Morphological and chemical assessment of juices and antimicrobial activity of peels from two varieties of pomegranates grown in the region of North Macedonia

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Abstract
The chemical composition of pomegranate juice in terms of organic acids, sugars, vitamin C, total phenolics, catechins and anthocynanins from two Macedonian varieties "Karamustafa" and "Hicaz" was first objective of this study. Furthermore, morphological and physical characteristics of pomegranate fruits from autochthonous "Karamustafa" and "Hicaz" variety were compared. The level of the anthocyanins was significantly higher to "Hicaz" pomegranate variety and it was in good correlation to the results of hue and color of the juice. The most statistically significant difference was obtained for ascorbic acid which was four time higher for freshly squeezed juice from "Hicaz" pomegranate variety. In addition, the variation of chemical composition of elements of fruit juices from both variety could be attributed to the difference in cultivar.

Finally, the last objective of our study was antibacterial activity of peel extracts from both varieties. The strong antibacterial activity against Staphylococcus aureus (ATCC 25923) was significant without statistical differences between both varieties.

Keywords: Morphological parameters, chemical composition of elements, antifungal and antibacterial activity.

Introduction

Pomegranate (*Punica granatum* L.) is one of the major sources of polyphenolic phytochemicals such as anthocyanins and catechins [1]. An excellent review of Kalaycıoğlu and Erim[2] quantitatively established the antioxidant activity, total phenolic content, anthocyanins, organic acids, sugars and other important ingredients in pomegranate juices obtained from cultivars from different regions.

In the work of Mena et al[3] 15 pomegranate cultivars were studied in order to demonstrate the wide diversity among the quality of Spanish pomegranates. According to their findings, "Wonderful" juices displayed large antioxidant activity and a polyphenol content with very high acidity. In contrast, 'Mollar de Elche' showed fewer anthocyanins although it had very superior organoleptic properties.

In addition to a high content in ellagitannins, 'Valenciana' juices had exclusive colour parameters[3]. Regarding total phenol content, rutin was predominant flavonoid from the pomegranates peel. The analysis of HPLC/ESI/MS in the work of Sepúlveda and his research group[4] allowed to identify punicalagin, punicalin and ellagic acid from the pomegranate husk by size exclusion chromatography.

The late-pomegranate fruits were rich in phytochemicals and could be of great interest to the juice industry[6,7]. Regarding total phenol content, rutin was predominant flavonoid from the pomegranates peel[8]. In the work of Atukuri et al,[9] the pomegranates treated with fludioxonyl at 600mg/L had the best quality regarding decay incidence, weight loss, total phenolics and sensory attributes. The procedure of isolation and NMR elucidation of two new ellagitannin oligomers, pomegraniins A (tetramer) and B (pentamer), and a new glucose ester of neolignan, pomegralignan, together with six known ellagitannins from the arils and pericarps of *Punica granatum* L. (pomegranate) was studied by Ito et al[10].

Urolithins were separated from the intestinal metabolites of pomegranate ellagitannins by high-speed counter current chromatography. The interest of these compounds increased in the last years due to their possibility to reduce the oxidative stress status in colon cancer by decreasing the intracellular ROS and malondialdehyde levels and increasing SOD activity in H_2O_2 treated Caco-2 cells[11].

The results from study of Tezcan et al[12] showed that commercial pomegranate juices had markedly high total phenolic contents and antioxidant capacity. In the six commercial pomegranate juices, in comparison to fruit juices reported in the literature, much higher total phenolic content and antioxidant capacities were observed with increased health benefits for the consumers[13]. The antioxidant activity of pomegranate aril juice attributed to a great extent to total phenols and anthocyanins by cyanidin-3,5-diglucoside was the major antocyanin in pomegranates[14,15].

The object of our study was study of freshly squeezed juice as well as extracts from pomegranate peel. The quality of

freshly squeezed juices from autochthons "Karamustafa" and "Hicaz" pomegranate varieties was examined by determination of morphological, chemical and composition of elements.

Finally, the antibacterial activity of extracts from the peel of both pomegranate varieties was studied against gram-positive bacterial strain *Staphylococcus aureus* (ATCC 25923) and gram-negative bacterial strain *Escherichia coli* (ATCC 25922) and for antifungal activity *Candida albicans* (ATCC 1023) was used. The quality of freshly squeezed juices from autochthons "Karamustafa" and "Hicaz" pomegranate varieties was examined by determination of morphological, chemical and composition of elements.

Material and Methods

Sample preparation: The sampled fruits were selected randomly in order to separate three replicates for analysis, using 10 kg per replicate and cultivar. Pomegranates were weighed, cut in halves, and arils were hand separated from the pith avoiding contamination by components in membranous walls (septum). Juices of each cultivar were obtained by pressure of arils and were weighed to determine the juice yield. Samples of freshly prepared juice were stored frozen (−20ºC) until analyzed[16].

Determination of titratable acidity, total soluble solids, pH: For determination of titratable acidity (TA), 2 ml of fresh juice was diluted with 70 mL of distilled water and titrating with 0.1 M NaOH to an end point of pH 8.2 using a Metrohm 862 compact titrosampler (Herisua, Switzerland). The results were expressed as percentage of citric acid (% CA). Total soluble solid (TSS, °Brix) was measured using a digital refractometer (Atago, Tokyo, Japan) calibrated with distilled water. The pH values were determined at room temperature using a calibrated pH meter (Crison, Model 00924, Barcelona, Spain).

Determination of organic acids, sugars and ascorbic acid: For determination of ascorbic acid, 20 mL of test preparation of fresh juices from both varieties was transferred to a 250-ml conical flask and dissolved in a mixture of 50 ml water and 15 mL H_2SO_4 (1:5). After addition of 1 mL starch, the samples were titrated with 0.1 mol/L (I_2) of solution to a blue endpoint.

The percentage of ascorbic acid was measured by following calculation:

$$mg\ Ascorbic\ ac./100ml = \frac{Vs\ x\ N\ x\ F\ x\ 100}{W}$$

where VS is Titrant volume consumed by sample (ml), N is actual normality of the titrant (mEq/ml), F is equivalency factor, 8.805 mg/mEq and W is volume of juice (mL).

Chromatograph Agilent technologies 1200 series, with Jasco AS-950 sampler, an auto injector (20 µl injection volume) and refractive index detector were used for analyses of sugars. Separation of the sugars was performed by using a LiChrospher 100 NH_2 (5µm x 250 x 4) column. The eluents, acetonitrile and water were mixed in ratio (78:22). The mobile phase flow rate was 1.0 mL/min and the temperature was 40°C.

The percentages of sugars were measured by following calculation:

$$sugar\% = \frac{At}{As}\ x \frac{Cs}{Ct} x\ potency\ x\ 100$$

where At is the area of test sample, As is the area of standard, Cs is the concentration of standard in mg/mL and Ct is the concentration of test in mg/mL.

For determination of organic acids, Chromatograph Agilent technologies 1200 series, with Jasco AS-950 sampler, an auto injector (10 µl injection volume) and refractive index detector were used. Separation of organic acid was performed on AMINEX HOX-87 (H 300 x 7.8) column. The eluent was prepared by mixing 75µl H_2SO_4 in 250 mL H_2O. The mobile phase flow rate was 0.6 mL/min and the temperature was 55°C.

The percentages of organic acids were measured by following calculation:

$$mg\ organic\ ac./100ml = \frac{At}{As}\ x \frac{Cs}{Ct} x100$$

where At is the area of test sample, As is the area of standard, Cs is the concentration of standard in mg/mL and Ct is the concentration of test in mg/mL.

Determination of total phenolic compounds, total catechins and total antocynanins: The total phenolic content of pomegranate juices was determined with Folin–Ciocalteau reagent. Determination of total phenolics was performed by the colorimetric method of Singleton and Rossi[17]. For determination of total catechins, the modified method of Atamossa and Gholap[18] was used. In brief, the pomegranate juices from both varieties were dissolved in water (1:5) and measurements of total catechins was performed using UV-VIS spectrometer in the spectral range of 200 to 500 nm. The measurements were performed in triplicate.

Multi-element characterization: Inductively coupled plasma with mass spectrometry (ICP-MS, model 7500cx Agilent Technologies, USA) with a glass concentric nebulizer was used for analyses of the elements content. In this study, five step set or combination of power, pressure, and time conditions for microwave-assisted digestion were applied (Table 1). Microwave-assisted digestion conditions involved the digestion of 0.5 g of the sample with 5 mL HNO_3 and 2 mL of H_2O_2 in the microwave digestion system CEM model MARS 5 (CEM Corporation, Matthews, NC, USA).

After digestion, the vessels were allowed to cool until the pressure of the vessel was reduced to below 50 psi and temperature was below 40 °C. The caps of each vessel were then carefully removed and the contents were filtered using 2 µm filter paper diluted to 25 mL in a volumetric flask using deionized water, and stored in polyethylene vial prior to the final determination of the elements' concentration. Total of 35 elements were analyzed using the ICP-MS technique (Ag, Al, As, B, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, Ge, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, Sb, Se, Sn, Sr, Ti, V and Zn).

The ICP-MS system was optimized under typical tuning conditions for high and variable sample matrices.

Antimicrobial assays: The samples of peel extracts from two varieties of pomegranate were investigated for their "*in vitro*" antibacterial and antifungal properties using a disk-diffusion method in Petri dishes. The pomegranate peel extracts were tested for antibacterial activity against one gram-positive bacterial strain *Staphylococcus aureus* (ATCC 25923), and against one gram-negative bacterial strain *Escherichia coli* (ATCC 25922) and for antifungal activity using *Candida albicans* (ATCC 1023).

In brief, each suspension of microorganisms was suspended in Mueller Hinton (MH) broth. Furthermore, the suspension of microorganisms is diluted by using the McFarland scale. An inoculum equivalent to the nº 1 of the McFarland scale was prepared and diluted approximately to 10E6 colony forming unit (cfu)/mL. They were "flood-inoculated" onto the surface of MH agar and MH Dextroxe Agar (MDA) and then dried. Six-millimeter diameter wells were cut from the agar using a sterile cork-borer, and 60 µL of each sample of juices was delivered into the wells. The plates were incubated at 37 °C and the diameters of the growth inhibition zones were measured after 24 h. Gentamicin (20 µg/well), nalidixic acid (30 µg/well), ciprofloxacine (5 µg/well) and erytromicine (15 µg/well) were used as positive control. The controls were performed with only sterile broth and with only overnight culture and 10 µL of 70% ethanol.

The antibacterial and antifungal activity tests of pomegranate juices from two varities are shown in table 7. The antibacterial activity ranked from no activity (-: inhibition diameter < 10 mm), low (+: inhibition diameter between 10 and 15 mm), moderate (++: inhibition diameter between 15 and 20 mm) and high activity (+++: diameter inhibition ≥ 20 mm). All tests were performed in triplicate and clear halos greater than 10 mm were considered as positive results.

Statistical analyses: The level of significance in differences between anthocyanin content and total phenolic content was determined by 5% by one-way ANOVA using Tukey's test. The results from statistical analyses were classified using letters (different letters means significant differences among results). The letters are a,b,c,d,e and f according to the decrease of the result values. SPSS v.16.0 software, IBM corporation, USA was used for the applied statistical treatment.

The data for the elements contents were statistically processed in order to test the variability of the elemental composition between the two examined samples. For that issue t-test (using dependent samples testing origin with significance measuring at $p < 0.050$) was applied (StataSoft Version 11, StataCorp., USA).

Results and Discussion

Results from morphological and physical characterization of pomegranates from "Hicaz" and "Karamustafa" varieties are presented in table 2.

The pomegranates from "Hicaz" variety had higher average fruit weight. Percentages of grain and peel were higher for "Karamustafa" variety as well as weight of 100 arils and percentage of skin. The percentage of carpels was more than double for "Hicaz" variety as well as equatorial and calix diameter and fruit height with and without calix. Pomegranates from "Karamusfata" variety had higher percentage of juice and the differences in color of the juice were in correlation to the results from color intensity and hue.

The average fruit weight of pomegranates from "Hicaz" variety was the same as "Chioukhi" variety of pomegranates from Morocco. On the other hand, calix diameter for "Hicaz" variety of pomegranates was similar to Moroccanish "Ounk Hmam" variety and calix for "Karamusfata" variety was more similar to some Spanish varieties of pomegranates[19]. As we can see, there is significant difference between all measured parameters with exception of pH of the juices and percentage of fructose (Table 3).

Table 1
Microwave digestion program for digestion of pomegranate juices

Step	Initial *T* (°C)	Final *T* (°C)	Power (W)	Time (min.)
1	25	150	800	15
2	150	150	800	10
3	150	180	1600	5
4	180	200	1600	10

Table 2
Morphological and physical characteristics of pomegranate fruits from "Hicaz" and "Karamustafa" varieties

Variety	Average fruit weight (g)	Grain (%)	Peel (%)	Weight of 100 seeds (g)	Weight of 100 arils (g)	Skin (%)	Carpels (%)
Hicaz	359.6±25.7[a]	50.1±1.2[b]	26.9±2.2[b]	3.6±0.1[a]	28.6±2.7[b]	1.2±0.1[b]	13.8±2.0[a]
Karamustafa	260.1±29.1[b]	56.6±9.9[a]	29.7±7.4[a]	2.6±0.5[b]	31.8±3.9[a]	1.4±0.1[a]	6.2±0.8[b]
	Equatorial diameter (mm)	**Calyx diameter (mm)**	**Fruit height without calix (mm)**	**Total fruit height (mm)**	**Juice (%)**	**Color of fruit**	**Color of juice**
Hicaz	89±4[a]	23±2[a]	79±5[a]	91±3[a]	45.8±11.3[b]	Reddish	Dark pink
Karamustafa	81±6[b]	12±2[b]	76±3[b]	83±4[b]	49.0±10.4[a]	Reddish yellow	Pink

Table 3
Chemical parameters of pomegranate juice

Samples of fresh juice	Brix	pH	Total acids g/L	Malic acid g/L	Citric acid g/L	Total phenolics	Total catechines	Total antocynains
Hicaz	16.0±1.2[a]	3.04±0.2[a]	29.6±3.4[a]	0.19±0.11[b]	0.29±0.08[b]	2403±228[a]	26.7±9.8[b]	373.1±39.6[a]
Karamustafa	17.0±0.9[a]	3.05±0.3[a]	5.4±1.9[b]	0.83±0.52[a]	0.75±0.16[a]	2386±321[a]	29.1±5.0[a]	117.3±28.4[b]
	IC	**Hue**	**A_{420}**	**A_{520}**	**A_{620}**	**Vitamin C mg/100 mL**	**Glucose mg/100mL**	**Fructose mg/100mL**
Hicaz	2.98±0.21[a]	0.23±0.07[a]	16.9±0.12[b]	73.1±7.13[a]	9.9±0.9[b]	17.5±1.6[a]	7.6±1.1[a]	16.6±2.1[a]
Karamustafa	1.03±0.34[b]	0.30±0.05[a]	20.3±0.22[a]	66.5±9.18[a]	13.1±1.8[a]	4.1±0.9[b]	6.3±1.4[a]	16.9±2.1[a]
	Fructose/Glucose F/G ratio							
Hicaz	2.18[a]							
Karamustafa	2.68[a]							

However, the total sugar in pomegranate juices from both varieties was significantly higher than data of USDA database (12.65%)[20]. The level of malic and citric acid was significantly lower in comparison to commercial pomegranate juices from Turkey.

However, the amounts of total phenolic compounds in both variety were very similar to the same samples[13]. The level of monomeric anthocyanins was three times higher for "Hicaz" in comparison to "Karamustafa" variety. Furthermore, the amount of monomeric anthocynains from "Hicaz" variety was very similar to the results published for pomegranate juices from the working group of Jaiswal et al[1]. The intensity of the color was higher for "Hicaz" variety which was expected due to the higher level of total phenolic compounds and monomeric anthocyanins (Table 2). Moreover, the higher intensity of red color can be linked to the dominance of cyanidin-3-glucoside as the major anthocyanin in pomegranate juice[14,15,21]. Opposite, yellow and blue color was more intense for "Karamustafa" pomegranate variety. The most significant difference was detected for ascorbic acid with amount of 17.5 mg/100 mL for "Hicaz" variety in comparison to 4.1 mg/100 mL for the pomegranate juice form "Karamustafa" variety.

On the other hand, citric and malic acids were the predominant organic acid in the examined pomegranate juice from "Karamustafa" variety. The acids attributed to the formation of ester upon the reaction of predominant citric acid with some juice compounds. They affect the taste of the juice and generate sourish taste of "Karamustafa" variety in comparison to sweet taste of juice from "Hicaz" variety[22].

The fructose/glucose ratio in both varieties was higher than 2 in favor of fructose. However, the results published by different authors indicated almost equivalent amount of fructose and glucose in pomegranate juice. Our results were similar to those published in the review of Kalaycıoğlu and Erim,[2] for juices from 76 pomegranate accessions selected from the Eastern Mediterranean Region (Hatay province) of Turkey.

The results for amounts of total phenolic compounds obtained from Macedonian varieties are similar to some varieties published by Mena et al[3]. According to their findings, total phenolic content in WSN sample had similar value as Macedonian "Hicaz" variety. They stated that Folin–Ciocalteu values were not the sum of polyphenols which can be detected by HPLC (EA derivatives, punicalagins and anthocyanins) as other compounds, such as flavanols, flavonols, phenolic acids, proanthocyanidins and hydrolysable tannins that are different to punicalagins and also contribute to the phenolic profile[3].

The pomegranate juice from Macedonian "Hicaz" variety had higher value for total phenolic components than Valencia variety but significantly lower value than Akko, Hershkovitz and Wonderful varieties[1]. The amount of total phenolic component in pomegranate juice of two Macedonian varieties is similar to total phenolic content in Spanish pomegranate juice which was in range from 170-270 mg/mL gallic acid equivalent[6].

Evidence literature review showed that major volatile compounds and flavour descriptors in pomegranate such as the terpenes (limonene, α-terpineol, β-pinene and β-caryophyllene), the alcohol group (trans-3-hexen-1-ol, 1-hexanol, 3-hexanol-1-ol and hexanol) and ketones (2-octanone and 2-nonanone) had the most significant role to the overall flavor of pomegranate juice Additionally, changes in VOCs reported in literature further emphasized the need for a shift from the traditional assessment of chemical attributes (total soluble solids (TSS), titratable acidity (TA) and pH) and physical attributes (colour, firmness, juiciness and presence/absence of decay), to a holistic assessment which include the change in VOC as a component of fresh juice[23].

The elemental analysis of fresh juices from two varieties (presented in Table 4) clearly showed that potassium was predominant macro-element in fresh fruit juice from "Hicaz" variety.

Magnesium and phosphorus were the major macro-elements presented in fruit juice from "Karamustafa" variety. Regarding the abundance of micro-elements, the quantity zinc was almost double in "Karamusfata" variety.

Moreover, strontium and barium as micro-elements were present in higher amount in the fresh juice from "Hicaz" variety.

Table 4
Multi-element content characterization of pomegranate peel extracts

Element	Unit	Fruit juice from "Hicaz" variety	*RSD* (%)	Fruit juice from "Karamustafa" variety	*RSD* (%)
Li	µg/L	0.45	2.55	0.62	3.21
B	µg/L	17.5	1.08	9.53	1.56
Na	mg/L	8.26	1.44	11.2	1.12
Mg	mg/L	13.2	0.78	28.3	0.95
Al	mg/L	0.95	0.96	0.16	1.7
P	mg/L	12.3	3.45	24.6	2.26
K	mg/L	22.3	3.66	18.9	1.44
Ca	mg/L	4.56	2.78	9.33	1.23
Ti	µg/L	22.5	2.22	16.5	0.47
Cr	µg/L	18.2	1.19	15.6	4.2
Mn	µg/L	21.6	0.55	9.80	1.61
Fe	mg/L	0.94	1.49	0.52	2.14
Co	µg/L	3.26	3.26	5.36	1.06
Cu	µg/L	13.5	2.09	56.3	2.55
Zn	µg/L	124	1.34	253	3.08
Ge	µg/L	2.44	0.85	1.84	1.02
Se	µg/L	0.25	1.25	0.086	1.47
Rb	µg/L	55.3	1.46	74.1	0.88
Sr	µg/L	89.3	1.92	55.6	0.65
Mo	µg/L	7.19	0.86	3.69	0.78
Cd	µg/L	0.17	2.73	0.23	1.55
Cs	µg/L	15.9	0.69	10.1	1.28
Ba	µg/L	93.6	1.1	65.8	0.96
Tl	µg/L	0.84	1.36	0.55	2.36
Pb	µg/L	0.77	0.75	0.33	3.55
Bi	µg/L	1.23	3.44	0.58	1.28
$\sum_{\text{total element}}$	mg/L	63.0		93.5	
T-Test*	N	Diff.	t	df	p
	27	-4.52	-0.83	26	0.41

*T-test for Dependent Samples, significant at $p < .0500$

Table 5
Antimicrobial activity of pomegranate peel extracts

Samples	Dosage	*Staphylococcus aureus* (ATCC 25923)	*Escherichia coli* (ATCC 25922)	*Candida albicans* (ATCC 1023)
Karamustafa	100 μL	39 mm	10 mm	11 mm
Hicaz	100 μL	41 mm	9 mm	10 mm
Positive control				
Gentamycin	20 μg	14 mm	25 mm	6 mm
Nalidixic acid	30 μg	14 mm	22 mm	6 mm
Ciprofloxacin	5 μg	25 mm	35 mm	6 mm
Erythromycin	15 μg	30 mm	8 mm	6 mm

The conducted t-test has showed that there is no-significant variation (p=0.41) between the analyzed pomegranate peel extracts comparing as dependent samples for the total content of the analyzed elements.

The results for antimicrobial activity are presented in table 5. The highest antimicrobial activity was detected for *Staphylococcus aureus* (ATCC 25923), weak antibacterial activity against *Escherichia coli* (ATCC 25922) and weak antifungal activity against *Candida albicans* (ATCC 1023). The extraction of the peel from pomegranates by mixture of methanol and water led us to conclusion that polar compounds such as ellagic acid and punicalagin a and b might be responsible to antimicrobial activity against *Staphylococcus aureus* (ATCC 25923).

The explanation attributed to the phenolic toxicity is caused by perforation and/or reduction in membrane fluidity with final cytoplasmatic membrane damage[2]. Opposite to the results for many researchers, we did not find significant antifungal activity of pomegranate peel extracts from both variety.

Conclusion

Based on the explanation above, we can summarize that pomegranate juice from "Hicaz" variety is richer source with polyphenolic compounds in particular monomeric anthocyanins and total phenolic compounds. Regarding potential antibacterial activity against *Staphylococcus aureus* (ATCC 25923), further investigation of peels extracts from both varieties against other pathogens is highly recommended.

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