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TOTAL PHENOLS, FLAVONOIDS, ANTHOCYANINS AND ANTIOXIDANT ACTIVITY OF WILD POMEGRANATE (*Punica granatum* L.) BIOWASTE FROM MONTENEGRO

SUMMARY

In this paper, the content of total phenols, flavonoids, anthocyanins as well as antioxidant activity of 80% methanolic extracts of fruit peel and seeds of wild pomegranate (*Punica granatum* L.) collected from different localities of Montenegro were analyzed.

The obtained results showed a high level of presence of these bioactive substances with a high level of antioxidant activity. Among the samples, the dominant higher level of these bioactive substances was determined in the samples collected in the coastal region, while the smallest amount was detected in the samples collected from the locality Carev Laz, in that with higher altitude.

There was a negative correlation between the content of total phenols and flavonoids in the peel of wild pomegranate ($r = -0.57$), as well as a strong positive correlation between the amount of total phenols and antioxidant activity determined by the FRAP method ($r = 0.81$). In contrast to peel samples, in seed samples there was a positive correlation between the content of total phenols and flavonoids ($r = 0.67$). A high correlation was observed between antioxidant activity (FRAP method) and anthocyanin content ($r = 0.96$) as well as between antioxidant activity (DPPH method) and total phenols content ($r = 0.84$).

Keywords: *Punica granatum* L., phenols, flavonoids, anthocyanins, antioxidant activity, biowaste

INTRODUCTION

The organic part of municipal solid waste, also known as bio-waste, includes food scraps from households and garden waste (leaves, branches, grass).

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By developing strategies for its utilization, bio-waste can be an additional source of fertilizer for plants, but also a valuable source of secondary metabolites that can contribute to human health. Its recycling would reduce disposal costs and reduce its impact on air pollution (Vieira and Matheus, 2019; Mahro and Timm, 2007). In addition to the primary metabolites responsible for the growth, development and reproduction of each plant (Erb and Kliebenstein, 2020), plants also produce metabolites specific to each plant species. The production of secondary metabolites provides plants biotic and abiotic factors protection (Mazid, 2011). Phenols are a group of secondary metabolites that are distinguished by the level of biological capacity due to pronounced antioxidant, antimicrobial, antifungal and even anticancer activity (Viuda-Martos *et al*., 2010, Braga *et al*., 2005, Singh *et al*., 2018, Albrecht *et al*., 2004). Many studies have confirmed that the peel and seeds of wild pomegranate, as its biowaste, contains a large amount of phenols, flavonoids and anthocyanins (Radunić *et al*., 2017, Gadže *et al*., 2012, Jacob *et al*., 2018, Kam *et al*., 2013). Tzulker *et al*. (2007) suggest that punicalagin is one of the main bioactive substances that contributes to the total antioxidant capacity of wild pomegranate fruit (*Punica granatum* L.). According to Gil *et al*. (2000), the wild pomegranate juice shows up to eight times stronger antioxidant activity than the juice of grapes, cranberries and oranges, and three times stronger than the activity of red wine and green tea. Derakhshan *et al*. (2018) in their study presented a positive correlation between phenolic composition and antioxidant activity. They also proved that temperature and environmental conditions have an impact on the amount of phenol and antioxidant activity.

The aim of this study was to determine the content of total phenols, flavonoids, anthocyanins, as well as antioxidant activity of peel and seeds of wild pomegranate (*Punica granatum* L.) collected from different localities of Montenegro.

MATERIAL AND METHODS

Plant material

For the purposes of this research, the fruits of wild pomegranate *(Punica granatum* L.) were collected in the period of October 2020 at six different localities of Montenegro: Bar (Šušanj), Budva, Cetinje (Carev Laz), Kotor (Škaljari), Kotor (Dobrota) and Podgorica (Brdo Gorica) (Table 1).

Table 1. Location of the sampling station

Locality	*Coordinates*	*Altitude*
Šušanj	42.11896N 19.10660E	71m
Budva	42.285978N 18.854130E	25m
Carev Laz	42.2220N 19.0610E	142m
Škaljari	42.416544N 18.768540E	11m
Dobrota	42.458932N 18.765057E	5m
Brdo Gorica	42.44832N 19.26640E	76m

Extraction of bioactive compounds

1g of each comminuted sample was dissolved in 10ml of 80% methanol in a test tube. Extraction was performed according to Sharayei *et al*. (2018), with certain modifications, in an ultrasonic water bath (ViMS Electric Serbia), at a constant temperature of 50°C with a frequency of 50KHz in a thirty-minute time interval. The extracts were filtered through Whatman filter paper and stored in sealed tubes in a refrigerator at 4° C until research.

Total phenolic content (TPC)

Determination of TPC was performed by the Folin-Ciocalteu method (Singleton and Rossi, 1965), according to Sarkhosh *et al*. (2009) by adding 10µl of extract, 100µl of Folin-Ciocalteu reagent and 1ml of distilled water to glass tubes. After 3 minutes, 0.5 ml of saturated sodium carbonate solution was added. The reaction mixture was vortexed and incubated in a water bath for 25-30 minutes at 50 ° C. Absorbance is measured spectrophotometrically at a wavelength of 765nm. The results are presented as milligrams of gallic acid equivalent per 100 grams of dry matter (mgGAE/100gDW).

Total flavonoids content (TFC)

Determination of TFC was performed by aluminum chloride colorimetric test according to Shams Ardecani *et al*. (2011) with certain modifications. Quantitative determination of total flavonoids were done by adding 1.2 ml of extract and 1.2 ml of 2% solution of aluminum chloride to glass tubes. The reaction mixture was incubated for 60 minutes at room temperature. Absorbance was measured spectrophotometrically at a wavelength of 420nm. An extraction solvent (80% methanol) was used as a blank. Results were presented as milligrams of quercetin equivalent per milliliter (mgQE/ml).

Total anthocyanins content (TAC)

Determination of total anthocyanins was performed by pH differential method according to Turfan *et al*. (2011). Two test tubes should be prepared for each test sample. 0.5ml of extract was added to the tubes in, and then 2ml of pH 1 buffer was added to one tube and 2mL of pH 4.5 buffer to the other. The reaction takes place for 20-30 minutes, at room temperature, in daylight. After that, the absorbance of each solution was measured at wavelengths of 500nm and 700nm. The results were presented as cyanidin-3-glucoside equivalents per gram (mgC3GE/g).

Antioxidant activity determined by FRAP method

Determination of antioxidant activity by FRAP (ferric reducing antioxidant power) method was performed according to Benzie and Strain (1996), with certain modifications (Sadeghi *et al*., 2009). The working (FRAP) reagent was incubated for 10 minutes at 37°C. Then 1ml of distilled water was added to one tube and the tubes were thermostated for 10 minutes at 37˚ C. 100µl of extract

and 3 ml of heated working reagent (FRAP) were added to the tubes in succession. The mixtures were vortexed, thermostated for 10 min at 37° C, and then their absorbance was measured at 593 nm. The results are presented in μmol/l FRAP.

Antioxidant activity determined by DPPH method

Determination of antioxidant activity by DPPH method was performed according to Akpinar-Bayizit *et al*. (2016). 1ml of extract, 1ml of methanol and 0.5ml of DPPH solution were added to the glass tubes. The reaction takes place for 30 min in the dark, after which the absorbance is measured spectrophotometrically at a wavelength of 517 nm with a blank which is solvent for extraction (80% methanol). Due to background turbidity, the absorbance of the solution was measured first before the addition of DPPH, and then after the addition and development of a thirty-minute reaction, so the values are subtracted to make the results as accurate as possible. The results were presented as milligrams of trolox equivalent per 100 grams of dry matter (mgTE/100DW).

RESULTS AND DISCUSSION

Table 2 presents the content of total phenols, flavonoids, anthocyanins, as well as the antioxidant activity of the extract of the peel and seeds (*Punica granatum* L.). with a standard deviation of three measurements.

Table 2. Content of total phenols, flavonoids, anthocyanins and antioxidant activity of wild pomegranate peel samples (*Punica granatum* L.)

	Phenols (mgGAE/100gDW)	***Flavonoids (mgQE/ml)***	***Anthocyanins (mgC3GE/g)***	***FRAP (μmol/l FRAP)***	***DPPH (mgTE/100DW)***
Šušanj	701.54 ± 5.76	317.56 ± 1.53	0.45 ± 0.04	5714.28 ± 4.14	118.5 ± 3.23
Budva	750.66 ± 1.60	291.29 ± 8.93	0.90 ± 0.10	4859.34 ± 31.75	225.16 ± 2.24
Carev Laz	695.28 ± 0.88	275.11 ± 1.35	1.2 ± 0	4600 ± 5.35	205.16 ± 4.67
Škaljari	956.98 ± 4.80	236.88 ± 4.78	0.41 ± 0.008	6505.49 ± 3.53	223.5 ± 1.12
Dobrota	808.21 ± 1.83	304.52 ± 4.53	1.65 ± 0.27	5978.02 ± 8.41	226.83 ± 9.84
Brdo Gorica	905.05 ± 3.85	295.7 ± 1.35	0.53 ± 0.01	6109.89 ± 5.90	216.83 ± 1.26

According to the presented results, in methanolic extracts of wild pomegranate peel (*Punica granatum* L.), the highest amount of total phenols was recorded in the peel extract of Škaljari (956.98 ± 4.80 mgGAE/100gDW), while the lowest amount of total phenols was recorded in Carev Laz peel extracts – (695.23 ± 0.88 mgGAE/100gDW). The obtained results differ from the results of research on the content of total phenols in ethanolic extracts of wild pomegranate peel (*Punica granatum* L.) from the area of Iran, where the obtained results of research by Sarkhosh *et al*. (2009) and Derakhshan *et al*. (2018) range from 276 to 413 mgGAE/gDW and from 50.73 to 103.83 mgGAE/100gDW.

The most dominant content of total flavonoids in methanolic peel extracts was determined in the extract of the Šušanj locality, and the lowest in the peel extract of the Škaljari locality. The results range from 236.88 ± 4.78 to 317.77 ±

1.53 mgQE/g. The results obtained in this study differ from the results obtained in the study of Hajimahmoodi *et al*. (2013) where the values of determination of total flavonoids in aqueous extracts of fruit peel were presented and range from 11.46 to 23.06 mgCE/gDW. The obtained results were significantly lower compared to the results of this research, which is the result of the weak ability of water to allow the extraction of bioactive materials, as well as the use of conventional extraction methods. The obtained results of the content of total anthocyanins are in agreement with the results obtained in the research of Zhao *et al*. (2012) where the obtained values are in similar intervals. In their study, methanol was used as the extraction solvent in the same volume ratios as the test material as in this study.

The antioxidant activity of the tested extracts of wild pomegranate fruit peels was determined by two methods: FRAP and DPPH. Both methods have shown that the antioxidant capacity is at a high level. Determined by the FRAP method, the antioxidant activity of methanolic peel extracts ranges from 4600 ± 5.35 to 6505.49 ± 3.53μmol/l FRAP. The antioxidant activity of these results is relative to the study of Sharayei *et al*. (2018), where results range from 287 to 1950 μmol/l FRAP, significantly more dominant. The extraction was performed in the same way, with the ultrasonic type of extraction, with a ratio of solvent and test material of 1:10. However, water was used as the solvent. Determined by the DPPH method, the antioxidant activity of the tested extracts ranges from 118.5 to 226.83 mgTE/100gDW and is in accordance with the research of Kam *et al*. (2013) where the results range from 46 to 283 mgTE/100gDW.

Table 3 presents the content of total phenols, flavonoids, anthocyanins, as well as the antioxidant activity of wild pomegranate seed extract (*Punica granatum* L.). with a standard deviation of three measurements.

Table 3. Content of total phenols, flavonoids, anthocyanins and antioxidant activity of wild pomegranate seed samples (*Punica granatum* L.)

	Phenols (mgGAE/100gDW)	***Flavonoids (mgQE/ml)***	***Anthocyanins (mgC3GE/g)***	***FRAP (μmol/l FRAP)***	***DPPH (mgTE/100DW)***
Šušanj	120.12 ± 7.60	23.88 ± 1.18	4.09 ± 0.06	473.92 ± 0.02	106.83 ± 2.86
Budva	110.64 ± 1.75	31.18 ± 0.98	3.54 ± 0.20	326.53 ± 0.40	23.5 ± 4.10
Carev Laz	89.07 ± 2.52	12.11 ± 0.15	2.73 ± 0.39	260.77 ± 3.45	45.16 ± 0.85
Škaljari	189.77 ± 7.81	39.61 ± 1.58	4.34 ± 0.56	476.19 ± 2.86	161.83 ± 4.06
Dobrota	120.12 ± 5.30	15.05 ± 0.43	3.47 ± 0.38	349.2 ± 8.61	105.16 ± 1.02
Brdo Gorica	142.05 ± 3.17	15.2 ± 0.30	3.99 ± 0.19	453.51 ± 1.84	101.83 ± 3.40

As in determining the content of total phenols in peel extracts, the largest and smallest amounts are recorded in samples from the same localities, which leads to the conclusion that with increasing height, the amount of total phenols decreases. The obtained results are in accordance with the results of the research of Gözlekçi *et al*. (2011) where the obtained values of the research range from 125.3 to 177.4 mgGAE/100gDW, while the amount of total phenols compared to

the research of Peng *et al*. (2019) where the obtained values range from 62 to 68 mgGAE/100gDW, more dominantly expressed.

The amount of total flavonoids in seed extracts ranges from 12.11 ± 1.58 to 39.61 ± 0.15 mgQE/g, where the highest amount of flavonoids was recorded in the extract of the locality Škaljari, and the lowest in the extract of the locality Carev Laz, as well as the content of total phenols. As in the case of total phenols and flavonoids, the highest amount of total anthocyanins was recorded in the extract of the locality Škaljari, and the lowest in the extract of the locality Carev Laz and the results are in the range from 2.73 ± 0.56 to 4.34 ± 0.39 mgC3GE/g. In relation to the research of Parseh and Shahablavasani (2019) where the content of total anthocyanins of wild pomegranate seed extract is 28 mgC3GE / g, the obtained results of this research deviate and the content of total anthocyanins is lower.

The antioxidant capacity of the seed extract determined by the DPPH method shows values ranging from 23.5 ± 4.10 to 161.83 ± 4.06 mgTE/100gDW. The strongest antioxidant capacity was recorded in the extract of the locality Škaljari, and the lowest in the extract of the locality Budva. The antioxidant capacity of the Carev Laz extract is 45.16 mgTE/100gDW, so it is again concluded that the antioxidant capacity, as well as the amount of total phenols, decreases with increasing altitude. Determined by the antioxidant method FRAP, the strongest antioxidant capacity was recorded in the seed extract of Škaljari, and the lowest in the extract of Carev Laz and Budva. The results range from 260.77 to 476.19 μmol/l FRAP and deviate from the results presented in the study by Sadeghi *et al*. (2009). Sadeghi *et al*. examined the antioxidant capacity of the seeds of six different varieties of wild pomegranate from Iran. Results range from 2.76 to 3.45 μmol/l FRAP. However, in this study the ratio of solvent to test sample was 1: 100 and water and ethanol, weaker solvents compared to methanol, were used as solvent.

Comparing the presented results with the results of Radunić *et al.* (2017) paper which talks about the content of phenols, flavonoids and anthocyanins of wild pomegranate (*Punica granatum* L.) juice from the territory of the Mediterranean part of Croatia, which states that the content of total phenols is 679.6 mg/100gDW, total flavonoids 393.6 mg/100g DW and total anthocyanins 81.06 mg/100gDW we conclude that the peel is a part of the fruit that is really rich in secondary metabolites, specifically phenols and that according to the above work it can match the juice.

Table 4 presents a correlation analysis of the content of total phenols, flavonoids, anthocyanins, as well as the antioxidant activity of samples of wild pomegranate fruit peel (*Punica granatum* L.).

There was a negative correlation between the content of total phenols and flavonoids in the fruit peel of wild pomegranate ($r = -0.57$), as well as a strong positive correlation between the amount of total phenols and antioxidant activity determined by FRAP method ($r = 0.81$).

Table 5 presents a correlation analysis of the content of total phenols, flavonoids, anthocyanins, as well as the antioxidant activity capacity of samples of wild pomegranate seeds (*Punica granatum* L.).

Table 4. Pearson's correlation coefficient ($p < 0.05$) for total phenols, flavonoids, anthocyanins and antioxidant capacity of wild pomegranate peel samples

	Phenols	***Flavonoids***	***Anthocyanins***	***FRAP***	***DPPH***
Phenols	1				
Flavonoids	-0.57	1			
Anthocyanins	-0.35	0.24	1		
FRAP	**0.81***	-0.21	-0.37	1	
DPPH	0.52	-0.5	0.39	0.04	1

*Bold values represent strong correlations ($p < 0.05$)

Table 5. Pearson's correlation coefficient ($p < 0.05$) for total phenols, flavonoids, anthocyanins and antioxidant capacity of wild pomegranate seed samples

	Phenols	***Flavonoids***	***Anthocyanins***	***FRAP***	***DPPH***
Phenols	1				
Flavonoids	0.36	1			
Anthocyanins	0.83	0.63	1		
FRAP	0.77	0.45	**0.96***	1	
DPPH	**0.84***	0.35	0.74	0.79	1

*Bold values represent strong correlations ($p < 0.05$) between the content of total phenols and flavonoids ($r = 0.67$). A high correlation was observed between antioxidant activity (FRAP method) and anthocyanin content ($r = 0.96$) as well as between antioxidant activity (DPPH method) and total phenol content ($r = 0.84$).

CONCLUSIONS

Comparing the results of total phenols, flavonoids, anthocyanins and antioxidant activity of samples of fruit peel and seed of wild pomegranate (*Punica granatum* L.) collected from six different localities of Montenegro, it is noted that a larger amount of these bioactive substances contains samples collected at lower altitudes, in fact those collected from coastal localities from the territory of Škaljari and Šušanj predominantly, and the smallest amount collected from higher altitudes, from the locality Carev Laz. According to the results obtained in this study, along with numerous other studies with similar research point, it is concluded that wild pomegranate biowaste is a valuable source of bioactive substances and that further research should focus on testing its effects in vivo.

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