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Research Article

Bioactive compounds of *Arctostaphylos uva-ursi* wild-growing populations from Bulgaria

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Abstract: *Arctostaphylos uva-ursi* (L.) Sprengel (bearberry), Ericaceae is a valuable medicinal plant with diuretic and uroantiseptic action which is mainly due to arbutin. In Bulgaria the species is considered as rare. Content assessment of bioactive compounds of *A. uva-ursi* leaves from three natural populations from Bulgaria was the aim of the present study. Leaf samples were collected from Pirin, Vitosha, and Rhodope Mountains. Bioactive compounds in the methanolic extracts of the samples were analysed by GC/MS. Total phenolic content was determined using Folin–Ciocalteu reagent. Arbutin, quinic acid and gallic acid were detected in the highest amounts. Catechine, 4-hydroxybenzoic acid, chlorogenic acid, triterpenes (α- and β-amyrin, uvaol and lupeol) and other primary and secondary metabolites were found, also. Differences in the content of individual compounds between samples of different origin were established. The highest total phenolic (182.98 mg GAE g^{-1}) and arbutin (8.4%) content was found in the sample from Vitosha Mountain. The presented data characterizes the profile of bioactive compounds in the Bulgarian bearberry raw material for the first time.

1. INTRODUCTION

Arctostaphylos uva-ursi (L.) Sprengel (bearberry), Ericaceae is a valuable medicinal plant that occurs in large areas in Europe, Asia, North America and Greenland. However in Bulgaria the plant is considered as rare species and is included in the Red List of Bulgarian vascular plants with the category "vulnerable" (Petrova and Vladimirov, 2009). Leaves of *A. uva-ursi* are used mainly as a diuretic, antimicrobial, anti-inflammatory and skin-whitening agent (EMA, 2016; Shamilov *et al*., 2021). The main active substances of *Arctostaphylos uva-ursi* leaves and its preparations are simple phenols (hydroquinone derivatives such as arbutin, methylarbutin and pyroside), phenolic acids (gallic and ellagic) flavonoids (myricetin, hyperoside and quercetin), iridoid glucosides (asperuloside, monotropein and unedoside (EMA, 2016; Kurkin *et al*., 2018; Shamilov *et al*., 2021). Chemical profile of the species is complemented also by the presence of corilagin (ellagitannin), picein, penta-O-galloyl-β-D-glucose, ursolic acid, tannic acid, p-

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coumaric acid, syringic acid, p-hydroxybenzoic acid, ferulic acid, caffeic acid, lithospermic acid, and proanthocyanidins (Sugier *et al*., 2021; Stefkov *et al*., 2021).

Arbutin (hydroquinone glucoside) is the main compound of *A. uva-ursi* leaves with antioxidant, anti-inflammatory, anticancer, antiparkinsonian, hypoglycemic, and antipyretic activities **(**Bhalla *et al*., 2023). The compound is used as a depigmentation agent also (Boo, 2021). The most commonly used method of arbutin content evaluation in plant material is by HPLC (Song *et al*., 2021; Stefkov *et al*., 2021). However, the GC/MS method is also determined as suitable for the analysis of this compound (Jurica *et al*., 2015). A good comparability of the data obtained by both methods was established (Lamien-Meda *et al*., 2009)

Accumulation of aubutin and other metabolites in the *A. uva-ursi* leaves depends on many factors: genetic, ontogenetic and environmental such as temperature, altitude, radiation, nature of the soil (Asensio *et al*., 2020; Sugier *et al*., 2021; Stefkov *et al*., 2021). The highest content of arbutin has been determined in the samples collected in the autumn after fructification (Stefkov *et al*., 2021). Asensio *et al*., (2020) have reported higher arbutin content in plants growing in the northern locations and at relatively higher altitudes. Sugier *et al*., (2021) have found that samples of bearberry collected from the pine forest populations contain significantly higher arbutin than those from heathlands.

Although the phytochemical profile of the species is known and analyzed in populations from different geographical regions, including Iberian Peninsula, Catalonia (Parejo *et al*., 2002; Asensio *et al*., 2020), Asiatic part of Russia (Olennikov and Chekhirova, 2013), Northern Macedonia (Stefkov *et al*., 2021), and Poland (Sugier *et al* 2021) data on the content of bioactive compounds in Bulgarian populations of the species is missing. That is why the assessment of bioactive compounds with an emphasis on the arbutin content of *A. uva-ursi* wild-growing populations in Bulgaria was the aim of the present study.

2. MATERIAL and METHODS

2.1. Plant Material

Leaf samples of *A. uva-ursi* were collected from three natural populations of Pirin, Vitosha, and Rhodope Mountains of Bulgaria at the beginning of June, 2021. The species was identified by authors (Prof. Petar Zhelev and Dr. Ina Aneva) according to Kozhuharov (1992). Voucher specimens are deposited at the Herbarium, Institute of Biodiversity and Ecosystem Research (SOM), Bulgarian Academy of Sciences, Bulgaria.

Details of the localities of the studied populations are presented at Table 1.

Table 1. Origin of the studied *A. uva-ursi* samples.

Sample code	Mountain	SOM	Geographic Coordinates Altitude m asl
AU1	Vitosha	178670	42° 31' N, 23° 16' E, 1800-1900, open areas above the upper limit of the forest
AU2	Rhodope	178671	41° 35' N, 24° 26' E, 1200-1300, sub-Mediterranean pine forests
AU3	Pirin	178672	41° 50' N, 23° 23' E, 1300-1400, sub-Mediterranean pine forests

2.2. Extraction

Methanolic extract was prepared from 100 mg powdered plant material macerated with 1 mL methanol in 2 mL Eppendorf tubes. 50 µL of 3,5 dichloro-4-hidroxybenzoic acid (1 mg/mL) were placed at the beginning of the extraction procedure as an internal standard. After 24 h of extraction at room temperature, an aliquot of 500 µL of each sample was transferred to a glass vial and was dried.

2.3. Derivatization

100 µL pyridine and 100 µL of N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) were added to the dried samples of the extract (50 mg) and heated at 70 °C for 2 h. After cooling, 300 µL of chloroform were added and the samples were analyzed by GC/MS.

2.4. Gas-Chromatography-Mass Spectrometry Analysis (GC-MS)

GC/MS analysis of methanolic extracts was recorded on a Thermo Scientific Focus GC coupled with Thermo Scientific DSQ mass detector operating in EI mode at 70 eV. A DB-5MS column (30 m x 0.25 mm x 0.25 µm) was used. The conditions of the analysis were described by Berkov *et al*., 2021. The compounds were identified by comparing their mass spectra and retention indices (RI) with standard compounds from the National Institute of Standards and Technology (NIST) and home-made MS databases. Relative quantification for metabolomics was calculated from total ion chromatogram peak area integration of single metabolite and internal standard (3,5 dichloro-4-hidroxybenzoic acid).

2.5. Quantification of Arbutin

Arbutin was quantified after the construction of a calibration curve by plotting the ratio of the peak areas of arbutin (50, 100, 150, 200, 250 and 300 µg/mL) versus that of 50 µg internal standard 3,5-dichloro-4-hydroxybenzoic acid. Arbutin content was expressed as a percentage of DW of the sample.

2.6. Total Phenolic Content

Total phenolic content of methanol extracts was determined using Folin-Ciocalteu reagent and gallic acid as standard. Methanolic extracts were diluted to a concentration of 2 mg/ mL, and aliquots of 0.200 mL were mixed with 2 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 1.8 mL of Na_2CO_3 (7.5%). After 1 h at room temperature, the absorbances of the samples were measured at 765 nm on spectrophotometer versus blank sample. Total phenols were determined as gallic acid equivalents (mg GA) per gram of extract.

2.7. Statistical Analysis

Statistical analysis was carried out using Excel. All experiments were performed in triplicate. Results are presented as a value ± standard deviation (SD). Significant levels are defined at $p < 0.05$ as analyzed by t-test.

3. RESULTS

3.1. Gas-Chromatography-Mass Spectrometry Analysis

The GC-MS analyzes of methanolic extracts of *A. uva-ursi* samples from three populations revealed primary and secondary metabolites including phenolic acids, flavonoids, fatty acids, sterols, triterpenes, saccharides, and polyols. The results are presented in Table 2. The most abundant primary metabolites in the methanolic extracts were the monosaccharides fructose and glucose, as well as disaccharide sucrose. Arbutin and gallic acid were found in the highest amounts of the secondary metabolites. Quinic acid, catechin, triterpenes (α- and β-amyrin, uvarol) were identified as main bioactive compounds in the studied samples. GC/MS chromatograms of the extracts from the studied samples are presented in Figure1.

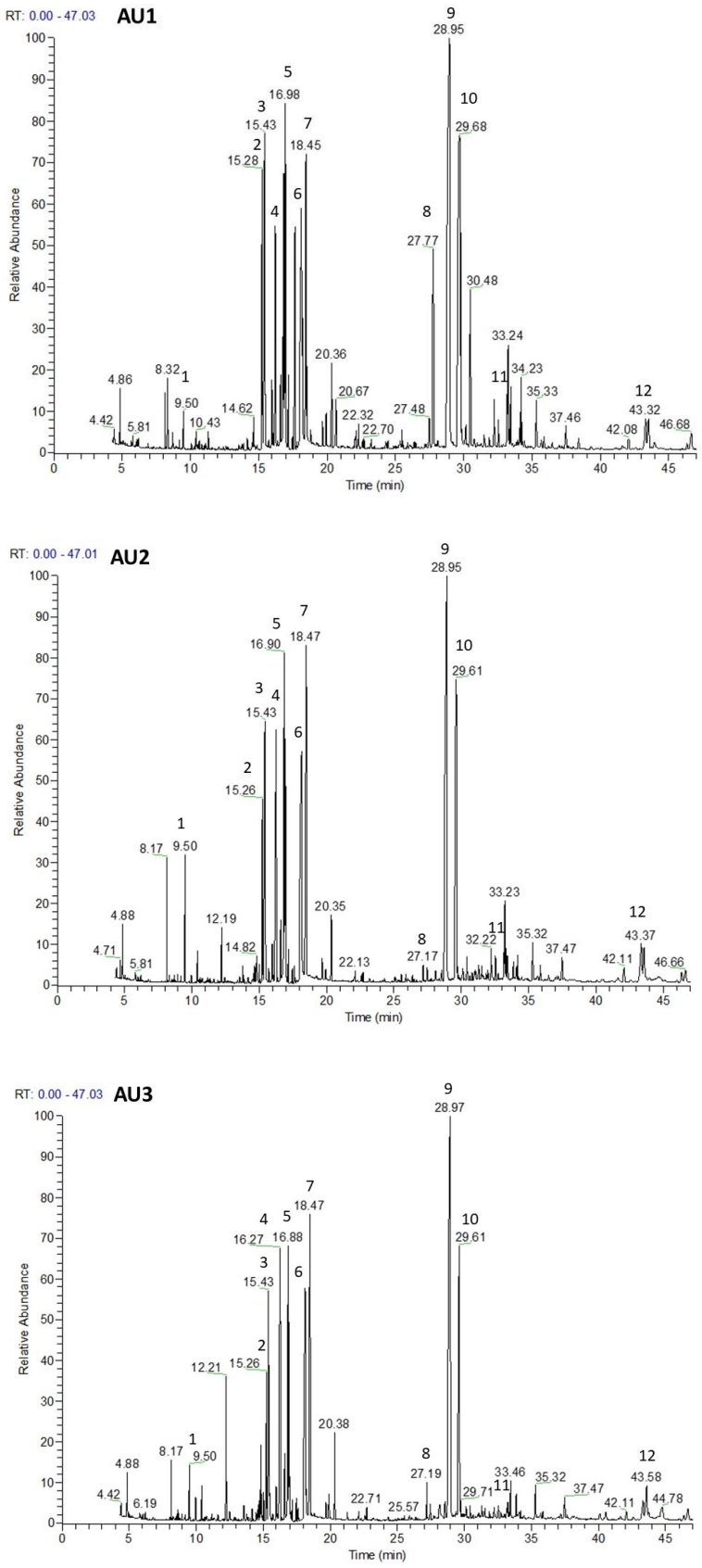

Figure 1. GC/MS chromatograms of methanolic extracts of the samples from Vitosha (AU1), Rhodope (AU2) and Pirin (AU3); 1. Hydroquinone; 2. Fructose 1; 3. Fructose 2; 4. Quinic acid; 5. Glucose; 6. Gallic acid; 7. Monosaccharide; 8. *Disaccharide*; 9. Arbutin; 10. Sucrose; 11. Catechine 12. α-Amyrin.

Quality differences in the content of individual compounds between samples of different origins were found. Monosaccharides, sugar alcohol - inositol and especially sucrose were found in significantly greater amounts in the Vitosha sample (AU1). With regard to lipid compounds, no significant quantitative differences between the samples from the different populations were found. The content of gallic acid as a major component also showed no differences between samples from the three populations. The amounts of arbutin and catechin were found to be the highest in the sample from (AU1) sample again.

Table 2. Identified compounds in the methanolic extracts of the studied samples of *Arctostaphylos uva-ursi* *

Identified compounds	RI	AU1	AU2	AU3
Phenolic compounds				
Hydroquinone	1396	63.5±29	04.6±132	559±172
4-Hydroxybenzoic acid	1637	2.7±0.8	3.2±1.2	1.3±0.3
Protocatechuic acid	1811	0.9±0.3	1.0±0.4	2.8±0.6
Quinic acid	1846	253.9±63	163.0±35	287.6±44
Syringic acid	1888	trace	0.2±0.1	0.4±0.1
4-Hydroxycinnamic acid	1934	0.2±0.1	0.2±0.1	0.4±0.1
Gallic acid	1976	628.3±86	650.4±73	652.5±105
Caffeic acid	2142	trace	trace	3.4±2
Arbutin	2561	3944.7±412	3226.6±378	3209.8±402
Catechin	2861	30.7±9	10.5±2	15.7±6.2
Lipid compounds				
Hexadecanoic acid	1929	16.5±4	19.9±5	12.5±4
Octadecanoic aicd	2132	2.9±0.6	2.7±0.8	1.1±0.4
β-Sitosterol	2614	6.0±1.6	10.7±4	4.1±2.7
β-Amyrin	3335	30.3±0.9	28.3±8.6	21.5±7.2
α-Amyrin	3382	111.6±3.5	153.5±14	76.4±18
Lupeol	3434	99.2±11	108±16	117.7±25
Uvaol	3716	60.9±2.2	41.7±9.3	50.8±14
Saccharides and polyols				
Fructose 1	1793	561.1±75	277.0±25	264.4±98
Fructose 2	1830	600.2±137	502.9±98	352.9±102
Glucose	1889	519.9±216	425.5±132	441.5±111
myo Inositol	2080	92.5±33	65±13	33.8±12
Sucrose	2712	1025.9±298	592.0±94	366.2±77

*Relative quantification for metabolomics was calculated from total ion chromatogram peak area integration of single metabolite and standart

3.2. Quantification of Arbutin and Total Phenolics

The content of arbutin in the leaves of the studied samples was determined by GC/MS and that of total phenolics by spectrophotometric assay. The results of quantitative analyses are presented in Table 3. Total phenolic content in the studied samples ranged from 137 to 182 GAE/g extract. The level of arbutin in the studied populations fluctuated from 6.8% to 8.4 %.

The highest content of arbutin and total phenols was found from the Vitosha Mountain (AU1), but a significant difference (p<0.05) among the studied samples was not found.

Table 3. Total phenol and arbutin content in the studied samples of *A. uva-ursi*.

Title	Total phenolic content [mg GAE/g extract]	Arbutin content [%, DW of the sample]
AU 1	182.98±6	8.4±2.6
AU 2	138.25±12	7.0±1.1
AU 3	137.55±12	6.8±2.3

4. DISCUSSION and CONCLUSION

Arbutin, gallic acid, quinic acid and triterpenoids were identified as the main bioactive compounds in the *A. uva ursi* leaf samples from Bulgarian populations which is consistent with previously reported data for the chemical composition of the species (Kurkin *et al*., 2018; Shamilov *et al*., 2021; Song *et al*., 2021). Among minor components considering the toxicological profile of free hydroquinone (de Arriba *et al*., 2013), it is worth noting that its content is significantly lower in the sample (AU1) from Vitosha. The established quantitative differences in the chemical profiles of the studied samples are probably determined by the local influence of environmental factors. It has been found that environmental factors such as latitude, altitude, UV-radiation, habitat types (heathlands or pine forests), the date of collection influenced on the accumulation of phenolic compounds of *A. uva-ursi* (Asensio *et al*., 2020; Stefkov *et al*., 2021; Sugier *et al*., 2021). Sugier *et al*., (2021) have reported that leaves of the species collected from the heathland population are characterized by higher total flavonoid content in comparison with the pine forest population. In the present study higher catechin (flavonoid) content was found in the samples from population (AU1) located in open areas above the upper limit of the forest than in the samples collected from population in the pine forest (AU2 and AU3). Accumulation of disaccharides is important and possibly best-known reaction of plans in response to water stress (Ingram and Bartels, 1996) furthermore Oliver *et al*., (2001) have reported that non-disaccharide compounds such as arbutin also accumulated in the condition of water deficit. The open areas of the locality (AU1) probably lead more often to water deficit and this is reflected in the higher disaccharide content in the samples from this region.

The amount of arbutin determined in the samples of the present study most closely approximates the data reported for the samples from Catalan Pyrenees, Spain (Parejo *et al*., 2002). It should be kept in mind that samples in the present study were collected at the beginning of June, and many studies reported higher arbutin content in leaves of the species collected in autumn (Parejo *et al*., 2002; Stefkov *et al*., 2021). Asensio *et al*., (2020) and Sugier *et al*. (2021) have been concluded in their comprehensive studies that *A. uva ursi* samples of populations from northern latitudes and at higher altitudes showed frequently high arbutin content. The present results are consistent with these conclusions. The sample (AU1) collected from the highest altitude had the highest arbutin content.

In conclusion, a comparative analysis of bioactive compounds of *A. uva ursi* plant material from three Bulgarian populations was done by GC/MS for the first time. The highest amounts of arbutin, total phenolics, sucrose and catechin were found in the leaves of the plant from Vitiosha Mountain. The study adds to the knowledge of the content of arbutin in bearberry leaves within the southern limits of its natural distribution. We recommend that samples be analyzed by HPLC so that the content of more polar substances can be compared.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Milena Nikolova: experimental design, chemical analysis, manuscript writing; **Ina Aneva** and **Petar Zhelev**: Collection and identification of plant material; **Strahil Berkov**: Chemical analysis; **Elina Yankova-Tsvetkova**: Conceptualization, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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