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RESEARCH ARTICLE

Physicochemical Characterization and Bioactive Profile of Wild Demir Apple (*Malus sylvestris*) From the Eastern Black Sea Region

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ABSTRACT

This study characterized the physicochemical properties, bioactive composition, and antioxidant capacity of the wild Demir apple (*Malus sylvestris*), an endemic genotype from the Eastern Black Sea region. Samples collected from Artvin were analyzed for physical and chemical attributes, including total soluble solids (13.87%), titratable acidity (0.43% malic acid), ascorbic acid (5.93 mg/100 g FW), and total sugars (11.94%). High-performance liquid chromatography (HPLC–PDA) revealed chlorogenic acid, epicatechin, and catechin as the predominant phenolic compounds. The total phenolic and flavonoid contents were 103.98 mg GAE/100 g FW and 2.09 mg QUE/100 g FW, respectively, while antioxidant capacity, assessed via FRAP and DPPH assays, was 2635.91 µmol $FeSO_4$/g and 7.75 mg/mL. These findings highlight the wild Demir apple as a rich source of bioactive compounds with strong antioxidant potential, supporting its value for nutritional and functional food applications.

1 | Introduction

Apple (*Malus* spp.) is one of the most widely cultivated fruit trees worldwide. Today, more than 7000 apple varieties have been identified, and these varieties are classified as commercial, local (traditional), and wild species. Commercially produced apple varieties around 100 and include common varieties such as Golden Delicious, Red Delicious, Granny Smith, Fuji, Gala, and Braeburn [1].

Local and wild apple species contribute to the conservation of genetic diversity, especially in regions such as the Caucasia, Central Asia, and Europe, and are an important source for the development of new cultivars. Although *Malus domestica* is the most common cultivated apple species, wild species such as *Malus sylvestris*, *Malus sieversii*, and *Malus asiatica* are also among the genetic ancestors of modern apple varieties [2]. In Turkiye, the most produced apple varieties are Starking, Golden, Amasya, and Granny Smith [3].

Anatolia is one of the centres of apple genetic diversity, and a wide variation and richness, including local genotypes, are observed in this region [2, 4]. Amasya apple and Gümüşhane apple are among the important local apple varieties, while Yomra apple and Demir apple are important winter apple varieties grown in the Eastern Black Sea Region. Demir apple is a variety with high resistance to ecological conditions and has medium-sized fruits. The surface exposed to the sun is pink, red in color, while the part in the shade has greenish tones. Demir apple genotypes are characterized by a firm skin, juicy texture, and a balanced flavor that is both sweet and slightly tart. Considered a diabetes-friendly fruit due to its low sugar content, this apple is grown with traditional organic

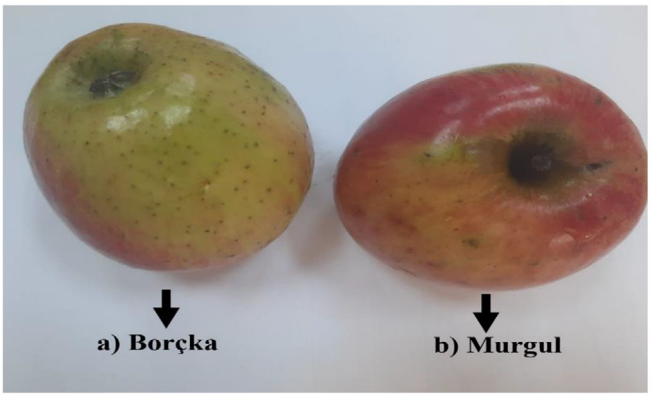

FIGURE 1 | Apple samples and their geographical collection sites: (a) Borçka—41.361480° N, 41.670149° E; (b) Murgul—41.272284° N, 41.563814° E.

farming methods and can be kept for about nine months without the need for cold storage. Another remarkable feature of the Demir apple is that the tree does not bear fruit every year, but leaves it fallow for a year in certain periods [5]. These endemic apple species exhibit a high level of resistance to diseases and adverse climatic conditions. However, scientific studies on this species are quite limited.

In this study, it was aimed to add the distinctive features in terms of nutritional chemistry to the literature by examining the physicochemical properties, phenolic composition, and antioxidant properties of Demir apples obtained from two different districts of Artvin Province.

2 | Materials and Methods

2.1 | Samples and Extractions

In October 2024, apple samples were harvested from the Borçka and Murgul districts of Artvin Province (Figure 1). The physical properties, acidity, and soluble solids of the harvested fruits were determined as soon as they arrived at the laboratory. The remaining fruits were stored at +4°C for analysis. The apples were peeled and cut into thin slices and blended in 80% methanol. The mixture was left in an ultrasonic bath for 2 h and then extracted in a shaker at room temperature for 24 h. Filtration was performed in two steps: initially, a coarse filtration was conducted, followed by fine filtration using high-quality, fine-pored filter paper. The resulting clear extract was stored in a closed container in the refrigerator for use in the study.

2.2 | Physicochemical Properties

The total soluble solid (TSS), namely, Brix%, was determined following the guidelines established by AOAC [2]. This measurement was conducted on the juice extracted from each sample using an Abbe-3 L refractometer (Tokyo, Japan) at a controlled temperature of 20°C (Table 1).

The titratable acidity (TA) of the apple extract was measured using a titration technique. Initially, the aqueous extract was centrifuged, and the supernatant was diluted 1:20 with distilled water. The prepared solution was then titrated with 0.01 M sodium hydroxide (NaOH) solution and phenolphthalein was used as an indicator until a stable pink color appeared. TA was calculated as a percentage of malic acid equivalents [6].

2.3 | Total Phenolic Content

Total phenolic content (TPC) of methanolic apple extracts was determined according to the Folin–Ciocalteu spectrophotometric method [7]. For this purpose, 50 µL of extract was mixed with 400 µL of 0.2 N Folin–Ciocalteu reagent and diluted with 680 µL distilled water. The mixture was incubated at room temperature for 3 min, then 400 µL of 10% Na_2CO_3 solution was added and incubated at 25°C for another 2 h, after which the absorbance was measured at 760 nm. After a calibration curve was prepared according to the gallic acid standard, the amounts of TPC expressed as (mg GAE/100 g) were calculated according to the absorbance of the samples (Table 2).

2.4 | Total Flavonoid Contents

Total flavonoid contents (TFC) of methanolic apple extracts were analyzed by the spectrophotometric method of aluminum nitrate reaction with a small modification [8]. In this determination, 50 µL of the extract was mixed with 50 µL of 10% aluminum nitrate $Al(NO_3)_3$ and 50 µL of 1.0 M ammonium acetate (NH_4CH_3COO). The mixture was diluted to a final volume of 3.0 mL with 99% ethanol and incubated at 25°C for 45 min. After that, the absorbance was measured at 415 nm. A calibration curve was prepared according to the quercetin standard, and the results were calculated as (mg QUE/100 g) (Table 3).

2.5 | Total Antioxidant Capacity

Total antioxidant capacity (TAC) of methanolic extracts was determined according to the ferric reducing antioxidant power (FRAP) method [9]. In this method, 3 mL of FRAP reagent was mixed with 100 µL of extract and incubated at 37°C for 4 min. After that, absorbance was read at 595 nm. $FeSO_4 \cdot 7H_2O$ standards were diluted from 1000 to 31.25 µmol/mL at a 1:1 ratio to create a calibration curve for $FeSO_4 \cdot 7H_2O$. According to the graph, the FRAP value of the samples was calculated as µmol $FeSO_4 \cdot 7H_2O$/100 g.

2.6 | DPPH Radical Scavenging Activity

DPPH radical scavenging activity of methanolic extracts was measured by the spectrophotometric method [10]. One milliliter of the methanolic solution of commercial DPPH radical (0.04 mg/mL) was taken, 1.0 mL of the extract was added, and incubated at 25°C in the dark for 45 min, and then the absorbance was measured at 517 nm. The SC_{50} (mg/mL) value, which is the amount of extract that scavenges 50% of the radical, was determined using six different extract concentrations.

TABLE 1 | Physical properties of the Demir apple samples.

	Borçka ($n = 10$)	Murgul ($n = 10$)	Mean ($n = 20$)
Weigt (g)	83–104 (8.51)	77–121 (11.7)	93.15 ± 2.25
	92.70[a]	93.6[a]	
Length (mm)	48–57 (4.27)	51–63 (4.08)	54.20 ± 0.14
	54.40[a]	55.00[a]	
Width (mm)	49–68 (5.54)	58–70 (3.54)	60.45 ± 1.41
	57.40[a]	63.5 ± 4.64[a]	
Titratable acidity (TA) (malic acid%)	0.40–0.50 (0.05)	0.35–0.45 (005)	0.43 ± 0.00
	0.45[a]	0.40[a]	
Total soluble solid TSS (%)	14.00–14.50 (0.25)	13.00–14.00 (0.50)	13.87 ± 0.18
	14.02 ± 0.35[a]	13.50[a]	
Ascorbic acid (mg/100 g FW)	5.30–6.40 (0.57)	5.70–6.4 (0.36)	5.93 ± 0.15
	5.77[a]	6.10[a]	

Note: Values are presented as min–max (standard deviation).
Different letters (a) in the same lines are significantly different at the 5% level ($p < 0.05$)

TABLE 2 | Sugar composition of the Demir apple samples.

g/100g	Borçka ($n = 10$)	Murgul ($n = 10$)	Mean ($n = 20$)
Fructose%	9.10 ± 0.58[a]	8.46 ± 0.46[a]	8.78 ± 0.51
Glucose%	0.28 ± 0.04[a]	0.29 ± 0.05[a]	0.29 ± 0.05
Sucrose%	3.20 ± 0.12[a]	2.55 ± 0.30[b]	2.88 ± 0.23
Total sugar content%	12.58 ± 4.49[a]	11.30 ± 0.68[a]	11.94 ± 2.24

Different letters (a-b) in the same lines are significantly different at the 5% level ($p < 0.05$)

2.7 | Sugar Compositions and Total Sugar Amount

The sugar profiles of the methanolic extracts were analyzed using high-performance liquid chromatography (HPLC) equipped with a refractive index detector (RID). Analysis was performed with HPLC–RID pre-calibrated against eight different sugar standards (ribose, fructose, glucose, sucrose, maltose, trehalose, melibiose, and melezitose). Ten-milliliter methanolic extract was filtered, and the filtrate was evaporated using a rotary evaporation (IKA-Werke, RV 05 Basic, Staufen, Germany) to dryness. The residue was reconstituted in 2 mL ultrapure water and analyzed by injection. In this process, a reverse-phase-NH_2 column (EC 250/4 Nucleosil, Macherey-Nagel) with an isocratic mobile phase of 80% acetonitrile and 20% ultrapure water was used. The injection volume was 20 µL, with a mobile phase flow rate of 1.5 mL/min and a column temperature of 30°C [11]. Sugar contents were calculated in mg/100 g by evaluating each peak area. Total sugar concentration was calculated in g/100 g FW relative to the total of sugars detected (Table 4).

2.8 | Phenolic Components

In this study, individual phenolic components of methanolic extracts were measured by HPLC–PDA (Shimadzu Liquid Corporation LC 20AT) system using a photodiode detector (Shimadzu Liquid Corporation LC 20AT) and a C18 column (250 mm × 4,6 mm, 5 µm; GL Sciences).

The methanolic apple extracts were analyzed after enrichment by liquid–liquid extraction. For the extraction, 10 mL of methanolic extract was evaporated at 40°C using a rotary evaporator. Ten milliliters of distilled water was added to the residue, and the pH was adjusted to 2 with 2 N HCl. After the mixture was vortexed, it was extracted with diethyl ether and ethyl acetate three times consecutively [12].

After evaporation of the organic solvents, the remaining residue was dissolved in 2 mL of methanol, filtered with a 0.45-µm RC membrane, and prepared for analysis. A total of 26 phenolic standards were analyzed using a pre-calibrated method [13]. The mobile phase consisted of two solvents: (A) 2% acetic acid in water and (B) a 70:30 mixture of acetonitrile and water. Both sample and standard injections were set to 20 µL, the column temperature was maintained at 30°C, and the flow rate was 1.0 mL/min.

2.9 | Statistics

All experiments were conducted in triplicate, and the results are presented as the mean ± standard deviation. Pearson correlation analysis, a parametric test ($p < 0.05$), was used to assess relationships between variables.

3 | Results and Discussions

3.1 | Physicochemical Properties

Table 1 presents the measured physical properties of weight, length, and width of Demir apple samples collected from two distinct districts of Artvin Province. According to the averages taken from 10 apple samples, it was determined that the samples

TABLE 3 | Phenolic composition of the Demir apple samples.

Phenolic content (μg/g)	Borçka	Murgul
p-OH Benzoic acid	<LOD	<LOD
Gallic acid	<LOD	<LOD
Protocatechuic acid	<LOD	<LOD
Chlorogenic acid	52.05 ± 0.02	65.19 ± 0.05
Caffeic acid	<LOD	<LOD
Syringic acid	<LOD	<LOD
Vanillic acid	<LOD	<LOD
Ellagic acid	<LOD	<LOD
p-Coumaric acid	1.43 ± 0.03	0.34 ± 0.01
Ferulic acid	<LOD	<LOD
Catechin Hydrate	30.53 ± 0.08	23.29 ± 0.04
p-OH Benzoic acid	<LOD	<LOD
t-Cinnamic acid	<LOD	<LOD
Epicatechin	50.90 ± 0.08	60.44 ± 0.09
Rutin	<LOD	<LOD
Chrysin	0.73 ± 0.07	3.23 ± 0.02
Myricetin	<LOD	<LOD
Daidzein	<LOD	<LOD
Luteolin	<LOD	<LOD
Quercetin	<LOD	<LOD
Naringenin	<LOD	<LOD
Apigenin	<LOD	<LOD
Hesperetin	0.39 ± 0.02	<LOD
Rhamnetin	<LOD	<LOD
Pinocembrin	0.88 ± 0.03	3.20 ± 0.02
CAPE	0.19 ± 0.01	0.78 ± 0.03
Galangin	0.10 ± 0.00	1.74 ± 0.02

Abbreviation: LOD, limit of detection.

from the Borçka region had a weight of 92.70 ± 8.51 g and the samples from the Murgul region had a weight of 93.60 ± 11.70 g. The mean values for weight, length, and width were determined as 93.15 ± 2.25 g FW, 60.45 ± 1.41 mm, and 54.20 ± 0.14 mm, respectively. There was no statistical difference between the Demir apple samples from the two regions. A study examining apple varieties grown in the Çoruh Valley, which includes Artvin Province, reported that the Demir apple has physical characteristics of 92.35 ± 7.00 g in weight, 62.56 ± 0.20 mm in width, and 51.84 ± 0.20 mm in length [5]. It has been shown that the average weight of Demir apple samples collected in the Yomra district of Trabzon varied between 100 and 123 [14]. A study examining apple varieties grown in the Çoruh Valley, which includes Artvin Province, reported that the Demir apple has physical characteristics of 92.35 ± 7 g in weight, 62.56 ± 0.2 mm in width, and 51.84 ± 0.2 mm in length [5]. Our findings revealed similar physical properties. When compared with different apple species, it is seen that the Iron apple does not have a significant difference in size from other apples. In fact, it has been reported that the weight of the Eskişehir region apples, called summer apples, varies between 80 and 86 g, the length varies between 39 and 49 mm, and the width varies between 51 and 60 mm [15].

The apples, with an average TSS of 14.02%, have a TA of 0.45% in terms of malic acid. The soluble solids are measured in degrees Brix (°Brix) or as a percentage. One degree Brix corresponds to 1 g of sucrose dissolved in 100 g of an aqueous solution, representing the concentration as a percentage by mass. This measurement assumes that all dissolved solids present are sucrose [16]. It was reported that the soluble solid content of four different summer apple varieties ("Vista Bella," "Summer Red," "Williams Pride," and "Jersey Mac") grown in the Eskişehir region of Turkiye ranged between 11.87% and 12.19%, while their TA (%TA) was also within this range [15]. A study reported TSS content of 10.48% in delicious apples, 13.40% in the Golden Delicious variety, and 13.84% in the Fuji variety [17]. The vast majority of the TSS content of apples is due to soluble sugars and organic acids. Apples contain many organic acids such as sorbitol, ascorbic acid, malic acid, citric acid, and succinic acid [3, 17, 18].

One of the important antioxidant compounds found in apples is ascorbic acid. The average ascorbic acid content in Demir apple genotypes was determined as 5.77 mg/100 g FW. Vitamin C, an important organic acid, also plays a role in the shelf life and durability of apples. In a study on summer apple cultivars, ascorbic acid contents were reported to vary between 2.24 and 4.79 mg/100 mL [15]. In a large-scale study, ascorbic acid levels in 428 cultivated apples and 29 wild apple species were compared. The results showed that the vitamin C content of wild apple species was about two times higher than that of cultivated apples, with an average ascorbic acid level of 78.42 μg/g FW [18].

3.2 | Sugar Compositions

Sugar compositions of apple samples were analyzed by HPLC–RID. The sugar composition of the samples is given in Table 2. The presence of fructose, glucose, and sucrose was identified in accordance with eight established standards. Ribose, maltose, trehalose, melibiose, and melezitose were not detected in the samples. Fructose was the major sugar, followed by sucrose and glucose. Total sugar content was determined as 12.53% in Borçka Demir apple and 11.30% in Murgul Demir apple. Similar to our study, the soluble sugar profile in apples has been reported to consist of fructose, sucrose, and glucose. However, the quantitative composition of these sugars varies depending on the apple variety and the climatic conditions of the growing region. In the Golden Delicious cultivar, the fructose content was reported to be 6.89% [3]. A study conducted on some of the most commercially consumed apple varieties reported that the total sugar content ranged between 8% and 12.5% [17, 19]. As no study on the sugar profile and sugar content of the Demir apple variety was found, a direct comparison within the same variety could not be made. However, no significant differences were observed between the sugar composition of Demir apples and that of widely consumed apple varieties.

In a previous study conducted on *M. domestica* Borkh. cv. Bravo de Esmolfe, the total sugar content composed of fructose, glucose, and sucrose was reported to be 28.8%, which is approximately

TABLE 4 | Total phenolic contents and antioxidant capacities of the Demir apple samples.

	Borçka ($n = 10$)	Murgul ($n = 10$)	Mean ($n = 20$)
TPC (mg GAE/100 g)	106.06 ± 3.42[a]	101.89 ± 2.75[a]	103.98 ± 3.95
TFC (mg QUE/100 g)	2.03 ± 0.03[a]	2.58 ± 0.09[b]	2.31 ± 0.39
FRAP (mM $FeSO_4$/g)	2.53 ± 0.69[a]	2.75 ± 0.23[a]	2.64 ± 0.16
DPPH-SC_{50} (mg/mL)	9.36 ± 0.19[b]	6.13 ± 0.06[a]	7.75 ± 2.28

Different letters (a-b) in the same lines are significantly different at the 5% level ($p < 0.05$)

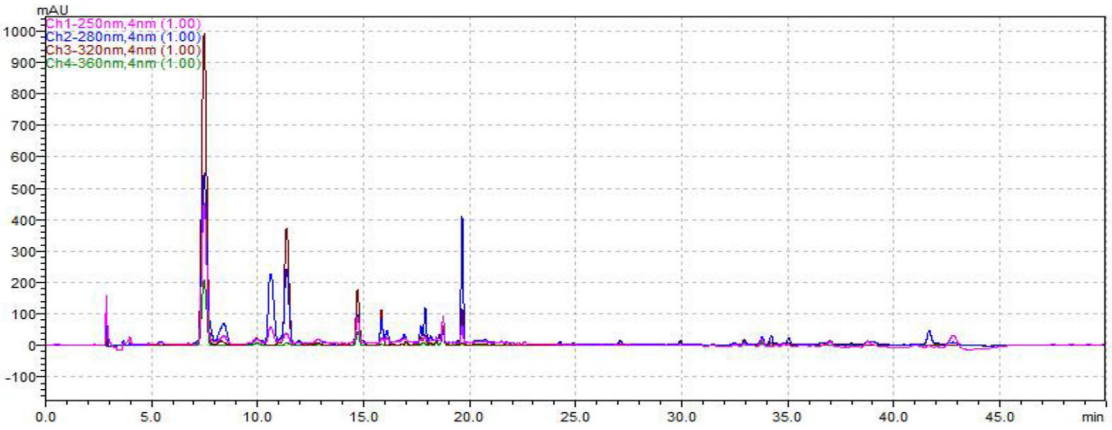

FIGURE 2 | The chromatogram of HPLC–PDA of Borçka Demir apple.

twice as high as that of the Demir apple genotype (*M. sylvestris*) [20]. Apple varieties with lower soluble sugar contents are known to exhibit lower glycemic index values. Furthermore, their high polyphenolic content has been suggested to contribute to significant antidiabetic potential by inhibiting key carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase [21]. Therefore, the combination of low sugar density, high vitamin levels, and rich polyphenolic composition indicates that the Demir apple may represent a naturally antidiabetic apple genotype.

3.3 | Phenolic Compositions

The phenolic profile of the apple samples was analyzed using 26 phenolic standards, and the findings are summarized in Table 3. In addition, the HPLC chromatograms of the methanolic extracts of both apple samples are presented in Figures 2 and 3.

In Demir apple samples from both regions, chlorogenic acid, epicatechin, and catechin were identified as the major polyphenols, while lower concentrations of *p*-coumaric acid, chrysin, pinocembrin, galangin, and CAPE were detected. Hesperetin was found in very low amounts, exclusively in samples from the Borçka region. Similar to our study, chlorogenic acid has been reported as a major phenolic compound in some commercially available apple varieties [17].

In a Canadian study of 11 commercial apple cultivars, chlorogenic acid was shown to be the major phenolic component, and epicatechin, catechin, and *p*-coumaroylquinic acid were found in all apple cultivars [22]. Among the 26 phenolic standards analyzed, chlorogenic acid was identified as the most abundant compound in the Demir apple samples. As a derivative of hydroxycinnamic acid, chlorogenic acid is widely present in coffee, apples, pears, berries, and various vegetables. This bioactive compound is known for its potent antioxidant, anti-inflammatory, and metabolic regulatory properties [23]. Catechin and its cis isomer, epicatechin, are phenolic compounds belonging to the flavonoid class. They are potent antioxidants found predominantly in tea, cocoa, grapes, and various fruits [24].

In this study, lower amounts of phlorizin molecules were also found in all apples. Phlorizin, a dihydrochalcone polyphenol, is predominantly found in apples (*Malus* spp.) and apple tree bark. It exhibits antioxidant, antidiabetic, and anti-inflammatory properties. Notably, phlorizin regulates blood glucose levels by inhibiting SGLT1 in the intestine and SGLT2 in the kidneys, reducing glucose absorption and reabsorption. However, in this study, phlorizin, which is specific to apples, was not included among the standards [17, 25].

The total phenolic and flavonoid contents of the methanolic extracts of Demir apple samples are presented in Table 4. The amount of TPC ranged between 101.89 and 106.06 mg GAE/100 g FW. No significant difference was detected between the Demir apple samples from the two regions. In a study conducted on Summer Apple Cultivars, the TPC was reported to range from 25.92 to 87.14 mg GAE/100 g [15]. In another study, the TPC of the flesh from 11 different summer apple varieties was reported to range between 50.2 and 129.5 mg GAE/100 g. The study also demonstrated that the peel exhibited approximately five times higher TPC levels compared to the flesh. Among the analyzed

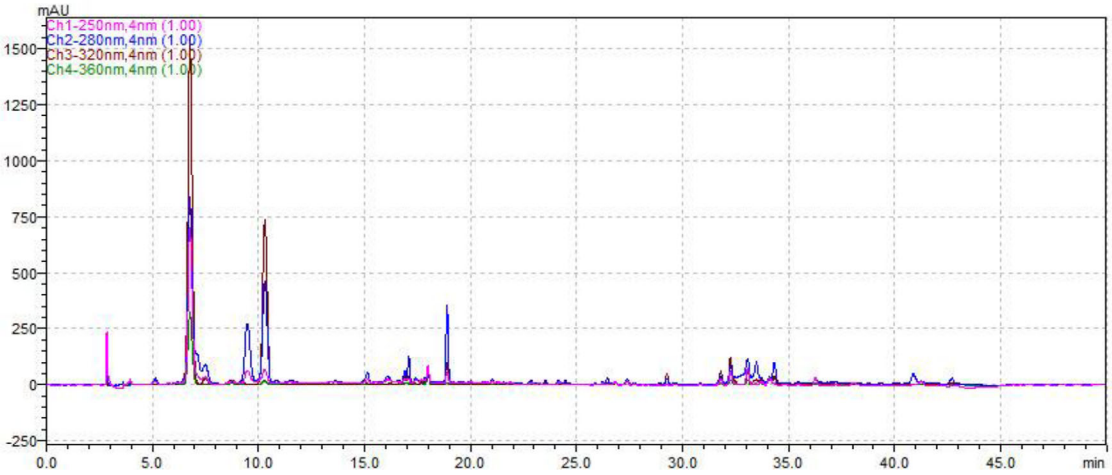

FIGURE 3 | The chromatogram of HPLC–PDA of Murgul Demir apple.

varieties, Reinette Russet was identified as the cultivar with the highest TPC value [22].

Flavonoids constitute a significant subclass of polyphenols, and the TFC was determined to be 2.09 mg QUE/100 g FW on average. Compared to TPC, the TFC values in Demir apple extracts were found to be considerably lower, accounting for approximately 2% of the total polyphenols. Since flavonoids are less soluble in water than phenolic acids, it is suggested that the Demir apple samples are richer in phenolic acids. This is further supported by HPLC-based phenolic profile analysis, which identified chlorogenic acid as the predominant compound.

In this study, the antioxidant properties of Demir apple extracts were evaluated using the FRAP and DPPH radical scavenging assays. A high FRAP value indicates strong antioxidant activity, and the mean FRAP value was determined in terms of mM $FeSO_4$/g. The mean FRAP value of Demir apple samples was determined as 2.64 ± 0.16 mM $FeSO_4$/g, with no significant differences observed among the samples. The DPPH radical scavenging activity was measured in terms of the SC_{50} value. The mean SC_{50} value was determined to be 7.75 mg/mL, with a range of 6.13 to 9.36 mg/mL. A lower SC_{50} value indicates higher antioxidant activity. While no significant differences were observed between Demir apple samples from the two regions, the samples from the Murgul region exhibited a higher radical scavenging capacity. This may be attributed to their higher ascorbic acid and TFC. Furthermore, phenolic composition analysis revealed that Murgul region samples contained higher levels of flavonoids, including pinocembrin, galangin, and chrysin. In the study conducted on 13 apple cultivars, the antioxidant activities of the apple varieties were analyzed using methods like those employed in our research, specifically FRAP, DPPH, and CUPRAC assays. The results indicated that there were no significant differences in antioxidant activities among the cultivars [26, 27].

4 | Conclusion

The findings of this study highlight the significant bioactive potential of Demir apple (*M. sylvestris*), a wild and resilient fruit native to the Eastern Black Sea Region. The high levels of phenolic compounds, flavonoids, and ascorbic acid, along with considerable antioxidant capacity, suggest that this fruit may offer valuable health benefits. The sugar profile, characterized by fructose, sucrose, and glucose, contributes to its unique taste and potential functional properties. Overall, these results indicate that Demir apple could be a promising natural source of antioxidants and bioactive compounds, supporting its potential utilization in functional food and nutraceutical applications. Further research on its phytochemical composition and health effects could provide deeper insights into its nutritional and pharmacological value.

Author Contributions

Zeynep Berin Celebi: in vitro studies. **Erol Tunca**: formal analysis. **Ali Murat Kesemen**: sampling and draft. **Sevgi Kolaylı**: Conceptualization, writing, and editing.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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