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Polyphenol profile and antioxidant activity of wild growing populations of *Nectaroscordum siculum* ssp. *bulgaricum* (Janka) Stearn in Bulgaria

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Abstract

Worldwide, studies of *Nectaroscordum siculum* ssp. *bulgaricum* (Janka) Stearn have been focused mainly on the botanical and taxonomic characteristics of the species resulting in scanty data about its polyphenol profile and biological activity. Therefore, the aim of the present work was to analyse and compare the polyphenol substances and antioxidant activity of four wild populations of *N. siculum* growing in Bulgaria. The total phenolic content of 70% ethanolic extracts of the analysed populations ranged between 15.62 ± 0.22 and 43.50 ± 0.50 mg Gallic acid equivalent (GAE) per g dry weight (DW) (mg GAE/g DW). The total flavonoid content varied between 5.66 ± 0.03 and 25.71 ± 0.17 mg quercetin equivalent (QE) per g DW (mg QE/g DW). Analysis of polyphenol profiles by High Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD) revealed the domination of sinapic acid (between 3.23 ± 0.02 and 6.37 ± 0.07 mg/g DW) and hyperoside (between 4.72 ± 0.02 and 22.94 ± 0.06 mg/g DW). All the analysed populations possessed antioxidant activity, determined by four most used *in vitro* methods, namely DPPH, ABTS, FRAP and CUPRAC. The highest radical scavenging and reduction ability was measured for the extract of the population from Eastern Stara Planina Mts. that could be explained by the highest amount of total and individual phenolic acids and flavonoids. The present work showed that the leaves of *N. siculum* are a valuable source of polyphenols and could potentially find applications in different food systems to improve their health benefits.

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Introduction

Nectaroscordum siculum ssp. *bulgaricum* (Janka) Stearn (*Allium siculum* subsp. *dioscoridis* (Sm.) K. Richt., *Allium bulgaricum*) is a traditional Bulgarian culinary spice (Alexieva *et al*., 2013). While the species *N. siculum* is distributed in the Mediterranean region from France to Turkey and Crimea; the subsp. *bulgaricum* is limited to Bulgaria, Romania, Turkey and Crimea. In Bulgaria, the plant could be found along the Black Sea coastal area, Strandzha Mts., North-eastern Bulgaria, Eastern Sredna Gora Mts. and in some areas of the Central and Eastern Stara Planina Mts. (Delipavlov *et al*., 1992; Radanova, 2006). The local name is "*samardala*" or "Bulgarian honey garlic". The leaves are characterised by a powerful and specific smell. Fresh and dried leaves of *N. siculum* are used for seasoning salads and in cooked vegetables and meat dishes by the populations of the Balkan Peninsula, mostly in Bulgaria (D`Antuono *et al*., 2012; Alexieva *et al*., 2013). Studies of the *N. siculum* have been focused mainly on the botanical

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and taxonomic characteristics of the species (Radanova, 2006; Friesen *et al.*, 2006; Krzymiñska *et al.*, 2008; Arcuş *et al.*, 2009). Arcuş *et al.* (2009) provided a comparative pharmacognostic study of the bulbs, leaves and stems of the Romanian population of *N. siculum*. Several analyses of certain sulphur-containing metabolites (dibutyl disulphide, 2-hexyl-5-methyl-2-hydropyran-3-one, S-methylcysteine sulfoxide, and butylcysteine sulfoxide) have also been reported. They are precursors of thiosulfinates responsible for its specific flavour and taste, as well as for the high anti-microbial and anti-inflammatory activities (Kubec *et al.*, 2002; Kürkcüoglu *et al.*, 2010; Alexieva *et al.*, 2012; 2013). Worldwide, in the scientific literature, the data describing the phenolic content and antioxidant activity of this plant species is quite limited (Alexieva *et al.*, 2013; Popova *et al.*, 2014a; 2014b), and there is no information about the polyphenol profile of *N. siculum*. Therefore, the aim of the present work was to analyse and compare the polyphenol profiles and antioxidant activity of four wild populations of *N. siculum* growing in Bulgaria.

Materials and methods

Plant material

Analysed samples of the four populations of *N. siculum* were collected in May 2017 during the flowering stage from their natural habitats in Bulgaria, namely: Strandzha Mts. (P1) - coordinates 41°59'53.76"N/27°49'32.56"E, Black Sea coast (P2) - coordinates 42°44'10.02"N/27°39'8.98"E, Eastern Stara Planina Mts. (P3) - coordinates 42°45'36.71"N/27°44'54.10"E, and Central Stara Planina Mts. (P4) - coordinates 42°45'51.57"N/25°12'16.53"E. In order to cover maximum area, transect method was used for establishing the distribution of *N. siculum* of the localities. Botanical identification was done by Aneva, I. (co-author of the current research) and the voucher specimen (SOM-1376) was deposited in the Herbarium of the Institute of Biodiversity and Ecosystem Researches, Sofia, Bulgaria. Leaves of the collected samples were dried in shade at ambient temperature and grounded by an electric mill.

Reagents

The following reagents were purchased from Sigma-Aldrich Chemie GmbH (Germany): Folin-Ciocalteu's phenol reagent, Gallic acid, sodium carbonate, aluminium nitrate, potassium acetate, quercetin, potassium persulfate, sodium acetate anhydrous, DPPH (2,2-diphenyl-1-picrylhydrazyl radical), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), TPTZ (2,4,6-tri(2-pyridyl)1,3,5-triazine), iron (III) chloride, neocuproine, copper (II) chloride, ammonium acetate, and HPLC-grade solvents (acetonitrile, methanol, ethanol, and acetic acid). Ultrapure water was obtained using water purification system Adrona B30 Integrity+ Bio (UAB Moris Technology, Vilnius, Lithuania).

Extraction procedure

Each of the analysed samples (1.0 g of dried leaves) was extracted three times with 10 mL 70% ethanol (v/v) under reflux-heat at 70°C for 20 min according to Ivanov *et al.* (2014). The residue of plant material was removed through filtration, and the combined extracts were stored in a refrigerator at 4°C for further analyses.

Analysis of total phenolic and total flavonoids content

Total phenolic content was determined spectrophotometrically using the Folin-Ciocalteu's reagent following the method of Popova *et al.* (2014b). Each sample (1 mL) was mixed with 5 mL of Folin-Ciocalteu's phenol reagent and 4 mL of 7.5% Na_2CO_3. The mixture was vortexed well and left for 5 min at 50°C. Following incubation, the absorbance was measured at 765 nm. The results were presented as mg Gallic acids equivalents (GAE) per g dry weight (DW) plant material.

Total flavonoid content was measured following a previously reported method (Ivanov *et al.*, 2014): Each obtained extract (0.2 mL) was added to test tubes containing 0.1 mL of 10% aluminium nitrate (Sigma), 0.1 mL of 1 M potassium acetate (Sigma) and 3.8 mL of ethanol. The reaction time was 40 min at ambient temperature. The absorbance was measured at 415 nm. The results were presented as mg quercetin equivalents (QE) per g dry weight (DW) plant material.

HPLC analysis

HPLC analysis of the flavonoids was performed according to Ivanov *et al.* (2014) by using Waters 1525 Binary Pump HPLC systems (Waters, Milford, MA, USA), equipped with Waters 2484 dual Absorbance Detector (Waters, Milford, MA, USA) and Supelco Discovery HS C18 column (5 µm, 25 cm × 4.6 mm) operated under control of Breeze 3.30 software. Quercetin and hyperoside (Sigma) were used for the construction of standard calibration curves in the range of 10 - 100 µg/cm^3. The detection of compounds was carried out at 370 nm. The results

were presented as mg of the respective flavonoid per g dry weight (DW) plant material.

HPLC analysis of phenolic acids was performed as previously described by Tumbarski *et al.* (2017) by using Elite LaChrome (Hitachi) HPLC system equipped with DAD and ELITE LaCHrome (Hitachi) software. Separation of the phenolic acids was performed by Supelco Discovery HS C18 column (5 µm, 25 cm × 4.6 mm), operated at 30°C under gradient conditions with mobile phase consist of 2% (v/v) acetic acid (solvent A) and acetonitrile (solvent B). Chlorogenic, caffeic, ferulic, *p*-coumaric, sinapic, and cinnamic acids (Sigma) were used for the construction of standard calibration curves in the range of 10 - 100 µg/cm^3. The detection of compounds was carried out at 280 and 320 nm, and the flow rate was 0.8 mL/min. The results were presented as mg of the respective phenolic acid per g dry weight (DW) plant material.

Analyses of the antioxidant activity

The antioxidant activity of the obtained extracts was measured by DPPH, ABTS, FRAP and CUPRAC assays (Popova *et al.*, 2014b).

DPPH assay was performed by mixing freshly prepared 4×10^{-4} mol methanolic solution of DPPH with the samples in a ratio of 2:0.5 (v/v). The light absorption was measured at 517 nm.

For ABTS assay, ABTS radical cation ($ABTS^+$) was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12 - 16 h before use. Afterward, the $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm and equilibrated at 30°C. After the addition of 1.0 mL of diluted $ABTS^+$ solution to 10 mL of samples, the absorbance was taken after 6 min at 30°C.

For FRAP assay, the FRAP reagent was prepared fresh and was warmed to 37°C prior to use. Next, 150 µL of plant extracts were allowed to react with 2,850 µL of the FRAP reagent for 4 min at 37°C, and the absorbance was recorded at 593 nm against a reagent blank.

The reaction for CUPRAC assay was initiated by mixing 1 mL of $CuCl_2$ solution (1.0×10^2 M), 1 mL of neocuproine methanolic solution (7.5×10^3 M), 1 mL of ammonium acetate buffer solution (pH 7.0), 0.1 mL of sample followed by 1 mL of water. Absorbance against a reagent blank was measured at 450 nm after 30 min.

The results for antioxidant activity were expressed as µM Trolox® equivalents (TE) per g dry weight (DW) plant material.

Statistical analysis

Three independent extracts from each of the analysed populations were prepared and each extract was analysed in triplicate for total phenolic and flavonoid contents, individual flavonoids and phenolic acids, and antioxidant activity. The presented values are means with standard deviations (± SD). Correlation analysis was performed by Microsoft Office Excel ® 2000.

Results and discussion

Analysis of total phenolic and total flavonoid contents

The total phenolic content varied between 15.62 ± 0.22 and 43.50 ± 0.50 mg GAE/g DW, while the total flavonoid content was in the range of 5.66 ± 0.03 and 25.71 ± 0.17 mg QE/g DW (Table 1). P3 was found to yield the highest amount of phenolic and flavonoid compounds, while P1 the lowest.

The total phenolic content (10.39 ± 0.17 mg GAE/g DW) of 70% ethanolic extract of dried leaves of *N. siculum* evaluated by Popova *et al.* (2014a) is lower than the total phenolic content found in all the analysed populations in the present work. These differences could be due to the different climatic and geographical conditions of the localities of the species.

Alexieva *et al.* (2013) studied the total phenolic in decoction extracts of fresh leaves of wild *A. bulgaricum*, where the determined values range from 0.22 mg GAE/g fresh weight (for 20 min decoction duration) to 0.67 mg GAE/g fresh weight (for 15 min decoction duration). Similar result (0.41 ± 0.03 mg GAE/g fresh weight) was obtained for 70% ethanolic extract under reflux-heat at 70°C (Popova *et al.*, 2014b). In the present work, we preferred to analyse dried leaves of the species because this is how the leaves are usually stored due to their limited availability (only during May and June). The dried and fresh leaves are most commonly used in traditional Bulgarian cuisines. Thus far, there are no reports on the total flavonoid content of *N. siculum*. The results for total phenolic content (in the range of 10.27 and 21.11 mg GAE/g DW) and total flavonoid content (in the range of 2.50 and 20.00 mg QE/g DW) in dried leaf extracts of different ecotypes of *Allium ursinum* L. reported by Pejatović *et al.* (2017) were similar to the data for *N. siculum* presented in the present work.

Table 1. Total phenolic and total flavonoid contents of wild populations of Nectaroscordum siculum.

Sample	Total phenolic content (mg GAE/g DW)	Total flavonoid content (mg QE/g DW)
P1	15.62 ± 0.22	5.66 ± 0.03
P2	32.64 ± 0.42	20.88 ± 0.15
P3	43.50 ± 0.50	25.71 ± 0.17
P4	31.12 ± 0.38	19.55 ± 0.13

P1: Strandzha Mts., P2: Black Sea coast, P3: Eastern Stara Planina Mts., P4: Central Stara Planina Mts. Data are means of triplicates (*n* = 3) ± standard deviation (SD).

HPLC analyses of some phenolic acids and flavonoids

The HPLC analyses of the extracts of the investigated populations aimed to determine the type and quantity of the individual phenolic acids and flavonoids. The analyses confirmed the presence of chlorogenic, caffeic, *p*-coumaric and sinapic acids, hyperoside and kaempferol (Table 2). In addition, ferulic and cinnamic acids were identified and quantified in P3 extract. The dominant polyphenol compounds in all the analysed populations were sinapic acid (between 3.23 ± 0.02 and 6.37 ± 0.02 mg/g DW) and hyperoside (between 4.72 ± 0.02 and 22.94 ± 0.06 mg/g DW).

The obtained results are of great importance because of the valuable biological activities of these substances. Numerous studies have reported that hyperoside possesses anti-viral, anti-nociceptive, anti-inflammatory, cardioprotective, hepatoprotective, gastricmucosalprotective and neuroprotective activity (Liu *et al.*, 2005; Wu *et al.*, 2007; Zeng *et al.*, 2011). Kaempferol was found in lower quantities, but many preclinical studies have shown that this flavonol has a wide range of pharmacological activities, including antioxidant, anti-inflammatory, anti-microbial, anti-cancer, cardioprotective, neuroprotective, anti-diabetic, anti-osteoporotic, estrogenic/anti-estrogenic, analgesic and anti-allergenic (Calderón-Montaño *et al.*, 2011). Sinapic acid was reported to have an anti-inflammatory effect by suppressing the production of some pro-inflammatory mediators (Yun *et al.*, 2008), and p-coumaric acid to protect DNA from oxidative damage (Guglielmi *et al.*, 2003). The high content of phenolic acids and flavonoids found in the investigated populations of *N. siculum* identifies them as valuable sources of biologically active substances with health benefits.

Analyses of the antioxidant activity

Polyphenols are the major plant compounds with antioxidant activity due to their redox properties, such as scavenging and neutralising free radicals, quenching singlet and triplet oxygen, and decomposing peroxides (Calderón-Montaño *et al.*, 2011). Therefore, the high content of polyphenolic compounds evaluated in the analysed populations could suggest high antioxidant activity. In order to determine the complex antioxidant ability of the extracts, different mechanisms were conducted, including scavenging of free radicals (DPPH and ABTS assays) and reduction capacity (FRAP and CUPRAC assays).

According to the results tabulated in Table 3, P3 extract yielded the highest potential to scavenge the free DPPH and ABTS radicals, followed by P2 extract. The highest reduction ability determined by FRAP and CUPRAC assays was also recorded from P3 extract. The differences observed in the polyphenol profiles and antioxidant activity of the extracts of the analysed populations of *N. siculum* could be explained by the different climatic and geographic conditions of their natural habitats.

Table 3. Antioxidant activities of wild populations of *Nectaroscordum siculum* as determined by DPPH, ABTS, FRAP, and CUPRAC assays.

Sample	DPPH	ABTS	FRAP	CUPRAC
P1	18.46 ± 0.16	53.02 ± 1.27	56.80 ± 0.42	75.36 ± 1.08
P2	52.06 ± 0.22	83.94 ± 0.80	139.49 ± 1.46	171.08 ± 3.14
P3	55.70 ± 0.29	94.99 ± 1.17	157.85 ± 1.84	186.44 ± 3.19
P4	49.34 ± 0.23	73.35 ± 1.12	128.82 ± 1.31	155.29 ± 3.32

P1: Strandzha Mts., P2: Black Sea coast, P3: Eastern Stara Planina Mts., P4: Central Stara Planina Mts. Data are means of triplicates (*n* = 3) ± standard deviation (SD). The results are presented in μM TE/g DW.

Table 2. HPLC analyses of the polyphenol profiles of wild populations of *Nectaroscordum siculum*.

Sample	Chlorogenic acid	Caffeic acid	Ferulic acid	p-Coumaric acid	Sinapic acid	Cinnamic acid	Hyperoside	Kaempferol
P1	0.86 ± 0.01	0.03 ± 0.01	n.d.	1.04 ±0.01	3.23 ± 0.02	n.d.	4.72 ± 0.02	0.08 ± 0.01
P2	3.45 ± 0.02	0.50 ± 0.01	n.d.	3.03 ± 0.02	3.95 ± 0.02	n.d.	18.41 ± 0.05	0.12 ± 0.01
P3	4.35 ± 0.02	0.51 ± 0.01	2.14 ± 0.02	5.94 ± 0.03	6.37 ± 0.07	0.93 ± 0.01	22.94 ± 0.06	0.13 ± 0.01
P4	3.18 ± 0.02	0.54 ± 0.01	n.d.	2.70 ± 0.02	5.05 ± 0.04	n.d.	17.88 ± 0.05	0.11 ± 0.01

P1: Strandzha Mts., P2: Black Sea coast, P3: Eastern Stara Planina Mts., P4: Central Stara Planina Mts. Data are means of triplicates (*n* = 3) ± standard deviation (SD). The results are presented in mg/g DW. n.d.: not detected.

A positive linear correlation between the antioxidant activity (determined by DPPH, ABTS, FRAP, and CUPRAC assays), and total phenols and flavonoids was established (Table 4). These results suggested that the highest antioxidant potential of P3 extract could be associated with the highest amount of total and individual phenolic acids and flavonoids as compared to the other extracts. P1 extract yielded the lowest total phenols and flavonoids content and the lowest antioxidant activities as determined by all the testing methods. A similar correlation between the content of phenolic acids, flavonoids and antioxidant activity was also observed for P2 and P4 extracts. Several studies have demonstrated a positive correlation between the phenolic content and the antioxidant activity of the plant extracts (Kiselova *et al.*, 2006; Petkova *et al.*, 2017; Parzhanova *et al.*, 2018).

Table 4. Coefficients of correlation (r) between antioxidant activities (DPPH, ABTS, FRAP, and CUPRAC assays), total phenolic content, and total flavonoid content.

	DPPH	ABTS	FRAP	CUPRAC
Total phenolic content	0.919	0.994	0.972	0.967
Total flavonoid content	0.995	0.945	0.996	0.989

The radical scavenging activity of 70% ethanolic extracts of dried leaves of *N. siculum* determined by Popova *et al.* (2014a); through ABTS method (458.32 ± 1.28 μM TE/g DW) was higher than the radical scavenging activity found in the present work, but their reduction ability measured by CUPRAC assay (45.59 ± 0.69 μM TE/g DW) was lower than that of the present work. These differences could be due to the different types and concentrations of the biologically active substances responsible for antioxidant activity in the investigated populations.

The results of the analyses of the antioxidant activity of the extracts of *N. siculum* correlated with the type and quantities of phenolic acids and flavonoids (Table 2), as the identified hydroxyacinnamic acid derivatives dominated over the hydroxybenzoic acid derivatives. Hyperoside do not contain the 5'-OH-group but it was in significant concentrations in the analysed populations of *N. siculum* and contributed to the evaluated high antioxidant potential.

Conclusion

To the best of our knowledge, this is the first report of detailed analyses on polyphenol profiles of wild growing populations of *N. siculum*. The present work showed that the leaves of *N. siculum* are a valuable source of phenolic acids and flavonoids, and that the observed antioxidant activity could provide the basis for their further inclusion in different food systems for health improvement.

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