11.969		
2 vs 3	7.865	5.257	2.597	0.000	Yes	3.979	11.751		
3 vs 1	0.218	0.146	2.597	0.988	No	-3.667	4.104		
ABTS (mg trolox equivalents/g fresh weight) – solvent water									
2 vs 1	11.805	11.884	2.597	< 0.0001	Yes	9.225	14.385		
2 vs 3	8.042	8.095	2.597	< 0.0001	Yes	5.461	10.622		
3 vs 1	3.763	3.788	2.597	0.005	Yes	1.183	6.344		
ABTS (mg trolox equivalents/g fresh weight) – solvent ethanol									
2 vs 3	18.282	18.227	2.597	< 0.0001	Yes	15.676	20.887		
2 vs 1	6.883	6.863	2.597	< 0.0001	Yes	4.278	9.489		
1 vs 3	11.398	11.364	2.597	< 0.0001	Yes	8.793	14.004		

Legend: *1, 2, and 3 are the sampling dates

The extracts of Blackthorn fruits show high content of polyphenolic compounds and strong antioxidant activity, consistent with the findings by Veličković et al. (2014), who determined the contents of phenolics, flavonoids, and anthocyanins, as well as the antioxidant activity, using spectrophotometric methods.

Spearman's correlation coefficient (ρ) confirmed only a positive and very strong correlation (0.870) with $p = 0.003$, observed in the third sampling period for the water solvent between flavonoids and phenols (Figure 3). The higher total flavonoids content correlating with increased total phenols levels may be linked to ecological conditions favoring biosynthesis of these compounds or to greater stress factors (Kolarov et al., 2021). Other correlations were not statistically significant at $p < 0.05$, and the correlations between different solvents were not relevant.

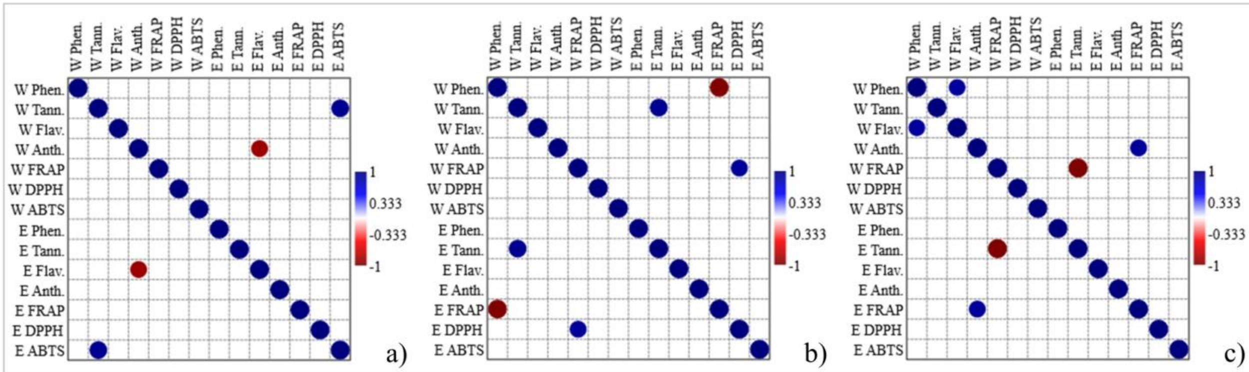

Figure 3. Spearman's correlation coefficients (p<0.05) for phenols (Phen.), tannins (Tann.), flavonoids (Flav.), anthocyanins (Anth.), and antioxidant tests FRAP, DPPH, and ABTS: a) late summer 2024, b) early winter 2024/2025, and c) early spring 2025 for solvents water (W) and ethanol (E)

To identify the effects of sampling time and solvent on the biochemical characteristics of Blackthorn fruits, as well as the observed differences in previous tests, the LS means test (least squares means) was applied. LS means estimate the average values as if the data were balanced and indicate the significance of the analyzed parameter's response. The graphical representations of the values by sampling time and solvent are shown in Figure 4, with statistical significance detailed in Table 6.

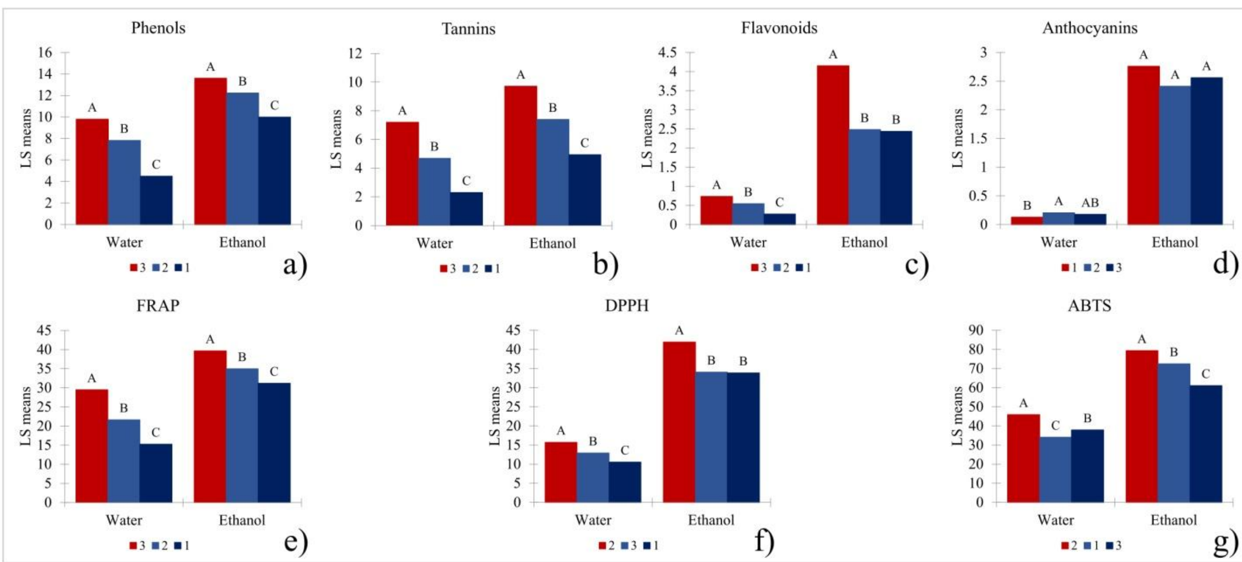

Figure 4. Visual representation of LS means: a) total phenols (mg quercetin equivalents/g fresh weight), b) total tannins (mg quercetin equivalents/g fresh weight), c) total flavonoids (mg quercetin equivalents/g fresh weight), d) total anthocyanins (mg cyanidin-3-glucoside equivalents/g fresh weight), e) FRAP (mg trolox equivalents/g fresh weight), f) DPPH (mg trolox equivalents/g fresh weight), and g) ABTS (mg trolox equivalents/g fresh weight)

Based on the LS means results, the statistical significance between sampling periods was confirmed for all the parameters and solvents, except for anthocyanins extracted with ethanol, where no statistically significant differences between sampling periods were observed (Table 6). The variability patterns indicate the appropriate sampling periods for the extraction of bioactive components and can serve as a basis for strategies aligned with climate change predictions.

Table 6. Statistical significance of the LS means results for the polyphenolic compounds and antioxidant tests of Blackthorn fruits, by sampling time and solvent, at significance level $p < 0.05$

Solvent/Parameter	Phenols	Tannins	Flavonoids	Anthocyanins	FRAP	DPPH	ABTS
Water - Pr > F(Model)	0.0000	0.0000	0.0000	0.044	0.0001	0.0001	0.0001
Ethanol - Pr > F(Model)	0.0000	0.0000	0.001	0.669	0.0001	0.0001	0.0001
Water - Significant	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ethanol - Significant	Yes	Yes	Yes	No	Yes	Yes	Yes
Water - Pr > F(Group)	0.0000	0.0000	0.0000	0.044	0.0001	0.0001	0.0001
Ethanol - Pr > F(Group)	0.0000	0.0000	0.001	0.669	0.0001	0.0001	0.0001
Water - Significant	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ethanol - Significant	Yes	Yes	Yes	No	Yes	Yes	Yes

The potential effects of bioactive compounds

Polyphenolic compounds

The phytochemical analyses of ethanol and water extracts of Blackthorn fruits confirmed that the ethanol solvent consistently exhibited higher values of polyphenolic compounds compared to the water solvent across all the sampling periods, with the following percentage (%) differences between the sampling terms: total phenols (55.0% in the first, 36.0% in the second, and 27.8% in the third term), total tannins (53.4% in the first, 36.5% in the second, and 25.8% in the third term), total flavonoids (88.7% in the first, 78.1% in the second, and 82.3% in the third term), and total anthocyanins (95.4% in the first, 91.4% in the second, and 93.1% in the third term). Considering that the standard deviation, as a descriptive statistic element, indicates the variation of individual measurements within the group from the mean, these differences were further confirmed through the confidence intervals for both solvents and all three sampling periods at a 95% confidence level (Figure 5).

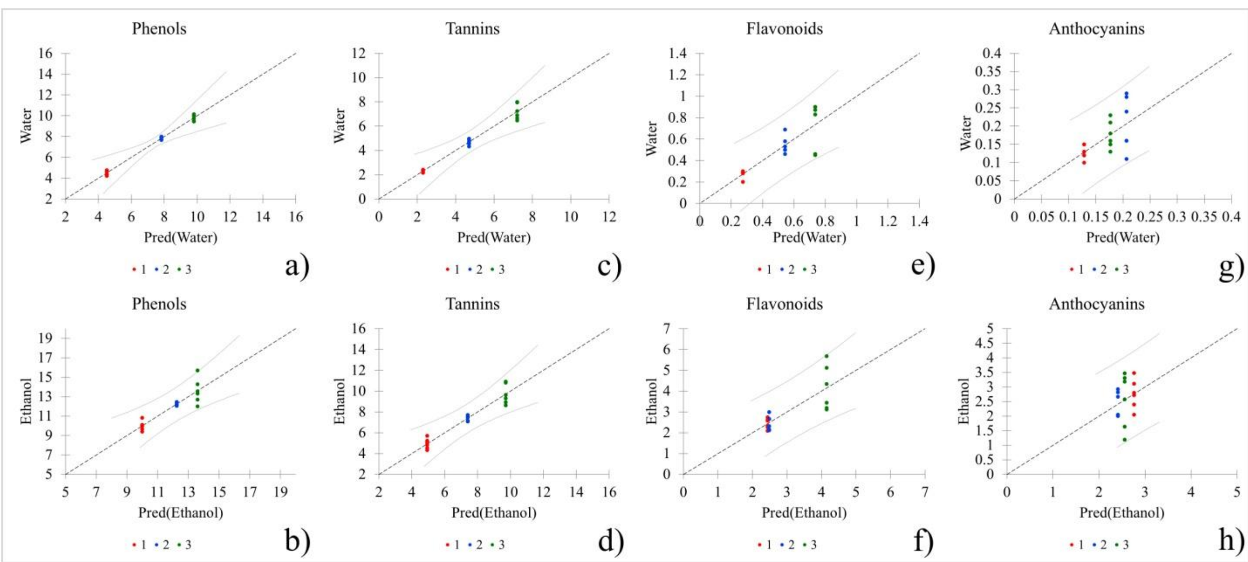

Figure 5. Graphical representation of the confidence intervals for polyphenolic compounds (mg quercetin equivalent/g fresh weight): a) total phenols for water solvent, b) total phenols for ethanol solvent, c) total tannins for water solvent, d) total tannins for ethanol solvent, e) total flavonoids for water solvent, f) total flavonoids for ethanol solvent, and (mg cyanidin-3-glucoside/g fresh weight) for g) total anthocyanins for water solvent and h) total anthocyanins for ethanol solvent

(Legend: *1, 2, and 3 in the legend represent the sampling terms)

Different contents of the polyphenolic compounds were observed between the sampling periods (Figure 5). For the water solvent, the highest contents of total phenols, tannins, and flavonoids were recorded in the third sampling period, being 54.1%, 68.1%, and 62.6% higher than in the first period, respectively, whereas the total anthocyanins peaked in the second period with a 37.9% increase compared to the first period. A decrease in the total anthocyanin content of 17.0% was noted in the third period compared to the second, which is considered the optimal period. For the ethanol solvent, the phenolic compound contents favor the third period, where the total phenols, tannins, and flavonoids were 26.5%, 49.1%, and 41.2% higher than in the first period, respectively, and the total anthocyanins were 5.9% higher than in the second period. The study findings confirm the positive influence of low air temperatures during November 2024 (second period) and February 2025 (third period) on the bioactive components of the fruits, consistent with Olesinska et al. (2019), who reported that freezing samples during storage enhances the ability of phenols to reduce iron ions. Overall, it was confirmed that Blackthorn fruits are rich in polyphenolic compounds, as also shown by Blagojević et al. (2022), who highlight the total phenols and anthocyanins. However, this study's novelty lies in identifying the most suitable harvest time as either early spring or early winter. Further research should promote the use of ethanol extracts of Blackthorn fruits and their encapsulated forms for the development of new functional foods and dietary supplements (Blagojević et al., 2022), cosmetic products with enhanced photoprotective properties (Cefali et al., 2019), as well as natural food preservatives and colorants (Backes et al., 2020).

This study makes a special contribution by confirming that the harvest time does not affect the anthocyanin content, thus promoting the commercial value of Blackthorn fruits for developing ethanol-based anthocyanin extracts for natural dyes. This supports previous research by Leichtweis et al. (2019), which indicated that Blackthorn fruits can be used for industrial dye production.

Antioxidant Activity

The strength of antioxidant activity was analyzed using the FRAP test (ferric reducing antioxidant power) and radical scavenging activity was measured by DPPH and ABTS tests. It was confirmed that the ethanol solvent, in all the sampling periods, exhibits higher values compared to the water solvent, with the following percentage (%) differences between the periods: FRAP (51.1 in the first, 38.2 in the second, and 25.7 in the third period), DPPH (68.9 in the first, 62.5 in the second, and 62.1 in the third period), and ABTS (53.0 in the first, 42.2 in the second, and 38.0 in the third period). These differences are also clearly reflected in the confidence intervals, representing the ranges within which the true mean values lie for the sampling periods, for both solvents, at a 95% confidence level (Figure 6).

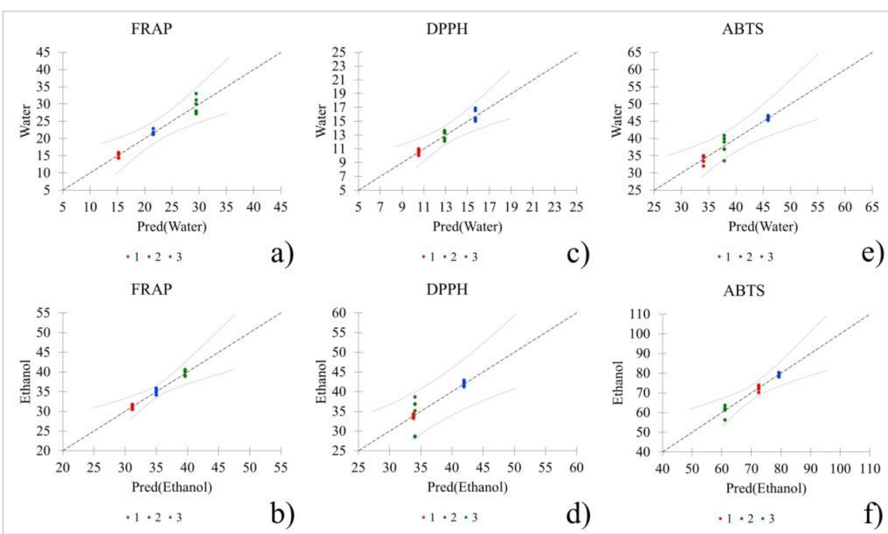

Figure 6. Graphical representation of the confidence intervals for antioxidant tests (mg Trolox equivalents/g fresh weight): a) FRAP for water solvent, b) FRAP for ethanol solvent, c) DPPH for water solvent, d) DPPH for ethanol solvent, e) ABTS for water solvent, and f) ABTS for ethanol solvent

(Legend: *1, 2, and 3 in the legend represent the sampling terms)

In Figure 6, different antioxidant activities can be observed between the sampling periods and solvents, with ethanol showing a stronger effect. For the water solvent, the highest antioxidant activity measured by the FRAP test was recorded in the third sampling period, being 48.3% higher than the first. For the DPPH and ABTS tests, the highest activity was observed in the second period, with increases of 33.0% and 25.7% respectively, compared to the first period. However, in the third period, the DPPH and ABTS activities decreased by 21.9% and 21.2% compared to the second period, which is considered the optimal sampling time. A similar trend is seen with the ethanol solvent, but with significantly higher values. The highest FRAP antioxidant activity for ethanol was in the third period, 21.4% higher than the first. For DPPH and ABTS, the second period showed the highest activities, 19.3% and 8.7% higher than the first period, respectively. In the third period, DPPH and ABTS activities decreased by 23.1% and 29.9% compared to the second period, with ABTS also showing an 18.7% decrease compared to the first period.

Moreover, the ethanol extracts of Blackthorn fruits were richer in total phenols, tannins, flavonoids, and anthocyanins compared to water extracts, demonstrating that the content of polyphenolic compounds and antioxidant activity are closely related. This finding aligns with previous studies (Ruiz-Rodriguez et al., 2014; Veličković et al., 2021; Marčetić et al., 2022). Sikora et al. (2013) also reported a strong correlation between DPPH values and phenolic components in Blackthorn fruits. To the authors' knowledge, this study is the first to establish that antioxidant content in Blackthorn fruits is dependent on harvest time. Therefore, ethanol extraction is recommended in early spring when FRAP values are highest, and in early winter when DPPH and ABTS values peak, alongside very high FRAP values.

Based on all the results, a wider cultivation of Blackthorn is recommended as a species with traditional use of fresh fruits, as well as a fruit plant suitable for targeted use and the formation of sustainable, climate-smart landscapes, as highlighted by Čukanović et al. (2024). Specifically, the conducted analyses of ecosystem services highlight Blackthorn as a fruit plant within the context of ecological engineering, with beneficial impacts on landscapes and potential to mitigate the challenges of climate change by providing food, improving the environment, and enhancing biodiversity.

CONCLUSION

The effects of Blackthorn fruit extracts on human health have been the focus of numerous studies. However, despite extensive analysis of the content of bioactive compounds, the most suitable harvesting periods for extracting polyphenolic compounds and antioxidant capacity—key factors responsible for antibacterial, antiviral, and anticancer properties—have yet to be identified.

This study demonstrates that the biochemical composition of Blackthorn fruits is influenced by the location of the population, with climatic factors playing a pivotal role in stimulating the accumulation of secondary metabolites from late summer through early spring. Low temperatures were confirmed to have a positive effect on the content of bioactive compounds, and the ethanol extracts consistently exhibited higher levels of polyphenols. Specifically, the ethanol extracts obtained in early spring (third harvesting period) contained the total phenols, tannins, and flavonoids that were 26.5%, 49.1%, and 41.2% higher, respectively, than those from the late summer harvest, while the total anthocyanin content was 5.9% higher than in the early winter harvest. The most suitable harvesting times for the functional extraction of phenolic compounds and antioxidant activity are identified as early spring or early winter. The exception is the total anthocyanins, for which the harvesting time was found to have no impact on their content. The antioxidant activity measured by the FRAP test with ethanol as the solvent was the highest in early spring (21.4% higher compared to the late summer harvest), whereas for DPPH and ABTS it was the highest in early winter (19.3% higher compared to the early spring harvest and 8.7% higher compared to the late summer harvest, respectively).

Further research should focus on the targeted use of Blackthorn fruit extracts as sustainable alternatives to conventional pharmaceuticals and other therapeutic interventions, addressing the growing demand for effective plant-based treatments and environmentally friendly production practices. Moreover, the results indicate that anthocyanin extracts from Blackthorn fruits, irrespective of harvest time, have potential as stable natural colorants in functional foods and dietary supplements, contributing to the development of safe, chemical-free food products.

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