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Influence of *in vitro* gastrointestinal digestion on phytochemicals in pomegranate juice

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Abstract

Nowadays, healthy food is in vogue. The consumption of fresh fruits and vegetables has always been advised. Their health benefits are estimated based on the chemical composition without taking into account the possible transformations during digestion. Undoubtedly, those with the highest antioxidant content are highly favored. However, whether they are just as effective after passing through gastrointestinal tract is a question that science should answer experimentally. Therefore, the influence of *in vitro* gastrointestinal digestion on phytochemicals in pomegranate juice. The change in total phenolic content, total flavonoid content and antioxidant activity was monitored. A drastic loss of phenolic compounds was measured after the intestinal phase of the assay resulting in only 25% and 15.8% of the initial total phenolic content and total flavonoid content, respectively. The antioxidant activity was also affected. The results indicated an after digestion antioxidant activity in the range 19-29% by the measured activity of pomegranate juice. HPLC analysis of phenolic compounds confirmed the presence of gallic acid and DL-catechin at concentrations 96.3 μg/mL and 340 μg/mL, resp. After digestion a loss of 93% and 99% was calculated.

Keywords: antioxidant activity; bioaccessability; *in vitro* digestion; phytonutrients; pomegranate juice.

Practical Application: The paper provides some information about the bioaccessibility of fruit juice.

1 Introduction

Freshly prepared fruit juices have always been considered as excellent sources of vitamins, minerals, antioxidants and other beneficial nutrients. Their daily consumption helps the body retain and balance hydration levels and exert a cleansing effect on the blood and digestive tract. Red fruit juices are rich in phytochemicals, which is associated with higher antioxidant activity (Vilela & Cosme, 2016). Acute intake of phenolic-rich juices also improves body antioxidant status and has a protective effect against many chronic diseases and some forms of cancer (García-Alonso et al., 2006; Boivin et al., 2007; Vilela & Cosme, 2016).

Pomegranate (*Punica granatum* L.) belongs to the family *Lythraceae*. In recent years, pomegranate fruit has gained popularity due to its functional properties. Fresh juice contains 85.4% water and 15.6% dry substance. The edible part of the fruit (arils) is rich in sugars, organic acids, vitamins, minerals and antioxidants (Tehranifar et al., 2010). Phenolic compounds, together with flavonoids, anthocyanins, and tannins, are the main group of antioxidant phytochemicals that are important due to their biological and free radical scavenging activities (Elfalleh et al., 2011). Pomegranate is reported to have the highest antioxidant activity in the top seven fruits (Fu et al., 2011). However, the chemical composition among fruits differs significantly depending on the cultivar, growing region, maturity, cultivation practice, climate, and storage circumstances (Fawole & Opara 2013; Okatan et al., 2015).

Most of the food research studies are focused on food evaluation as health promoter. Based on the chemical composition and biological activities many foods are recognized as beneficial for consumption. However, not many studies are focused on the transformations that occur during food digestion. It is a complex process in which many factors are involved. Ideally, a large population *in vivo* study is needed but the higher cost and ethical considerations are serious obstacles (Sengul et al., 2014). That is why *in vitro* simulations are more and more used in order to examine interactions between different products in a simple, reproducible and standardized test procedure. Although it is not easy to reproduce the human digestion tract, efforts are already being made in this direction (Hur et al., 2011; Lucas-González et al., 2018; Kopf-Bolanz et al., 2012; Minekus et al., 2014). Minekus et al. (2014) proposing a standardized static *in vitro* digestion method suitable for food. With such an *in vitro* digestion model, the bioaccessibility and bioavailability of compounds from the food matrix during transit in the gastrointestinal tract can be investigated.

The aim of the current study is to evaluate the bioaccessability of phenolic compounds found in freshly prepared pomegranate juice by using *in vitro* gastrointestinal digestion model. No extraction method of phytochemicals was applied in order to simulate real conditions of juice consumption. The loss of phytochemicals and antioxidant activity was also calculated. HPLC-DAD analysis of individual polyphenol was also performed.

2 Materials and methods

2.1 Chemicals and materials

The pomegranate juice that was used in this study was purchased from local shop where it was freshly cold pressed. The juice was then immediately subjected to analysis. Simulated

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gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described by Minekus et al. (2014).

All reagents used in this study were of analytical grade and purchased from Merck Chemicals and Sigma-Aldrich (Germany).

2.2 In vitro gastrointestinal digestion procedure

The assay was performed according to the procedures described by Minekus et al. (2014) and Liang et al. (2016) with minor modifications. Only gastric and intestinal phase were included.

2.3 Gastric phase

5 mL of juice sample was mixed with 3.62 mL porcine pepsin stock solution (Pepsin from porcine gastric mucosa, P7000, Sigma); 5520 U/mL made up in SGF electrolyte stock solution.), 2.5 µL of 0.3 M $CaCl_2$ and 132 µL phospholipids (0.17 mM in the final digestion mixture). The pH of the mixture was corrected with 1 M HCl to pH 3.0 and the volume of the mixture was made up to 10 mL with distilled water. The mixture was then incubated at 37 °C with constant shaking in a shaking water bath for 2 h. The pH was regularly checked and re-adjusted with 1 M HCl when needed.

2.4 Intestinal phase

The 10 mL gastric chyme is mixed with 8 mL of a pancreatin solution 1.72 U/mL made up in SIF electrolyte stock solution based on trypsin activity (pancreatin from porcine pancreas, Sigma, P1750), 1.9 mL fresh bile extract (160 mM fresh bile salts in final mixture), 20 µL of 0.3 M $CaCl_2$, 1M NaOH to reach pH 7.0 and d. water to 20 mL total volume. The mixture was then incubated at 37 °C in a shaking water bath for 2 h. The pH was regularly checked and re-adjusted with 1 M NaOH during the process if needed.

For blank sample water was used instead of juice. The obtained values were subtracted from the sample values for each analysis. The digestion liquid was then centrifuged and stored at -20°C till further analysis but not exceeding 7 days.

2.5 Determination of Total Polyhenolic Content (TPC)

The TPC was analyzed using the method of Kujala et al. (2000) with some modifications. Each extract (0.1 mL) was mixed with 0.5 mL Folin-Ciocalteu reagent and 0.4 mL 7.5% Na_2CO_3. The mixture was vortexed and left for 5 min at 50 °C. After incubation, the absorbance was measured at 765 nm. The TPC was expressed as mg gallic acid equivalents (GAE) per mL juice.

2.6 Determination of Total Flavonoid Content (TFC)

The total flavonoid content was evaluated according to the method described by Kivrak et al. (2009). An aliquot of 0.5 mL of the sample was added to 0.1 mL of 10% $Al(NO_3)_3$, 0.1 mL of 1 M CH_3COOK and 3.8 mL of ethanol. After incubation at room temperature for 40 min, the absorbance was measured at 415 nm. Quercetin was used as a standard and the results were expressed as mg QE/mL.

2.7 Determination of antioxidant activity

DPPH· radical scavenging assay

The ability of the extracts to donate an electron and scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams et al. (1995) as described by Mihaylova et al. (2015). Freshly prepared 4×10^{-4} M solution of DPPH was mixed with the samples in a ratio of 2:0.5 (v/v). The light absorption was measured at 517 nm after 30 min incubation. The DPPH radical scavenging activity was presented as a function of the concentration of Trolox - Trolox equivalent antioxidant capacity (TEAC) and was defined as the concentration Trolox having equivalent antioxidant activity expressed as the µM per mL (µM TE/mL).

$ABTS^{\bullet+}$ radical scavenging assay

The radical scavenging activity of the extracts against 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was estimated according to Re et al. (1999). Briefly, ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution (7 mM) 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 $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ solution to 0.01 mL of samples, the absorbance reading was taken at 30 °C after 6 min. The results were expressed as TEAC value (µM TE/mL).

Ferric-reducing Antioxidant Power (FRAP) assay

The FRAP assay was carried out according to the procedure of Benzie & Strain (1999) with slight modification. The FRAP reagent was prepared fresh daily and was warmed to 37 °C prior to use. One-hundred and fifty microliters of plant extracts were allowed to react with 2850 µL of the FRAP reagent for 4 min at 37 °C, and the absorbance was recorded at 593 nm. The absorbance was recorded at 593 nm and the results were expressed as µM TE/mL.

Cupric Ion Reducing Antioxidant Capacity (CUPRAC) assay

The CUPRAC assay was carried out according to the procedure of Apak et al. (2004). One mL of $CuCl_2$ solution (1.0×10^{-2} M) was mixed with 1 mL of neocuproine methanolic solution (7.5×10^{-3} M), 1 mL NH_4Ac buffer solution (pH 7.0), and 0.1 mL of herbal extract (sample) followed by addition of 1 mL water (total volume = 4.1 mL) and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min. Trolox was used as a standard and the results were expressed as µM TE/mL.

HPLC analysis

The HPLC analysis was performed on ELITE La Chrome (Hitachi), equipped with a gradient solvent pump, coupled with Diode Array Detector. The data collection and analysis were carried out using the software ELITE LaChrome (Hitachi).

The detection of compounds was performed on Discovery® SHC18column (25 x 4.6 mm, 5 µm, Supelco), at 278, 306 and 370 nm. The chromatographic separation was performed as described by Özkan & Göktürk (2006), with slight modifications. The temperature of the column was set to 30°C and the flow rate to 1.0 mL min^{-1}, 20 µL of injection. The following detection wavelengths were used: at 278 nm – gallic acid, DL-catechin, syringic acid, cinnamic acid, hesperidin; at 306 nm - chlorogenic acid, caffeic acid, ferulic acid, resveratrol, p-coumaric acid; at 370 nm - rutin and quercetin. The gradient used for the separation was performed using 2% (v/v) acetic acid (A) and methanol (B) as shown on Table 1.

Statistical analysis

All tests were carried out in triplicate and the results were presented as mean ± standard deviation (SD).

3 Results and discussion

The phenolic compounds, together with flavonoids are among the main phytochemicals found in plants. They are important due to their biological activity and free radical scavenging activity, in particular (Elfalleh et al., 2011). Both, TPC and TF of pomegranate juice were analyzed prior to the digestion process. The results are presented on Table 2. TPC was 14.29 ± 0.05 mgGAE/mL. Similarly, Tezcan et al. (2009) reported that commercial pomegranate juices had markedly high total phenolic contents and antioxidant capacity. All the same, in their review Kalaycıoğlu & Erim (2017) summarized the total phenolic contents, antioxidant activities, and bioactive ingredients of juices from pomegranate cultivars worldwide. The average reported range was 1.40-4.45 mgGAE/mL which is far less than the reported values in this study. Hmid et al. (2017) also studied the TPC and TF of pomegranate juices from 18 different cultivars from Morocco. Lower TPC was reported with considerable variations among the samples; the values ranged from 1.385 to 9.476 mg GAE/mL of local cultivars and foreign cultivars ranged from 1.284 to 8.295 mg GAE/mL.

Table 1. HPLC gradient for phenolic compound analysis.

Time, min	A, %	B, %
0	100	0
3	95	5
18	80	20
20	80	20
30	75	25
40	70	30
55	60	40
60	50	50
70	0	100
80	0	100

Table 2. Total phenolic content (TPC), total flavonoid content (TF) and antioxidant activity (DPPH, ABTS, FRAP and CUPRAC assays).

Assay	Result
TPC, mgGAE/mL	14.29 ± 0.05
TF, mgQE/mL	1.73 ± 0.02
DPPH, µMTE/mL	99.00 ± 0.32
ABTS, µMTE/mL	101.13 ± 1.29
FRAP, µMTE/mL	171.09 ± 0.99
CUPRAC, µMTE/mL	140.23 ± 1.01

In respect to TF the measured value was 1.73±0.02 mgQE/mL (Table 2). In comparison, Li et al. (2015) reported much lower content of total flavonoids from 0.045 to 0.335 mgQE/mL of extracted phenolic compounds from pomegranate juices from 10 Chinese cultivars.

The antioxidant activity of pomegranate juice was evaluated by applying four reliable *in vitro* methods – DPPH, ABTS, FRAP, CUPRAC. The results are shown in Table 2. The AOA ranged from 99.00±0.32 to 171.09 ± 0.99 µMTE/mL. The highest activity was measured by ferric reducing antioxidant power (FRAP) assay, which involves reduction of Fe^{3+} to Fe^{2+} by the action of electron donating antioxidants. In comparison, Özgen et al. (2008) reported AOA in the range 4.63 - 10.9 µMTE/mL following the same method for cultivars from Turkey. Similarly, Mena et al. (2011) established 10-38 mMTE/L for Spanish pomegranates which is lower in comparison to those cited here. Toward the ABTS assay Zaouay et al. (2012) reported 11.24-21.85 µMTE/mL for Tunisia cultivars which is far less than the 101.13 ± 1.29 µMTE/mL detected in this study. The significant variation among the references is probably due to the various chemical compositions of the fruits depending on the cultivar, growing region, maturity, cultivation practice, climate, and storage circumstances already summarized by Kalaycıoğlu & Erim (2017).

The influence of *in vitro* gastrointestinal digestion model on phenolic compounds found in freshly prepared pomegranate juice was evaluated. No extraction method was applied in order to simulate real conditions of juice consumption and digestion. Oral phase was not included due to the liquid form of the sample. The results are shown on Figure 1. A drastic loss of phenolic compounds was noticed. Only 25% of TPC and 15.8% of TF were detected at the end of intestinal phase of the assay. Phenolic compounds are susceptible to pH change. Mild alkaline treatment favors the liberation of phenolic acids from the complex structure they are part of but more severe alkaline treatment leads to alkaline-induced degradation (Liu et al., 2013). Although the TPC and TF vary greatly among cultivars,

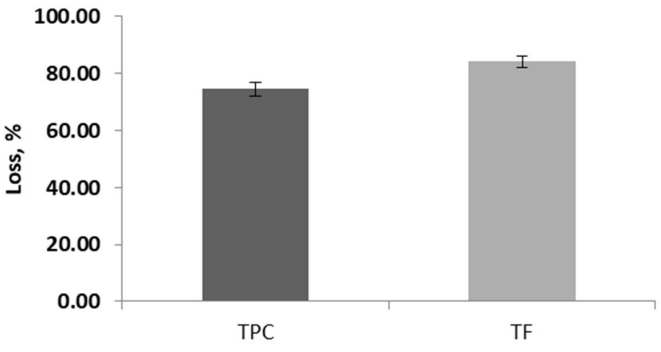

Figure 1. Loss of total phenolic and total flavonoid compounds after *in vitro* gastrointestinal digestion of pomegranate juice.

Table 3. HPLC analysis of both native and digested pomegranate juice.

Phenolic compound	Before digestion, μg/mL	After digestion, μg/mL	Loss, %
gallic acid	96.3	6.23	93.5
DL-catechin	340	2.6	99.2
syringic acid	-	-	-
cinnamic acid,	-	-	-
hesperidin	-	-	-
chlorogenic acid	-	-	-
caffeic acid	-	-	-
ferulic acid	-	-	-
resveratrol	-	-	-
p-coumaric acid	-	-	-
rutin	-	-	-
quercetin	-	-	-

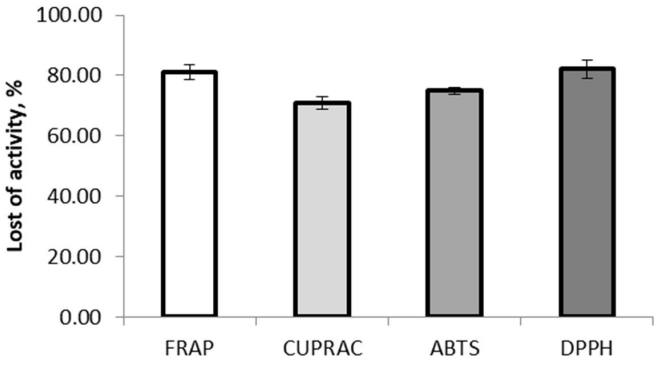

Figure 2. Loss of antioxidant activity after *in vitro* gastrointestinal digestion of pomegranate juice.

cultivation conditions, harvesting time etc. and comparisons are difficult to be made. The measured values in this study are still comparable to the average TPC and TF content reported for the native pomegranate juices by other researchers without treatment (Kalaycıoğlu & Erim, 2017). Unlike our study, Sengul et al. (2014) reported only 25% loss of TPC in pancreatic digestion but investigating methanolic extract of pomegranate juice which is not applicable in real conditions. In addition, the same authors studied the effect of food matrix and food components on the bioaccessibility of pomegranate. In general, the studied nutritional components have different effect on the bioavailability of phenolic compounds, mainly inhibitory or non-effecting, which once again proves the need for a detailed study of biologically active substances, their interactions with food and gastrointestinal transition.

The results from the AOA of digested juice are presented on Figure 2. The loss of phytochemicals also reflected on the antioxidant activity measured at the end of the experiment. Similarly, a significant decrease of biological activity was detected by all four methods of analysis. The results show that AOA varies in the range of 19-29% from the initial activity of pomegranate juice. Highest losses of phytochemicals are measured by DPPH (82.24%) and FRAP (81.05%) methods.

The HPLC analysis of both native and digested juice confirmed the presence of 96.3 μg/mL gallic acid and 340 μg/mL DL-catechin (Table 3). After digestion the measured concentrations of both compounds were 6.23 and 2.6 μg/mL for gallic acid and DL-catechin corresponding to 93.5 and 99.2% losses, respectively. Gallic acid, which is also part of the ellagitannin structure, is instable under alkaline conditions (Liu et al., 2013) thus explaining the dramatic loss after digestion.

Hmid et al. (2017) reported the presence of ellagic (not studied in this study), gallic, chlorogenic, caffeic and ferulic acids, as well as catechin, quercetin, rutin and phloridzin (not studied in this study) in pomegranate juices. Besides ellagic and gallic acids (12.42-88.51 mg/L) the other compounds were found in minor quantities. Fawole & Opara (2013) also confirmed the dominance of gallic acid among the phenolic compounds of pomegranate fruit arils. The authors reported significant variation of its concentration during ripening – four times lower (10.5 mg/L) at commercial harvest-165 days after full bloom (DAFB) compared to 52 DAFB. The inconsistency in concentration of individual phenolic acid in pomegranate juices was also confirmed by Li et al. (2015). The same authors reported the presence of catechin in the studied cultivars in range of 4.88-41.23 μg/mL.

4 Conclusion

An *in vitro* gastrointestinal digestion method was used to study the bioaccessibility of phytochemicals in pomegranate juice. Significant losses have been reported with regard to phytonutrients and antioxidant activity. Significant concentrations of gallic acid and catechin were detected in the juice prior digestion and over 93% loss was measured afterwards. Although an *in vitro* digestion method was used, the obtained results raise the question what amounts of food should be taken to produce beneficial health effects. Nevertheless, the consumption of freshly prepared fruit juices should be encouraged as well as more research on their possible impact after digestion.

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