

# Natural pigments, phenolic content and antioxidant activity in ironwort

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**Abstract.** The current study aimed to evaluate total carotenoids, chlorophylls, phenols, flavonoids, and antioxidant potential in ironwort (*Sideritis syriaca* L.) collected from different locations in Strandzha Mountain, Bulgaria, and from a single location in Turkey. We compared the results with cultivated samples from Momchilovtzi and Malko Tarnovo. The cultivated samples exhibited the highest levels of total carotenoids and chlorophylls, followed by those collected from the Turkish side of Strandzha Mountain. The samples from the Bulgarian side of Strandzha Mountain (Sredoka area) showed 1.5 to 2 times higher levels of total flavonoids and polyphenols, as well as twice the antioxidant activity, compared to cultivated ironwort samples. Therefore, the wild-growing samples accumulate more secondary metabolites, such as phenol and flavonoids, compared to cultivated ones.

## 1 Introduction

Ironwort (*Sideritis syriaca* L.), known also as Crimean tea, Siriiski mirizliv buren, Strandzhanski chaj, Strandzhanski bilkov chay is a perennial herb, 10-50 cm tall that grows on dry calcareous, stony terrains or on the karst rocky areas. This plant blooms in yellow during May-June, and its seeds ripen at the end of July-August [1]. It is distributed in Bulgaria, Crete, Lebanon, Syria, Turkey [2-4] and Italy [5]. However, Strandzhanski bilkov chay (*S. syriaca*) is a protected species included in the Red Book of Bulgaria, volume 1 - category "critically endangered" [2]. In Strandzha mountain it is distributed to the south of Malko Tarnovo town and in the Turkish part of the mountain [1, 2, 4]. A part of its population was existed in "Sredoka" and protected area "Dokuzak" [2]. However, due to its beneficial health properties it is grown in cultivated form in some farms in Bulgaria, especially in Malko Tarnovo and Sredetz [1,4]. Due mainly to the rich polyphenol composition ironwort demonstrates anti-inflammatory, antimicrobial, antioxidant and analgesic agent, protection against atherosclerosis, immunomodulation, anti-aging properties and many others [1,6-9].

To the best of our knowledge the studies about *S. syriaca* contained insufficient information about the phytochemical compounds and antioxidant potential especially in plants growing wild in the protected and unprotected area of Strandzha mountain. Some previous reports on *S. syriaca* were mainly focused on the analysis of phenolic compounds and antioxidant activity in decoction, 95% ethanol, methanol, ethyl

acetate, hexane, petroleum ether, acetone, butanol, diethyl ether and dichloromethane extract, as well as its essential oil [3, 7, 8, 9,10]. It was previously described that total polyphenol levels in *Sideritis* extracts sharply increased from 60 to 100% ethanol [3, 11]. In most of the papers DPPH was used as the main method for the evaluation of the antioxidant potential [4, 7, 9]. Goulas et al., (2014) [8] used DPPH, FRAP and phosphomolybdenum assay to evaluate the antioxidant potential of decoctions prepared with Greek *Sideritis syriaca*, while Koleva et al., (2003) evaluated methanol extract from Bulgarian *Sideritis syriaca* by DPPH method [7]. Bouloumpasi et al., (2024) analysed hydro ethanol extracts obtained by microwave irradiation from cultivated *S. syriaca* in Crete (Greece) as demonstrated the quantity of total phenols, flavonoids and antioxidant activity by DPPH, ABTS and FRAP method [3]. Most of the data about the polyphenol content and antioxidant potential were evaluated mainly by DPPH method and referred to the wild growing samples [5,7-10]. Until now, only one research was related with the cultivated population from village Varovnik, Sredets Municipality, Bulgaria compounds [4]. Therefore, there are many gaps comparing phenolic compounds accumulation in wild growing protected and cultivated samples of ironwort, especially in The Rhodopes and Strandzha mountain (Bulgarian and Turkish parts). The data about photosynthetic pigments and ratio between chlorophylls in plant material is still absent. Moreover, applying the multiple approaches for the evaluation of antioxidant activity in cultivated and wild growing plants from species *Sideritis syriaca* will reveal additional information about mechanism of action of some antioxidants accumulation in plant material. This

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provokes the need of evaluation of antioxidant activity by different method based on the different mechanisms.

Therefore, the aim of the current research was to evaluate the total carotenoids, chlorophylls, phenols, flavonoids, and antioxidant potential in ironwort (*Sideritis syriaca* L.) collected from different locations in Strandzha mountain, Bulgaria, and from a single location in the republic of Türkiye (Kuzulu), as well as to compare the results with cultivated samples harvested from Momchilovtzi and Malko Tarnovo, Bulgaria.

## 2 Material and methods

The fresh aerial parts of ironwort (*Sideritis syriaca* L.) were air-dried at 25 °C under the imperfect shade in Agriculture University (Plovdiv, Bulgaria). Then the dried samples were finely ground with a laboratory homogenizer Gorenje BN1200AL and sieved through 1.5 mm. The samples were placed in the tightly closed plastic containers and immediately used for further analysis.

### 2.1 Moisture analysis

The moisture of the aerial part of ironwort (%) was directly analysed on a moisture analyser balance Kern, DAB 100-3 (Germany).

### 2.2 The extract preparation

The ground ironwort samples (1 g) were placed in a plastic centrifuge tube of 50 ml and then extracted with 15 ml 95% ethanol using ultrasound-assisted extraction. The extraction was conducted in duplicate in an ultrasonic bath (IsoLab 621.05.001, Germany), with ultrasonic power 60W and ultrasonic frequency 40 kHz for 20 min, at 75°C. The 95 % ethanol extracts were filtered and combined for further analyses [12].

### 2.3 Analysis of photosynthetic pigments

Total chlorophylls and carotenoids in 95% ethanol extracts *Sideritis syriaca* were measured using spectrophotometer at three wavelength - 664, 648 и 470 nm, respectively. The concentrations of chlorophyll a (Chla), chlorophyll b (Chlb), total chlorophyll (Ca+b) and total carotenoids were calculated and presented as µg/g (dry weight), according to the equations (1-4), described by Lichtenthaler and Wellburn [13].

$$Chla (\mu\text{g/g}) = [(13.36 \times A_{664} - 5.19 \times A_{648}) \times V \times Kw] / p \quad (1)$$

$$Chlb (\mu\text{g/g}) = [(27.43 \times A_{648} - 8.12 \times A_{664}) \times V \times Kw] / p \quad (2)$$

$$Ca+b (\mu\text{g/g}) = [(5.24 \times A_{664} + 22.24 \times A_{648}) \times V \times Kw] / p \quad (3)$$

$$Total \text{ carotenoids } (\mu\text{g/g}) = [(1000 \times A_{470} - 2.13 \times Ca - 97.64 \times Cb) / 209] \times V \times Kw / p \quad (4)$$

where: p-weight of the sample, g; V- volume of extract, ml; and Kw – moisture coefficient = 100/(100-moisture content).

### 2.4 Analysis of phenols and flavonoids

The total phenols content in ironwort (*Sideritis syriaca* L.) extracts was evaluated using a five times diluted reagent of Folin–Ciocalteu, with a slight modification [12]. First 95 % ethanol ironwort extract (0.2 ml) was put into a disposable cuvette, then 1 ml Folin–Ciocalteu reagent and 0.8 ml 7.5% Na<sub>2</sub>CO<sub>3</sub> were added. After 20 min the absorbance was recorded at 765 nm against a blank sample. The results were expressed in mg equivalent of gallic acid (GAE) per g dry weight sample [14].

The total flavonoid content was determined by Al(NO<sub>3</sub>)<sub>3</sub> reagent with small modification [14]. The results were expressed as mg equivalents quercetin (QE)/g dry sample.

### 2.5 Analysis of antioxidant activity

The DPPH radical-scavenging ability, ABTS method, ferric reducing antioxidant power (FRAP) assay and cupric reducing antioxidant capacity (CUPRAC) assays of 95% ethanol extracts from *Sideritis syriaca* were performed.

DPPH (1,1-diphenyl-2-picrylhydrazyl) method was performed directly in a plastic cuvette where 0.15 ml 95 % ironwort extract was added to 2.85 ml freshly prepared 0.1 mM solution of DPPH- 2 mg DPPH dissolved in 50 ml methanol. After 15 min at 37 °C in darkness the absorbance was measured at 517 nm against methanol. The control sample was prepared as DPPH reagent was mixed with 0.15 ml 95 % ethanol – a solvent used for the extraction. Calculations of % inhibition were performed as previously described [14].

In the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) method [14], the reagent was freshly prepared and 2.85 ml was directly put in a plastic cuvette where 0.15 ml 95 % ironwort extract was added. After 15 min, the absorption was recorded at 734 nm, the inhibition of the ABTS radical cation (%) was calculated [14].

The FRAP reagent was immediately prepared just before analyses by mixing 100 ml of 0.3 M acetate buffer (pH 3.6), 10 ml of 10 mM 2,4,6- tripyridyl-s-triazine (TPTZ, Merck) in 40 mM HCl (Merck) and 10 ml of 20 mM FeCl<sub>3</sub>·6H<sub>2</sub>O (Merck) dissolved in distilled water. The reaction was performed in a disposable plastic cuvette where 0.1 ml of 95 % ironwort extract was mixed with 3.0 mL FRAP reagent. The blank sample contained 95 % ethanol instead of a sample. After 10 min at 25 °C in darkness and the absorbance was measure at 593 nm against blank [14].

The CUPRAC method

The reaction was carried out in a disposable plastic tube of 15 ml, where 1.0 ml 10 mM aqueous solution of CuCl<sub>2</sub>·2H<sub>2</sub>O, 1.0 ml methanol solution of 7.5 mM neocuproine, 1.0 ml 0.1 M ammonium acetate buffer (pH 7.0), 0.1 ml ironwort 95 % ethanol extract and 1.0 ml distilled water were added. A control sample was developed in the same way but with 0.1 ml 95 % ethanol instead of extract. The test tubes were placed in the water bath at 50 °C for 20 min. It was observed the change in the colour of the sample from light blue to

yellow-orange. The absorbance was measured at 450 nm against a control sample [14].

Antioxidant activity was expressed as mM Trolox equivalent (TE)/g dry weight sample.

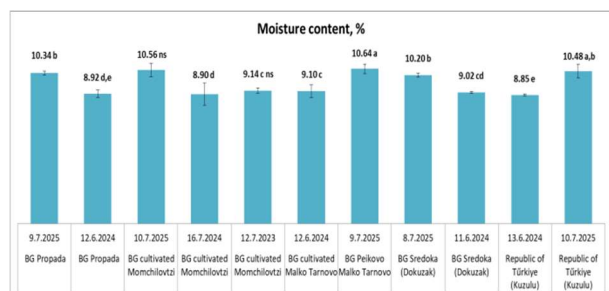
## 2.6 Statistical analysis

All experiments were performed in triplicate. Statistical analysis was performed using MS Excel 2010. One-way analysis of variance (ANOVA) combined with Tukey's test was applied to test for differences. The results were expressed as mean values  $\pm$  standard deviation (SD).

## 3 Results and discussion

### 2.1 Moisture content

The moisture content in *Sideritis syriaca* L. varied between 8.85 to 10.64 % (Fig. 2). In general the samples collected during 2024 demonstrated lower moisture content than these from 2025. The highest results for moisture content was found in samples from 2025 for the Bulgarian representatives collected from Peikovo, Malko Tarnovo, Momchilovtzi (Bulgaria) and the Turkish population (Kuzulu) - from 10.64% to 10.48%.



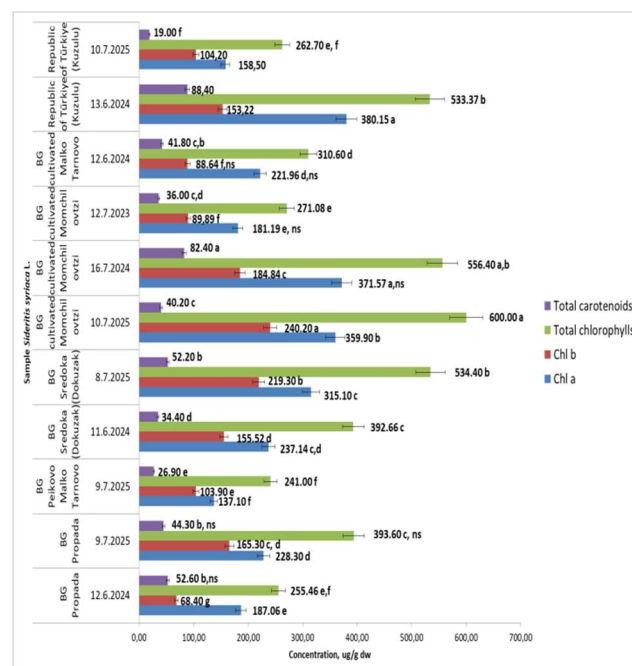
**Fig. 2.** Moisture content (%) in the aerial parts of *Sideritis syriaca* L. Different letters within each column indicate significant differences between treatments according to Tukey's test at  $p < 0.05$ ; ns - not significant

### 2.2 Photosynthetic pigments

The results for photosynthetic pigments (chlorophylls and carotenoids) were shown in Fig. 3.

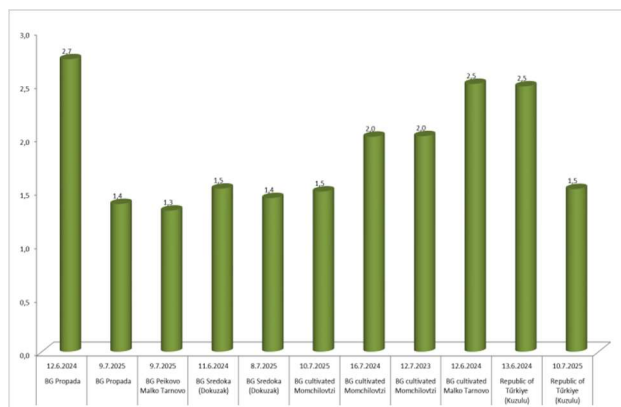
To the best of our knowledge this is the first detailed study about the content of chlorophyll a and b, total chlorophylls and total carotenoids in *Sideritis syriaca* L. collected from Bulgaria. The results showed that the total chlorophylls ( $600.0 \pm 0.5 \mu\text{g/g}$  dry weight) and the total carotenoids ( $82.4 \pm 0.5 \mu\text{g/g}$  dry weight) dominated in biologically grown cultivated field in Momchilovtzi (2025 and 2024, respectively). The comparable results for total chlorophylls was found in samples collected from Sredoka aeria (Doluzak), Strandzha mountain, Bulgaria during 2025 and Kuzulu (the Republic of Türkiye) for 2024 - 533-534  $\mu\text{g/g}$  dry weight. The lowest values of the total carotenoids were found in Turkish population of ironwort 2024 -  $19.0 \pm 0.6 \mu\text{g/g}$  dry. The lowest values of Chla, Chlb and total chlorophylls were found in samples from Propada

Strandzha mountain in 2024 and Kuzulu (the Republic of Türkiye) during 2025.



**Fig. 3.** Natural pigments in the aerial parts of *Sideritis syriaca* L. Different letters within each column with the same colour indicate significant differences according to Tukey's test at  $p < 0.05$ ; ns - not significant.

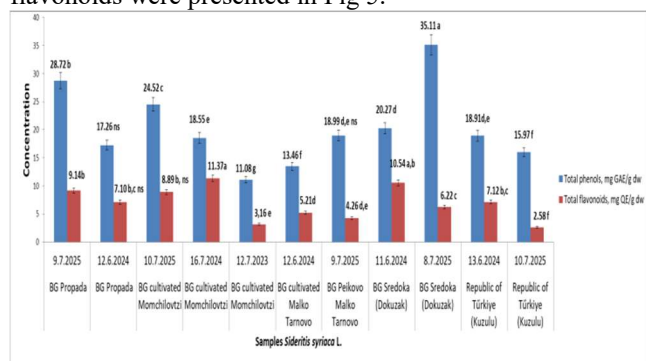
The foliar chlorophyll content is a good indicator of various biotic and abiotic stresses [15], that could explain the differences in chlorophyll content in cultivated and wild growing samples. In general, the total carotenoids were almost six to eight times lower than values of total chlorophylls in the investigated samples of *Sideritis syriaca* L. (Fig.3). In all samples of ironwort chlorophyll a dominated among chlorophyll b (Fig 3), as the ratio between Chla/Chlb was presented in Fig 4. This ratio is valuable indicator about stress conditions and environmental impact. In general the ration Chla/Chlb varies in the range of 1.5–3.0, where lower values indicate adaptation to stress by activating Chlb, while higher values are maintained under normal conditions [16]. In our case the ratio Chla/Chlb in the investigated *Sideritis syriaca* L. samples varies in the range from 1.3 to 2.7. The highest ratio was found in the cultivated samples from Momchilovtzi and Malko Tarnovo (Bulgaria) - 2.0-2.5 (2024), followed by the wild growing samples from Propada (2024) 2.7 and Kuzulu (2024) – 2.5. The lowest ratio 1.3-1.4 was found in 2025 for samples from Sredoka aria (Doluzak), Propada and Peikovo located in Strandzha mountain.



**Fig. 4.** Natural pigments ratio in the aerial parts of *Sideritis syriaca* L.

### 3.3. The content of phenols and flavonoids

The results for the total phenols content and total flavonoids were presented in Fig 5.



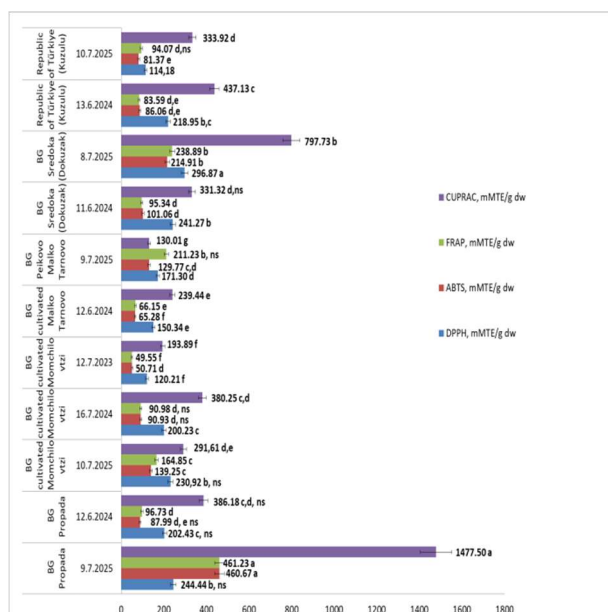
**Fig. 5.** Total phenols and total flavonoids in the aerial parts of *Sideritis syriaca* L. Different letters in the same colour columns showed that there was a significant difference at the corresponding probability level via Tukey's test ( $p < 0.05$ ).

In general, the wild growing samples from protected area in Strandzha mountain showed the highest content of total phenols. It was found that *Sideritis syriaca* from Sredoka (Dokuzak), Strandzha mountain (Bulgaria) contained the highest values of total phenols  $35.11 \pm 0.42$  mg GAE/g dw (2025). The highest total flavonoids  $11.37 \pm 0.42$  mg and  $10.54 \pm 0.25$  mg QE/g dw was observed in the samples from Bulgaria - Momchilovtzi (2025) and Sredoka (Dokuzak) (2025). The lowest values of total flavonoids were found in the samples from Kuzulu (2025) -  $2.58 \pm 0.15$  mg QE/g dw. In general, the total phenols were the highest in samples collected during 2025, especially these from Strandzha Mountain (Propada and Sredoka area). In cultivated samples of ironwort the total polyphenols content was in the range between 24.52 to 11.08 mg GAE/g dw. These values for a phenolic content was close to reported results for the infusions prepared from Greek *Sideritis syriaca* (Epirus, Greece) - 1863 mg of GAE per 100 g of dry material [8]. The total phenols obtained in the different extracts from cultivated hybrid *S. scardica*  $\times$  *S. syriaca* in Bulgaria varied between 8.8 to 35.1 mg CAE/g, while the total flavonoids was from 5.5 to 12.9 mg/g [11]. However, the values for the total phenols in the current study for wild growing samples were almost twice time high than the data of Prodanov [1] for these

places (Golyam Valog and Dokuzak) - 18.39 and 16.65 mg CAE/g, respectively. However, the results for total flavonoids were close to Prodanov [1], who reported the content of total flavonoids 5.73 and 5.65 mg/g, and 6.07 mg/g for cultivated samples *Syderitis syriaca* on limestone, from Malko Tarnovo. Moreover, its results for wild free growing samples from Golyam Valog and Dokuzak (2.79 and 3.35 mg RE/g) [1] were lower in comparison to all samples in our study collected from Strandzha mountain. In the current research cultivated samples from Malko Tarnovo coincided with above mentioned values for cultivated ironwort from Malko Tarnovo ( $5.21 \pm 0.12$  mg QE/g dw), while samples from Dokuzak was up to 3 times higher in comparison to data of Prodanov [1]. The quality and quantity of flavonoids and polyphenols in *Sideritis* species can be affected from different internal and external factors including genetic diversity, environmental conditions such as light intensity, humidity, temperature, soil structure, as well as solvent and type of extraction procedure [12, 17]. In general wild growing samples from the protected area in Bulgarian part of Strandzha mountain accumulated more polyphenols (Fig. 5) that could be explain with hard environmental conditions and poor sandy soils and rocks, what accelerate accumulation of the secondary metabolites preventing plants. Additionally, it was found that ethanol concentration was the most significant factor affecting the phenolic components in *Syderitis* species using microwave and ultrasonic irradiation [3, 11, 12]. Moreover, the phenolic content in this study was close to the data for polyphenols which varied between  $2404.6 \pm 30.7$  mg GAE/100 g dw for the *in vitro* *Syderitis scardica* shoots and 3929 mg GAE/100 g dw for its *in vitro*-obtained field grown samples [19].

### 3.4. Antioxidant activity

Until now, the information about antioxidant potential of wild growing plants from *Sideritis syriaca* from their natural habitat from Strandzha mountain in Bulgarian and Turkish part is faintly investigated. The first short report was published by our team for 2023 [12]. In the current research antioxidant activity of 95% ethanol extracts prepared with cultivated samples from Bulgaria and Turkish part of the Strandzha Mountain were presented in Fig 6.



**Fig. 6.** Total phenols and total flavonoids in the aerial parts of *Sideritis syriaca* L. Different letters within each column with the same colour showed significant differences according to Tukey’s test at  $P < 0.05$ ; ns - not significant

Four assays (DPPH, ABTS, FRAP and CUPRAC) based on different mechanism were employed to reveal the antioxidant potential of ironwort samples. In general, CUPRAC assays based on single electron transfer demonstrated the highest results for the antioxidant potential of samples, while ABTS method based on mixed mechanism showed the lowest values. The CUPRAC method is based on the ability of a substance with antioxidant activity to reduce  $Cu^{2+}$  by reactivating Cu (II)-neocuproine to  $Cu^+$  via single electron transfer (SET mechanism). From the investigated samples the highest antioxidant potential for four assays possessed samples *Sideritis syriaca* L. collected during 2025 from wild growing population in Propada and Sredoka area in Strandzha mountain, Bulgaria. The highest antioxidant activity by the DPPH method showed ironwort from Sredoka (Dokuzak) area 296.9 mM TE/g dw. The lowest values by this radical scavenging method were observed for samples 114.2 mM for Turkish population 2025 from Kuzulu. ABTS assay based on a mixed mechanism showed the highest results for samples collected from Bulgaria, Propada (2025) in Strandzha mountain – 460.7 mM TE/g dw. The lowest values by ABTS method were found in 50.7 mM TE/g dw (Momchilovtzi 2023) and Peikovo, Malko Tarnovo – 65.3 mM TE/g dw. Turkish population 2024–2025 from Kuzulu demonstrated close results 86.1–81.4 mM TE/g dw. FRAP assay, which is based on single electron transfer and metal ferric reducing properties showed similar results close to ABTS method. In general, the samples collected during 2025 from Strandzha mountain showed the highest values by FRAP assay, as follows: 461.2 mM TE/g dw (Propada), followed by samples from Sredoka (238,9 mM TE/g dw) and Peikovo (211,2 mM TE/g dw). The lowest activity by FRAP was found for samples 2024–2025 from Momchilovtzi (Bulgaria) 49.6–66.1 mM TE/g dw. CUPRAC assays based on single electron transfer and metal cupric reducing properties showed

the highest antioxidant potential for *Sideritis syriaca* L. collected during 2025 from Propada in Strandzha mountain – 1477.50 mM TE/g dw. This sample demonstrated approximately 2 to 4 times higher activity in comparison to other samples, except the samples from Peikovo. The lowest activity by CUPRAC method was found in Peikovo, Malko Tarnovo Strandzha mountain - 130,01 mM TE/g dw.

Veli et al. [4] used the DPPH method to evaluate the antioxidant potential of extracts prepared from cultivated *Sideritis syriaca* from the village Varovnik, Sredets Municipality, Bulgaria. These authors reported that methanol and ethanol extracts from cultivated Bulgarian *Sideritis syriaca* demonstrated – 579.3 and 524.3  $\mu$ M EVCAA/100  $\mu$ g extract by DPPH method, while Goulas et al., (2014) reported FRAP antioxidant activity for Greek *Sideritis syriaca* decoction (13.4 mmol AsA/100 g DM). In our early investigation 95% ironwort ethanol extracts specially from Propada Bulgaria gave the highest values  $163.09 \pm 2.11$  mMTE/g (DPPH assay) and  $45.27 \pm 0.21$  mM TE/g (FRAP assay) [12]. Extracts of *S. syriaca* and *S. scardica* had similar and insignificant ( $p > 0.05$ ) ABTS and FRAP value [3], as the similar observation was found in the current study (Fig.5). Some authors demonstrated values close to ours for cultivated in Greece *S. syriaca* [3]. ABTS values varied from 153.63 to 249.39 mg TE/g dw between *Sideritis* species, while DPPH and FRAP values ranged from 74.20 to 172.44 mg TE/g dw and 130.61 to 236.27 mg TE/g dw, respectively [3].

The correlation ( $r^2$ ) between the total antioxidant activity evaluated by DPPH, ABTS, FRAP and CUPRAC assays and total phenols, total flavonoids, total carotenoids and chlorophylls *Sideritis syriaca* 95 % ethanol extracts is shown (Table 1).

**Table 1.** Correlation between ( $r^2$ ) total phenols, total flavonoids, total chlorophylls, total carotenoids and antioxidant activity.

	DPPH	ABTS	FRAP	CUPRAC
Total phenols	0.8778	0.7155	0.7316	0.6936
Total flavonoids	0.6600	0.3014	0.2224	0.3285
Carotenoids	0.0630	0.0685	0.1371	0.0119
Chlorophylls	-0.4690	-0.4688	-0.4213	0.0119

The results showed the highest positive linear correlations between total antioxidant activities evaluated by DPPH and total phenols contents (coefficient of correlation  $r^2 = 0.8778$ ). Moreover, high correlation was observed between total phenols and the antioxidant method based on electron transfer – FRAP, as well as ABTS method. Total flavonoids are weakly correlated with antioxidant method based on electron transfer (FRAP and CUPRAC), while good correlated with the radical scavenging method DPPH  $r^2 = 0.6600$ . Total carotenoids weakly correlated with all antioxidant method, while total chlorophylls demonstrated negative results. Based on these findings, the radical scavenging

activity determined by DPPH method was most directly influenced by the amount of total phenols in ironwort. In general, a positive correlation between polyphenol content and antioxidant activity was observed. The current data was in a good agreement for the correlation between the total flavonoids and phenols in *Sideritis scardica* extracts [12, 17-19]. Dimaki et al. [20] reported that FRAP and DPPH values correlated strongly with phenolics and flavonoids in *Sideritis* extracts. In conclusion, the phenols and flavonoid content in ironwort 95% ethanol extracts demonstrated high radical scavenging and good metal chelating properties.

## 4 Conclusion

The current research expands the knowledge about the accumulation of natural pigments and phenolic compounds in *Sideritis syriaca* L. growing in natural habitats in the Strandzha Mountain on the territory of Bulgaria and Turkey, as well as in cultivated samples from Momchilovtzi (The Rhodopes mountain) and Malko Tarnovo (Strandzha mountain). The current research demonstrated that cultivated biologically grown samples from Momchilovtzi accumulated the highest values of chlorophylls comparable with wild-growing samples from Sredoka area (Bulgaria) and Kuzulu (Turkey). In general, the levels of total phenols, flavonoids and antioxidant potential were the highest during 2025, especially for ironwort samples collected from Sredoka and Propada. The highest positive correlation was found between the total phenols and DPPH method. To the best of our knowledge, this is the first detailed study about natural pigments, phenolic and flavonoid content, antioxidant potential in representatives collected from the protected area in Strandzha mountain (Bulgaria) and cultivated samples.

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Author contribution Tatyana Bileva manages the project, coordinated the work, participated in collecting of the plant material and in writing the manuscript, Nadezhda Petkova performed all chemical analysis and wrote the manuscript, Plamen Zorovski and Ekaterina Valcheva collected the samples.

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