

Antioxidant capacity of wild-growing bilberry, elderberry, and strawberry fruits

Radenka Kolarov¹, Marijana Peić Tukuljac¹, Aliksandr Kolbas², Natalia Kolbas², Goran Barać¹, Vladislav Ognjanov¹, Mirjana Ljubojević¹, Dejan Prvulović^{1*}

¹University of Novi Sad, Novi Sad, Serbia

²Brest State A.S. Pushkin University, Brest, Belarus

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Chemical properties (*L*-ascorbic acid and total sugars content, pH, titratable acidity, and dry solid content), phenolic compounds (total phenolics, tannins, flavonoids, anthocyanins, and flavan-3-ols) and antioxidant capacity were measured in ripe fruits of wild-growing strawberry, bilberry, and elderberry from eastern Serbia. All three selected fruits are rich sources of nutraceuticals: vitamin C, sugars, and different classes of phenolic compounds and their extracts expressed high antioxidant activity. Elderberry fruits possess highest concentration of all measured biomolecules.

Keywords: antioxidant activity, berry, fruits, phenolic compounds

1 Introduction

The antioxidant potential of phytochemicals in health maintenance has been increasingly recognized in recent years. Sufficient evidence has shown that free radicals play an important role in most major health problems such as cancer, cardiovascular disease, and degenerative diseases associated with aging (Zhang et al., 2015). Polyphenols are especially important antioxidants because of their high redox potentials allowing them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Kasote et al., 2015).

Red fruits, including different berries, are characterized by high amounts of bioactive molecules and rich sources of natural antioxidants: phenolic acids, tannins, carotenoids, vitamin A, C, E, folic acid, and minerals such as calcium, selenium, and zinc. These chemical compounds are secondary metabolites that prevent the fruit from environmental factors that could induce oxidation processes, such as air, oxygen, light, attacks of phytopatogens and herbivorous animals. Phenolic antioxidants interfere with the oxidation process as free radical terminators and sometimes also as metal chelators (Manganaris et al., 2013; Oanacea et al., 2015; Hidalgo & Almajano, 2017; Šapčanin et al., 2017).

Strawberry (*Fragaria* sp.) is a genus of flowering plant in the rose family, Rosaceae, commonly known as strawberry for their edible fruits. There are more than 20 described species and many hybrids and cultivars. While primarily valued for their taste and flavour, strawberries also have potential health benefits. Strawberries are high in the vitamins and mineral content, and they are also rich in different phenolic molecules, including anthocyanins, hydrolyzable tannins and phenolic acids (Liston et al., 2014). Vaccinium species: bilberries (*Vaccinium myrtillus* L.) and blueberries (*Vaccinium corymbosum* L.), have been proven to have high phenolic content and strong antioxidant potential (Bunea et al., 2011, Kevers et al., 2014). Bilberries and blueberries have multiple biological and health-promoting effects, including anticarcinogenic, anti-inflammatory, and antimicrobial effects (Johnson & Arjmandi, 2013; Salamon et al., 2021). *Sambucus nigra* L. is a species from the Adoxaceae family, known as a low growing tree. Elderberry is recognized for its therapeutic properties such as antidiabetic and antiviral effects, diuretic properties, and prevention of atherosclerosis, cardiovascular diseases, and cancer (Młynarczyk et al., 2018).

*Corresponding Author: Dejan Prvulović, Faculty of Agriculture, University of Novi Sad, trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia; phone: 381-21-485-3466, e-mail: dejanp@polj.uns.ac.rs

In the present study, the chemical composition, content of phenolic compounds, and antioxidant capacity of the three wild edible berry fruits from eastern Serbia region were assessed by different assays. An assessment of the correlation between the antioxidant activity of the fruits and the phenolic components contents was performed.

2 Material and methods

2.1 Plant material

Fresh fruits of wild-growing strawberries, bilberries, and elderberries were picked up manually at full maturity in a forested area near the town of Žagubica, Homolje Carpathian region, eastern Serbia. The samples were kept on ice during transportation to laboratory, divided into two subsamples for chemical and biochemical analysis, and stored. For all spectrophotometric analysis, the ThermoScientific Evolution 220 UV-visible spectrophotometer was used.

2.2 Chemical composition

Dry solid content of fruits was analysed by the evaporation method. Fresh fruits of known weights were placed in glass dishes and dried in laboratory oven at 80 °C until constant weight (Bradley, 2010). pH and titratable acidity were measured by methods described by Sadler and Murphy (2010). Results for titratable acidity were expressed as g citric acid 100 g⁻¹ fruit. Total sugar (carbohydrate) content was estimated by the phenol-sulfuric acid method (BeMiller, 2010). The standard curve was constructed using different concentrations of glucose, and the results were expressed as mg glucose equivalents (GE) per gram of fruit (mg.GE.g⁻¹). Content of vitamin C (ascorbic acid, AA) in fresh wild-growing strawberries, bilberries, and elderberries was determined by the 2,4-dinitrophenylhydrazine (DNP) method as described by Al-Ani et al. (2007). Calibration curve was conducted by solutions with different concentration of ascorbic acid and results were expressed as mg of ascorbic acid equivalent per g of fresh fruit (mg.AAE.g⁻¹).

2.3 Phenolic compounds

One gram of edible parts of each fruit was homogenized and extracted in 10 ml of 70% (v/v) methanol overnight. The extracts were filtered and centrifuged at 8000 rpm for 15 minutes. Extracts were kept in refrigerator and used for further biochemical analysis.

The content of total phenols (TP) was determined using a Folin-Ciocalteu reagent (Nagavani & Raghava Rao, 2010). The diluted Folin-Ciocalteu solution was mixed with 20 µl of the extracts. After five minutes 400 µl of 20% Na₂CO₃ solution was added. A series of standard dilution

of gallic acid was used to construct the calibration curve. After 60 min, the absorbance at $\lambda = 730$ nm was read on a spectrophotometer. The TP content of the extracts tested was expressed as mg of gallic acid (GA) equivalents per g of fresh fruit weight (mg of GAE.g⁻¹ FW). All samples were prepared and analysed in triplicate. The content of total tannins was determined by the same method as content of total phenolics after the removal of tannins by their adsorption on an insoluble PVPP (polyvinylpyrrolidone) matrix. The calculated values were subtracted from the TP content. The total flavonoid (TF) content in the obtained fruit samples was measured by the aluminium chloride spectrophotometric assay by the method described by Saha et al. (2013). Total flavonoid content was determined from the regression equation of the quercetin calibration curve and expressed as mg quercetin equivalent (QE) gram of fresh weight of selected fruits (mg of QE g⁻¹ FW). The content of total anthocyanins (TA) in the methanol extracts of three selected fruits was determined spectrophotometrically by measuring the difference in the absorbance between solutions of pH 1.0 and pH 4.5 at absorption on 510 and 700 nm (Giusti & Wrolstad, 2001). Values are expressed as mg cyaniding-3-glucoside (C3G) equivalents per gram of fresh fruit weight (mg C3GE.g⁻¹ FW). The content of flavan-3-ols in selected fresh fruits was measured by the vanillin assay using the daily prepared working solution of 4% vanillin in methanol (Laličić-Petronijević et al., 2016). The results were expressed in mg of (+)-catechin equivalents (CE) per g of fresh fruits weight (mg of CE.g⁻¹ FW).

2.4 Antioxidant capacity

Scavenging of free radicals was tested using a DPPH (2,2-diphenyl-1-picrylhydrazyl) acetone solution (Lai & Lim, 2011). The ferric-reducing antioxidant power (FRAP) assay was carried out according to the procedure described by Valentão et al. (2002). The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt) assay was based on a method described by Miller et al. (1993). A reducing power assay (total reduction capacity-TRC) was performed by the method of Saha et al. (2013). The standard curve for antioxidant tests (DPPH, FRAP, ABTS and TRC) was plotted using trolox solution and the results were expressed as mg trolox equivalents (TE) per g of the fresh plant material (mg TE.g⁻¹ FW). NBT (nitro blue tetrazolium) test or superoxide dismutase-mimetic (SOD-mimetic) activity was assayed according to the slightly modified method of Kalaskar and Surana (2014) by measuring fruit extracts ability to inhibit photochemical reduction of NBT. The reaction mixture contained 1 mL of 50 mM potassium phosphate buffer (pH 7.8) with dissolved NBT, EDTA (ethylenediaminetetraacetic acid),

L-methionine, riboflavin and 20 μL of the fruits' extract. It was kept under a fluorescent lamp for 10 min, and then the absorbance was read at 560 nm. One unit of the SOD activity was defined as the content of enzymes required to inhibit reduction of NBT by 50%. The activity of the extracts was expressed as IU SOD.g⁻¹ fresh plant material. NO generated from sodium nitroprusside (SNP) was measured according to the method of Marcocci et al. (1994). The reaction mixture containing 3 mL SNP phosphate buffered saline (pH 7.3), with or without 40 μL of fruit extract, was incubated at 25 °C for 180 min in front of a visible light source. The NO radical thus generated interacted with oxygen to produce the nitrite ion (NO₂⁻) which was assayed at 30 min intervals by mixing incubation mixture with an equal amount of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediaminedihydrochloride). The absorbance of the chromophore (purple azo dye) formed during the diazotisation of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylenediaminedihydrochloride was measured at 546 nm. The nitrite generated in the presence or absence of the fruit extract was estimated using a standard curve based on sodium nitrite solutions of known concentrations.

2.5 Statistical analysis

Results were expressed as a mean value of determinations of 3 independent samples made in triplicates. Statistical significance was tested by analysis of variance followed by comparison of means by the Duncan's multiple range test ($P < 0.05$) calculated using STATISTICA for Windows version 13.2 (StatSoft, Tulsa, OK, USA). Stepwise multiple regression analyses were used to determine correlation among variables.

3 Results and discussion

3.1 Chemical composition

Berries contain a wide variety of primary and secondary biomolecules that may help protect cellular systems from

oxidative damage and lower the risk of chronic diseases (Skrovankova et al., 2015). The content of dry solids, total sugars, pH values, titratable acidity, and ascorbic acid content of elderberry, strawberry and bilberry fruits are shown in Table 1. Dry solid content of three selected wild-growing fruits ranged from 10.83% for bilberry up to 22.27% for strawberry and 23.60% for elderberry. The pH of the bilberry pulp was 3.12, which was lower than the pH of strawberry (3.57) and elderberry (3.84). These results are in agreement with findings of other researchers who investigated properties of elderberry (Vujanović et al., 2020; Zhou et al., 2020) and bilberry (Bernal et al., 2014; Colak et al., 2016; Celik et al., 2018). Titratable acidity of all three investigated fruits was within the same range (1.22–1.28% citric acid) and is in good agreement with literature data (Vulić et al., 2008; Özgen et al., 2010; Colak et al., 2016; Celik et al., 2018). Sugar content in fruits is an important parameter. Consumers mostly prefer fruits with higher sugar content. There were no statistically significant differences in sugar content between all three selected fruits from this experiment. Total sugar concentration ranged from 5.17 g.100 g⁻¹ (elderberry) to 5.70 g.100 g⁻¹ (bilberry) and 6.59 g.100 g⁻¹ (strawberry). Similar findings are reported by other authors. For elderberry, Vujanović et al. (2020) reported 3.74 g of total sugars per 100 g of fruits, Elez Garofulić et al. (2012) reported 6.91 g.100 g⁻¹, while Vulić et al. (2008) measured 8.88 g.100 g⁻¹ in their samples. In plants, *L*-ascorbic acid (vitamin C) has several functions: as an enzyme cofactor, a radical scavenger, and a donor/acceptor in electron transport either in the plasma membrane or in the chloroplasts. In humans and animals, vitamin C functions as a cofactor in the enzymatic hydroxylation, preventing curvy and protects against different diseases. Currently, most of the daily intake of *L*-ascorbic acid for humans comes from fruits and vegetables (Fenech et al., 2019; Doseděl et al., 2021). The highest content of vitamin C was measured in elderberry fruits (0.30 mg AAE g⁻¹), while concentrations of vitamin C in strawberry and bilberry were 0.20 mg AAE g⁻¹ and 0.19 mg.AAE.g⁻¹, respectively. Literature data agrees with our findings (Vulić et al., 2008;

Table 1 Chemical composition of wild-growing strawberry, bilberry, and elderberry fruits

	Fruit		
	strawberry	bilberry	elderberry
Dry solid (%)	22.27 ± 0.36 ^a	10.83 ± 0.20 ^b	23.60 ± 0.69 ^a
pH	3.57 ± 0.01 ^a	3.12 ± 0.01 ^b	3.84 ± 0.03 ^a
Titratable acidity (% of citric acid)	1.26 ± 0.04 ^a	1.28 ± 0.06 ^a	1.22 ± 0.06 ^a
Ascorbic acid (mg.g ⁻¹)	0.20 ± 0.02 ^b	0.19 ± 0.00 ^b	0.30 ± 0.03 ^a
Total sugars (g 100.g ⁻¹)	6.59 ± 0.46 ^a	5.70 ± 1.56 ^a	5.17 ± 2.43 ^a

The data are presented mean values ± standard error; a–b values without same superscript within each row differ significantly ($P < 0.05$); 1%

Milivojevic et al., 2012; Poiana et al., 2012; Bernal et al., 2014).

3.2 Phenolic compounds

Results of the determination of the main phenolic compounds (TP, TT, TF, TA and flavan 3-ols) of wild-growing strawberry, bilberry and elderberry are shown in Table 2. Different parameters, such as fruit species, cultivar, maturity stage, harvesting time, postharvest conditions, extraction procedure and others affect the chemical and biochemical composition of fruits (Prvulović et al., 2016). Role of phenolics in fruits are numerous: they actively inhibit or stimulate physiological processes, represent defending system against pathogens and stress, and contribute to some of the quality properties of fruits (aroma, flavors, color, and astringency) (Sulusoglu, 2014). Significant differences in TP content among selected fruit species were recorded (Table 2). Elderberry contained the highest concentration of TP (17.40 mg.GAE.g⁻¹ FW), followed by strawberry (14.33 mg GAE.g⁻¹ FW) and bilberry fruits (11.14 mg.GAE g⁻¹ FW). The content of measured TP in our experiment was higher compared to literature data for elderberry (Özgen et al., 2010; Elez Garofulić et al., 2012; Vujanović et al., 2020; Zhou et al., 2020), strawberry (Wang & Lewers, 2007; Peñarrieta et al., 2009; Dyduch-Siemińska et al., 2015) and bilberry (Bunea et al., 2011; Milivojevic et al., 2012; Poiana et al., 2012; Bernal et al., 2014; Celik et al., 2018). Some researchers detected higher amount of TP in elderberry fruits compared to results obtained in our experiment (Tumbas et al., 2010; Colak et al., 2016).

Tannins are compounds with a relatively high molecular weight and could be divided into two groups: condensed and hydrolysable tannins. Condensed tannins (proanthocyanidins) are found in abundance in fruits and fruit products and are partly responsible for astringency and color of fruits (Skrovankova et al., 2015). Strawberry and elderberry fruits contained similar quantity of TT (13.41 mg.GAE.g⁻¹ FW and 13.24 mg GAE.g⁻¹ FW, respectively), while bilberry fruits had lower

amounts of these compounds (9.93 mg.GAE.g⁻¹ FW). Few researchers detected lower amount of TT in elderberry fruits compared to results of our research (ElezGarofulić et al., 2012; Najda et al., 2014; Dyduch-Siemińska et al., 2015; Vujanović et al., 2020).

Flavonoids has a variety of biological activities in plants: color and aroma of flowers and fruits, to attract pollinators, protect plants from different biotic and abiotic stresses and act as UV filters, detoxifying agents, signal molecules, allopathic compounds, phytoalexins, and antimicrobial defensive components. In addition, flavonoids have roles against frost hardiness, drought resistance and play a role in plant heat acclimatization and freezing tolerance (Panche et al., 2016). Significant differences of TF content among observed fruit species were confirmed. The highest levels of TF were found in elderberry fruits (8.09 mg.QE.g⁻¹ FW) followed by bilberry (2.53 mg.QE.g⁻¹ FW) fruits. The lowest levels of TF were measured in strawberry (0.081 mg.QE.g⁻¹ FW).

Anthocyanins are water-soluble phenolic compounds that serve as plant pigments responsible for red, purple or blue color of many plant organs. Seventeen anthocyanidins could be found in nature, whereas only six of them are present in most fruits: malvidin, pelargonidin, peonidin, petunidin, delphinidin and cyanidin. Anthocyanins are antioxidants that play a very important role in reducing risks of different human degenerative diseases (Hidalgo & Almajano, 2017). Elderberry showed the greatest total anthocyanins (TA) content of all three fruits (1.5.37 mg.C3G.g⁻¹ FW), followed by bilberry (3.95 mg.C3G.g⁻¹ FW) and the lowest TA content were found in strawberry fruits (0.29 mg C3G g⁻¹ FW). The present TA content values for elderberry (Özgen et al., 2010; Elez Garofulić et al., 2012; Zhou et al., 2020), bilberry (Bunea et al., 2011; Poiana et al., 2012; Bernal et al., 2014; Celik et al., 2018) and strawberry (Wang & Lewers, 2007; Dyduch-Siemińska et al., 2015) are in agreement with other studies.

Flavan-3-ols is one of the groups of plant phenolics that are distributed widely in plants and are synthesized via

Table 2 Phenolic compounds of wild-growing strawberry, bilberry and elderberry fruits

	Fruit		
	strawberry	bilberry	elderberry
Total phenolics (mg of GAE.g⁻¹ of FW)	14.33 ±0.66 ^b	11.14 ±0.70 ^c	17.40 ±0.85 ^a
Total tannins (mg of GAE.g⁻¹ of FW)	13.41 ±0.16 ^a	9.93 ±0.16 ^b	13.24 ±1.34 ^a
Total flavonoids (mg of QE.g⁻¹ of FW)	0.81 ±0.24 ^c	2.53 ±0.07 ^b	8.09 ±0.56 ^a
Total anthocyanins (mg of C3G.g⁻¹ of FW)	0.29 ±0.05 ^c	3.95 ±0.05 ^b	5.37 ±0.17 ^a
Flavan 3-ol (mg of CE.g⁻¹ of FW)	0.005 ±0.001 ^c	0.17 ±0.01 ^a	0.03 ±0.10 ^b

The data are presented mean values ± standard error; a–c values without same superscript within each row differ significantly ($P < 0.05$)

the phenylpropanoid and flavonoid pathways. Flavan-3-ols also have diverse biological activities, including protection against herbivorous and phytopatogens, and possess strong allelopathic activity (Enomoto et al., 2020). From the results shown in Table 2 it can be seen that bilberry fruits possess more flavan-3-ols (0.170 mg.CE.g⁻¹ FW) compared to fruits of elderberry (0.030 mg.CE.g⁻¹ FW) and strawberry (0.005 mg.CE.g⁻¹ FW).

3.3 Antioxidant capacity

Within the present study, the antioxidant capacity of three selected fruits was not uniform and depends on assay performed (Table 3). To date, antioxidant activities of plant extracts are measured using a panel of assays whereby each assay has its own advantages and limitations. It is not sufficient to use one *in vitro*

assay to claim antioxidant capacity of an extract (Sadeer et al., 2020). Elderberry extract expressed the strongest antioxidant activity in FRAP, ABTS and NO assays, strawberry extract in ABTS, DPPH, TAC and NBT assays, while bilberry extract performed the highest antioxidant capacity in TRC and NBT assays, compared with other two fruits' extracts. After comparing with literature data, it is obvious that all three fruits possess high antioxidant properties in comparison to many other fruits, crops, and vegetables. Results of our study are in agreement with previous works on strawberry (Wang & Lewers, 2007; Peñarrieta et al., 2009; Najda et al., 2014), elderberry (Elez Garofulić et al., 2012; Vujanović et al., 2020) and bilberry (Bunea et al., 2011; Milivojevic et al., 2012; Poiana et al., 2012; Bernal et al., 2014; Colak et al., 2016; Celik et al., 2018).

Table 3 Antioxidant capacity of wild-growing strawberry, bilberry, and elderberry fruits

Antioxidant tests	Fruit		
	strawberry	bilberry	elderberry
FRAP (mg TE.g ⁻¹ FW)	40.55 ± 6.03 ^a	32.54 ± 3.68 ^b	51.46 ± 2.40 ^a
ABTS (mg TE.g ⁻¹ FW)	36.23 ± 2.35 ^a	25.89 ± 1.98 ^b	36.98 ± 0.65 ^a
DPPH (mg TE.g ⁻¹ FW)	163.13 ± 6.46 ^a	92.51 ± 6.44 ^c	125.03 ± 9.59 ^b
TAC (mg TE.g ⁻¹ FW)	195.96 ± 2.60 ^a	102.20 ± 11.81 ^b	71.52 ± 0.83 ^c
TRC (mg TE.g ⁻¹ FW)	98.20 ± 2.06 ^b	108.01 ± 0.86 ^a	98.60 ± 1.16 ^b
NBT (IU SOD.g ⁻¹ FW)	256.05 ± 11.11 ^a	196.82 ± 6.64 ^b	151.70 ± 5.50 ^c
NO inhibition (% of inhibition of NO radicals)	78.30 ± 3.65 ^b	96.36 ± 0.23 ^a	95.79 ± 3.68 ^a

The data are presented via mean values ± standard error; a–c values without the same superscript within each row differ significantly ($P < 0.05$)

Table 4 Statistical analysis

Total phenolics	TAC	FRAP	ABTS	DPPH	NBT	TRC	NO inhibition
Correlation coefficient (<i>r</i>)	-0.2074	0.8925*	0.8793*	0.4625	-0.4114	-0.8335*	-0.0815
Coefficient of determination (<i>r</i> ²)	0.043	0.800	0.773	0.214	0.169	0.695	0.007
Total tannins							
Correlation coefficient (<i>r</i>)	0.2901	0.7164*	0.8702*	0.8226*	0.0994	-0.8607*	-0.4456
Coefficient of determination (<i>r</i> ²)	0.084	0.513	0.757	0.677	0.010	0.741	0.196
Total flavonoids							
Correlation coefficient (<i>r</i>)	-0.828*	0.716*	0.341	-0.2676	-0.9139*	-0.2536	0.6395
Coefficient of determination (<i>r</i> ²)	0.686	0.513	0.116	0.072	0.835	0.064	0.409
Total anthocyanins							
Correlation coefficient (<i>r</i>)	-0.994*	0.3286	-0.1793	-0.7136*	-0.972*	0.2698	0.9074*
Coefficient of determination (<i>r</i> ²)	0.988	0.108	0.032	0.509	0.945	0.728	0.823
Flavan 3-ol							
Correlation coefficient (<i>r</i>)	-0.9615*	0.4772	0.012	-0.566	-0.9872*	0.0821	0.8272*
Coefficient of determination (<i>r</i> ²)	0.925	0.228	0.000	0.320	0.974	0.007	0.684

* values marked with asterisk are statistically significant ($P > 0.05$)

3.4 Statistical analysis

Statistical correlation between different phenolic compounds and applied antioxidant assays are presented in Table 4. TP and TT revealed positive correlation with FRAP (0.8925 and 0.7164, respectively) and ABTS (0.8793 and 0.8702, respectively) assays, TF with FRAP assay (0.7160) while TA and flavan-3-ols expressed positive correlation only with NO radical scavenging test (0.9074 and 0.8272, respectively). TAC and NBT assays did not perform positive correlation with any of measured groups of phenolic compounds. It could be assumed that antioxidant capacity of extracts of selected fruits does not depend on phenolic compounds present only. Some other class of extracted molecules could play significant role in antioxidant activity of extracts.

4 Conclusions

In this work, the chemical properties, phenolic compounds and antioxidant capacity of wild growing strawberry, bilberry and elderberry from eastern Serbia were determined. All three fruits are high in ascorbic acid, sugars, and phenolics content and possess strong antioxidant activity. The elderberry fruits had the highest ascorbic acid and TT, TF and TA content. Antioxidant capacity of fruits appears to be largely influenced by TP and TT contents while ability of inhibition of NO radicals depends on the content of TA and flavan-3-ols in samples.

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