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Comparative Study of Some Physicochemical and *in vitro* Biological Properties of Juices of Four Pomegranate Cultivars

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ABSTRACT

The pomegranate (*Punica granatum* L.) juice, peels, and kernels are rich in secondary metabolites responsible for some biological properties that are important in healthy eating. Among them, anthocyanins present in the arils and, therefore, in juices are pigments with antioxidant and anti-inflammatory activities. In the Algarve region (southern Portugal), a century old variety *Assaria* is largely cultivated, but there is lack of information about its chemical and biological characteristics. In the present work, a comparative study of the pomegranate juice was made, over three years (2017-2019), between this cultivar and three other well-known cultivars (*Wonderful*, *Mollar de Elche*, and *Acco*). The variables studied were pH, titratable acidity, formol number, total phenol content, total monomeric anthocyanins, and polymeric color. The *in vitro* biological activities of pomegranate juices studied were antioxidant through the capacity for scavenging ABTS and NO free radicals; and inhibitory activities on lipoxygenase and α-glucosidase activities. Simultaneously, the biological activities of the main anthocyanins of pomegranate juice were also determined and compared with the juice samples. The results showed that *Assaria* had characteristics similar to *Mollar de Elche*, with lower amounts of total phenols (2.609 and 2.849 mg mL^{-1}, respectively) and monomeric anthocyanins (104.785 and 82.047 mg L^{-1}, respectively) and lower antioxidant and anti-inflammatory activities than the other two cultivars. *Wonderful* had the highest amounts of those metabolites (7.072 and 594.944 mgL^{-1}, respectively) and the best activities. 'The *in vitro* biological activities of individual anthocyanins were significantly higher than the juices. The juice activities of *Wonderful* cultivar were the closest to anthocyanin ones. The best activities of isolated anthocyanins lead to the hypothesis that those antagonistic effects can occur among the juices' constituents and decrease their activities.

Keywords: Anthocyanins, Antioxidant, Anti-α-glucosidase, cv. *Acco*, cv. *Assaria*, cv *Mollar de Elche*, cv *Wonderful*.

INTRODUCTION

The fruit of pomegranate (*Punica granatum* L.), native to Iran and the surrounding area (Varasteh *et al.*, 2012), is used since ancient times and, currently, is classified as a *super fruit* in the functional food industry (Arendse *et al*., 2021), mainly due to the presence of ellagitannins and gallotannins, hydroxybenzoic and hydroxycinnamic acids, anthocyanins, flavonoids, and organic acids, among other phytochemicals (Aarabi *et al*., 2008; Fadavi *et al*., 2005; Türkyılmaz *et al*., 2022). Also, these compounds, along with alkaloids, have led the pharmaceutical industry to be interested in extracting the bioactive compounds for using as dietary supplementation (Melgarejo-Sánchez *et al*., 2021).

Generally, pomegranate pulpy seeds are

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used for fresh consumption, or processed to obtain fruit juice (Tozzi *et al*., 2022), but also for alcoholic drinks, and dry rind for making infusions and jams (Melgarejo-Sánchez *et al.*, 2021). According to these authors, the biggest pomegranate germplasm collection can be found in Turkmenistan (Garrygala Research Station) with 1,117 accessions, followed by India, Russia, Iran, Ukraine, Turkey, China, USA, and Israel. In the European Union, the largest germplasm is located in Spain, with more than 140 accessions (Melgarejo-Sánchez *et al*., 2021). In Portugal, pomegranate was introduced by Arabs, namely, in the Algarve region, where the traditional variety *Assaria* is very appreciated for its big, red and sweet arils. Eighty-two accessions of pomegranate, mainly of different *Assaria* clones, are maintained in collection at the Direção Regional de Agricultura e Pescas do Algarve (DRAP Algarve).

The main goal of the present work was to compare some properties of the fruits of cultivar *Assaria* with those of some cultivars most produced and commercialized in Europe, namely, *Wonderful, Molar de Elche,* and *Acco*, all from the collection of DRAP Algarve, that is, in the same agroecological conditions, during a three-year period.

MATERIALS AND METHODS

Samples

In October, during a period of three years, fresh pomegranate fruits were picked in early morning according to the optimal maturation stage for each variety in the pomegranate germplasm collection of Centro Experimental de Tavira (Algarve, Portugal). The orchard has a calcic luvisol soil and the climate of the region is classified as Csa, according the Koppen classification (IPMA, 2022), described as a temperate climate with dry warmer summer. The annual temperature of the region was 17.3ºC and the annual precipitation of 509 mm during the period 1971-2000. The latest climatologic normal is available at IPMA (2022).

The seeds were extracted manually and the juice obtained by pressing the seeds trough a fine mesh clothe. Afterwards, the juices were freezed and maintained at -18ºC until the laboratorial analyses were performed.

Some Chemical Analyses of Juices

The pH values of juices were evaluated by means of a pH meter, at room temperature. Titratable acidity (g citric acid 100 mL^{-1}) was determined by a potentiometric method using titrant NaOH 0.1M up to pH 8.1 (Tinebra *et al*., 2021). Formol number was evaluated in this solution after addition of 10 mL of formaldehyde and new potentiometric titration was carried out using the same titrant up to pH 8.1. The results were then expressed as mL NaOH 0.1M 100 mL^{-1} (Šnurković, 2013). Total phenol content was determined with the modified Folin-Ciocalteu method (Zaouay *et al*., 2012). The total phenol content was calculated from a calibration curve, using gallic acid as standard. Results were expressed as mg Gallic Acid Equivalent (GAE) mL^{-1}. Total monomeric anthocyanin was determined by the pH-differential method (Giusti and Wrolstad, 2001). The monomeric anthocyanin content was expressed as cyanidin-3-glucoside L^{-1}, considering its molecular weight (445.2 g mol^{-1}) and extinction coefficient of 29.600. The polymeric anthocyanin content expressed as a percentage (%) was determined according to the method described by Giusti and Wrolstad (2001) and also used by Pala and Toklucu (2011) for pomegranate juices. The percent polymeric anthocyanin was calculated using the following equation:

% Polymeric anthocyanin= Polymeric anthocyanin/Color density (1)

Antioxidant Activity and Anti-α-Glucosidase Activity

The 2,2'-Azino Bis (3ethylbenzothiazoline-6-Sulphonic acid)

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(ABTS) free radical-scavenging and the Nitric Oxide (NO) scavenging activities of juice samples and individual anthocyanins (cyanidin-3-glucoside, cyanidin-3,5-diglucoside, delphinidin-3-glucoside, delphinidin-3,5-diglucoside, pelargonidin-3-glucoside, and pelargonidin-3,5-diglucoside) were evaluated following the protocol described by El-Guendouz *et al*. (2018). Briefly, 275 µL of ABTS solution previously prepared were added to 25 µL of different concentrations of samples and left at room temperature for 6 min. After this period, the absorbance was read at λ= 734 nm. The ability of samples for scavenging ABTS was determined through the following formula:

ABTS scavenging activity (%)= $[(A_0-A_1)/A_0]\times100$ (%) (2)

Where, A_0 is the absorbance of the control (water) and A_1 is the absorbance in the presence of the sample. The sample concentration able to inhibit 50% (IC_{50}) was obtained by plotting the inhibition percentage *versus* sample concentrations.

In the NO assay, 50 µL of different concentrations of samples were added to 50 µL of 10 mM sodium nitroprusside in Phosphate Buffered Saline (PBS) and left at room temperature for 90 minutes. After this period, 50 µL of Griess reagent and the absorbance was read at λ= 532 nm. The inhibition percentage was calculated using the following formula:

$[1 - (A_{sample} - A_{sample\ blank})/(A_{control} - A_{control\ blank})]x100$, (3)

Where, $(A_{sample}-A_{sample\ blank})$ is the difference in the Absorbance of a sample, with or without 10 mM sodium nitroprusside, and $(A_{control} - A_{control\ blank})$ is the difference in the Absorbance of the PBS control, with or without 10 mM sodium nitroprusside. The IC_{50} values were determined as aforementioned.

The inhibition of lipoxygenase and α-glucosidase activities of juice samples and individual anthocyanins were performed according to El-Guendouz *et al*. (2016). Briefly, 5 µL 5-lipoxygenase solution in borate buffer 0.005% were added to 937 µL borate buffer, 10 µL sample and 50 µL linoleic acid (0.001M). The enzymatic reactions were performed in the absence, or in the presence, of different concentrations of samples and their kinetics were compared, reading the absorbance at λ= 234 nm. The inhibition percentage of the enzyme was calculated as previously reported, and the IC_{50} values was determined.

A volume of 25 µL of different concentrations of samples, 30 µL of α-glucosidase (2.4 U mL^{-1}) and 100 mM phosphate buffer (pH 6.8) was left at room temperature for 10 min. After this period, 100 µL of 0.5 mM Pnitrophenyl-β-D-Glucopyranoside (PNPG) solution in phosphate buffer were added. The reaction mixture was incubated at 37 °C for 30 min, followed by the addition of 80 µL of sodium carbonate solution (0.4 mM). The absorbance was read at λ= 405 nm. The inhibition percentage of the enzyme was calculated as previously reported, and the IC_{50} values were determined.

Statistical Analysis

Statistical analysis (Principal Component Analysis and linear Pearson correlations) were determined using the PAST statistics version 4.02 software (2020) (Hammer *et al*., 2001). Statistical significance was set as $P< 0.05$. Correlations among titrable acidity, phenols, anthocyanins, NO, ABTS and lipoxygenase activity were achieved by Pearson's correlation coefficient (r) at a significance level of 95%.

RESULTS AND DISCUSSION

General Chemical Characterization of Pomegranates Juice

Some of the characteristics of the pomegranate juice such as pH, titratable acidity, and formol number are listed in

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Table 1. The pH values ranged from a minimal of 2.62, in *Wonderful* cultivar, to 3.65, in *Mollar* cultivar. The *Acco* cultivar presented the tightest range of pH as well as the highest minimal value of pH. In contrast, cultivar *Wonderful* presented the lowest values of pH with a mean of 2.72±0.09, which meant the highest titrable acidity (mean of 1.23±0.05). The mean titrable acidity of the *Wonderful* cultivar was about fivefold higher than that of *Mollar de Elche* cultivar (Table 1). The higher titrable acidity found for *Wonderful* cultivar than other cultivars was also reported by some authors (Tarantino *et al*., 2022; Tinebra *et al*., 2021). The percentages of titrable acidity found for all samples can be considered appropriate for the fresh market, since they are lower than 1.8% (Tinebra *et al*., 2021).

Formol number is related to the amino acid content in fruit juices, measured through a potentiometric titration, also estimates its purity, although sometimes needs supplementary assays (Esteve *et al*., 2005; Türkmen and Ekşi, 2011). The values found in several cultivars ranged from 57 mL NaOH 0.1M 100 mL^{-1} in both *Acco* and *Wonderful* cultivars, to 60 mL NaOH 0.1M 100 mL^{-1}, in the *Mollar de Elche* cultivar. Such values are much higher (10-100 higher) than those previously reported for diverse cultivars of pomegranates from diverse origins (Esteve *et al*., 2005; Türkmen and Ekşi, 2011; Ekşi and Özhamamcı, 2009; Poyrazoğlu *et al*., 2002; Rajasekar *et al*., 2012; Zaouay *et al*., 2014). The values found in the present work are similar to those reported for pomegranate juice from India (Confederation of Indian Industry, 2014). The variability found without any type of adulteration may be attributed to several factors such as cultivar, ripeness, manufacturing conditions, among others (Esteve *et al*., 2005). For example, an elimination of pulp and rind during refining of the juice gives rise to a lower formol number, since those parts of the fruits are richer in amino acids (Esteve *et al*., 2005).

The cultivars *Wonderful* and *Acco* had the highest amounts of total phenols, with 7.1 and 4.7 mg GAE mL^{-1}, respectively (Table 1), whereas *Assaria* and *Mollar de Elche* had similar amounts but much lower than those cultivars (2.6 and 2.8 mg GAE mL^{-1}, respectively). Tarantino *et al*. (2022) also reported that the *Wonderful* cultivar from Italy had more total phenols than *Acco* and *Mollar* cultivars, although with different amounts. This variability was also described by some authors (Zaouay *et al*., 2012; Akhavan *et al*., 2015) for Tunisian and Iranian cultivars, respectively. The monomeric anthocyanins were also present in higher concentrations in the juice of the cultivar *Wonderful* than in the remaining samples. In these three cultivars, the amounts of total anthocyanins were not statistically different, the means ranging from 82.0 mg cyanidin-3-glucoside equivalent L^{-1} in the *Mollar* cultivar, to 105.8 mg cyanidin-3-glucoside equivalent L^{-1} in the *Acco* cultivar (Table 1). As for the phenol content, the total monomeric anthocyanin content was revealed to be dependent on the cultivar, as already reported for other cultivars of Tunisian (Zaouay *et al*., 2012) or Iranian pomegranates (Alighourchi *et al*., 2008). These authors described a range of 50.5 and 490.4 mg L^{-1}.

Polymeric pigments are formed by the reaction of monomeric anthocyanins with condensed tannins or flavan-3-ols, such as catechin or epicatechin. These polymeric pigments have an important role in the maintenance of color stability (Türkyılmaz and Özcan, 2014). The juices of *Acco* and *Mollar de Elche* cultivars had higher polymeric colour percentages (46.3 and 37.5%, respectively) than *Assaria* and *Wonderful* cultivars (24.3 and 22.3%, respectively). Higher polymeric color percentages (> 10%) indicate prolonged storage of the fruits or vegetables. Nevertheless, and according to Türkyılmaz and Özcan (2014), this is attributable to the polymerization of anthocyanins with condensed tannins rather than anthocyanin degradation. The polymeric colour percentages are within the range reported by

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Türkyılmaz *et al.* (2013) for Turkish pomegranate juices, values of which range from 15 to 45%. The authors reported that the increase of polymeric colour coincided with the decrease of condensed tannins and monomeric anthocyanins. In addition, they also concluded that the formation of these pigments was affected by the structures of anthocyanins and polyphenols, pH, temperature, and concentrations of copigments.

The antioxidant activity was measured through the ability for scavenging the 2,2'-Azino-Bis (3-ethylbenzthiazoline-6-Sulphonic acid (ABTS) and Nitric Oxide (NO) free radicals. All samples were able to scavenge such free radicals, although with different strengths (Table 1). Since the results are presented as IC_{50} values, that is, the concentration of sample able to scavenge 50% of radicals, it means that lower values of IC_{50} correspond to higher activity. Therefore, regarding the ability for scavenging ABTS free radicals, the sample obtained from *Wonderful* had better antioxidant activity than the remaining samples. The lowest activities were observed for *Assaria* and *Mollar* with IC_{50} values of 3.8 and 4.2 mg mL^{-1}. The ability for scavenging ABTS radicals has been reported for other pomegranate juices from different origins. Although the results cannot be compared to ours, since they are represented as Trolox equivalent, all they describe is the activity that changes depending on the phenol amounts (di Stefano *et al*., 2019; Alsataf *et al*., 2021; Esposto *et al*., 2021; Wan *et al*., 2018). In our case, this correlation was also observed between the antioxidant activity and the amounts of phenols ($r= -0.89$; $P< 0.001$) (Table 2).The correlation is negative since higher activity has lower IC_{50} value. Moreover, a negative correlation was also observed between the capacity for scavenging the ABTS free radicals and total monomeric anthocyanins ($r= -0.88$; $P< 0.001$), in contrast to that observed by Esposto *et al*. (2021), who did not detect any correlation between anthocyanins and antioxidant activity measured through the capacity for scavenging ABTS radicals.

Nitric Oxide (NO) is a cell signaling-molecule in mammals and has an important role in the regulation of different physiological and pathophysiological processes, nevertheless, NO is highly reactive and has a few seconds lifetime, diffusing easily across membranes and large amounts can be toxic and pro-inflammatory (Guzik *et al*., 2003; Gülçin, 2012). Large amounts of NO along with superoxide anion radicals can form peroxynitrite ($ONOO^-$). This anion is able to induce DNA damage, oxidase Low-Density Lipoproteins (LDL), and inhibits mitochondrial respiration, among many other deleterious effects (Guzik *et al*., 2003). For this reason, samples with capacity for scavenging NO are considered as antioxidant and anti-inflammatory agents. As for ABTS scavenging activity, the juice samples of *Wonderful* cultivar had the best ability for scavenging NO radicals, in contrast to the *Assaria* and *Mollar* cultivars (Table 1). In this case, a negative correlation was also observed between phenol content or anthocyanin content and the NO scavenging activity, with lower r values ($r= -0.70$ and $r= -0.71$, respectively). These results indicate that, beyond anthocyanins, other phenolic compounds have also an important role in this activity.

Lipoxygenase catalyzes hydroperoxidation of unsaturated fatty acids originating leukotrienes, which are responsible for diverse inflammatory processes. Therefore, lipoxygenase inhibitors are important for preventing inflammation, but also oxidative processes, due to the decrease of hydroperoxides formation (Kurihara *et al*., 2014). Juice samples obtained from pomegranate cultivar *Wonderful* were the most effective anti-lipoxygenase in contrast to the samples of *Assaria* and *Mollar*, with IC_{50} values three-fold higher than 45 mg mL^{-1} (Table 1). Different parts of pomegranate plant (juice, fermented juice, cold-pressed seed oil, flowers, and rinds) of diverse cultivars showed the capacity of the extracts

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Table 1. Mean and Standard Deviation (STD) of the physicochemical and in vitro biological properties of pomegranate juices fom *Assaria*, *Mollar de Elche*, *Acco* and *Wonderful* cultivars grown in the same field.

	Assaria			*Wonderful*			*Acco*			*Mollar*		
	Minimum-Maximum	Mean	STD	Minimum-Maximum	Mean	STD	Minimum-Maximum	Mean	STD	Minimum-Maximum	Mean	STD
pH	2.67-3.63	3.15	0.20	2.63-2.81	2.723	0.090	3.02-3.52	3.327	0.315	2.97-3.65	3.420	0.390
Titrable acidity (%)	0.256-0.368	0.304	0.035	1.168-1.264	1.227	0.051	0.321-0.432	0.363	0.060	0.208-0.339	0.257	0.071
Formol number (mL NaOH 0.1M 100 mL^{-1})	35.0- 67.5	57.589	6.955	52.5- 60.0	56.667	3.819	52.5- 60.0	56.667	3.819	47.5- 70.0	60.000	11.456
Phenols (mg GAE mL^{-1})	1.172-4.087	2.609	0.764	5.515-8.982	7.072	1.760	3.844-5.754	4.677	0.978	2.433-3.243	2.849	0.405
Anthocyanins (mg Cy-3-Glu equivalent L^{-1})	18.619-685.656	104.785	118.222	493.452-657.936	594.944	88.744	109.712-321.175	105.752	21.666	59.866-106.956	82.047	23.663
Polymeric colour (%)	12.414-45.937	24.346	9.999	21.417-22.955	22.286	0.788	38.877-58.459	46.292	10.621	22.619-45.701	37.452	12.873
NO (IC_{50}, mg mL^{-1})	64.707-501.979	330.311	130.816	41.809-87.359	61.545	23.375	109.071-242.058	182.317	67.514	196.450-308.621	251.607	56.109
ABTS (IC_{50}, mg mL^{-1})	1.733-6.426	3.836	1.282	0.682-1.116	0.906	0.217	1.891-2.668	2.337	0.401	3.208-5.143	4.186	0.968
Lipoxygenase (IC_{50}, mg mL^{-1})	31.295-89.606	48.361	14.466	16.103-20.855	17.811	2.643	31.350-34.368	33.320	1.707	30.156-59.618	45.741	14.805
Glucosidase (IC_{50}, mg mL^{-1})	0.145-25.788	3.073	4.894	0.191-3.116	1.348	1.555	2.486-17.368	9.658	7.456	2.159-7.820	4.468	2.971

Table 2. Pearson correlation coefficients (r).

pH	Titrable acidity	Formol number	Phenols	Anthocyanins	Polymeric	NO	ABTS	Lipoxygenase	Glucosidase
pH	-0.78***	-0.08	-0.56	-0.74**	0.74**	0.55	0.69*	0.76***	0.23
Titrable acidity		-0.12	0.84****	0.94****	-0.50	-0.77***	-0.85****	-0.83****	-0.40
Formol number			-0.24	-0.19	-0.08	-0.19	0.34	0.23	0.29
Phenols				0.90****	-0.14	-0.70*	-0.89****	-0.83***	-0.31
Anthocyanins					-0.36	-0.71**	-0.88****	-0.84****	-0.23
Polymeric						0.10	0.26	0.34	0.42
NO							0.66*	0.67*	0.01
ABTS								0.95****	0.23
Lipoxygenase									0.17

* P< 0.05; ** P< 0.01; *** P< 0.005, ****P < 0.001.

to inhibit the activity of lipoxygenase (Bekir *et al*., 2013; Schubert *et al*., 1999; Sestili *et al*., 2007), but with different responses. In the present work, there was a negative correlation between the levels of total phenols or levels of total monomeric anthocyanins and the capacity for inhibiting the lipoxygenase activity ($r= -0.83$, $P< 0.005$; $r= -0.84$, $P< 0.001$, respectively). According to the authors, the activities found could not be attributed only to phenols, because fatty acids or phytosterols may have an important role on the lipoxygenase inhibitor activity.

α-Amylase and α-glucosidase are important enzymes of the digestive system, because they catalyze the hydrolysis of carbohydrates into smaller molecules easily absorbed through the gut wall. The inhibition of these enzymes will retard the carbohydrates digestion and, therefore, prevent type 2 diabetes mellitus (Catarino *et al*., 2019). The juice of cultivar *Wonderful* had the best activity with an IC_{50} value of 1.3 mg mL^{-1} (Table 1). The poorest activity was found for the juice of the *Acco*. In this assay, *Assaria* and *Mollar* had intermediate IC_{50} values. The anti-glucosidase activity was already reported by other authors for different plant parts of pomegranate (juice, natural or clarified; flowers; peel; seeds; waste), (Alsataf *et al*., 2021; Kam *et al.*, 2013; Çam and Içyer, 2015; Morittu *et al*., 2020). Generally, peels, mesocarp, and flowers had higher activity than juices (Alsataf *et al*., 2021; Kam *et al.*, 2013). Moreover, the activities found by these authors, presented as IC_{50} values, were lower than 1 mg mL^{-1}, therefore, better activities than our samples. A correlation between the activity and the amounts of phenols or anthocyanins was not found, as reported for the remaining samples (Table 2). According to some authors (Alsataf *et al*., 2021; Çam and Içyer, 2015; Kam *et al*., 2013), the α-glucosidase inhibitory activity could be attributed to the phenols, but particularly to Gallic acid or compounds with the presence of trihydroxybenzoic acid structure (Kam *et al*., 2013) such as punicalagin or their metabolites (e.g. urolithin A) formed during gastro-intestinal digestion (Bellesia *et al*., 2015; Les *et al*., 2018).

The inverse correlation between pH value and the total monomeric anthocyanins may result from the colorless hemiketal that forms at pH values of 4.5, therefore, as the pH increases, the formation of uncolored anthocyanis also increase. In these circumstances, a decrease of the absorbance value is observed at 510 nm and, consequently, the total monomeric anthocyanins that are measured at this wavelength (Giusti and Wrolstad, 2001). Moreover, the color changes of roselle extract in the pH range of 1-7 changed from dark red to light red at pH values of 1–4, near pH of 5, the extract presented as nearly colorless, whereas its color changed to blue at a pH of 7 (Wu *et al*., 2018). The effect of pH on the antioxidant activity of anthocyanins has also been reported for wine, roselle and *Hibiscus acetosella* (Wu *et al*., 2018; Lapidot *et al*. 1999; Muselík *et al*., 2007; Março *et al*., 2011). The presence of a catechol group generally improves the capacity for scavenging free radicals through the formation of a very stable semiquinone radical (Castañeda-Ovando *et al*., 2009). Cyanidin and delphinidin, in contrast to pelargonidin, present this catechol group in the ring C, which may explain the inverse correlation between the amounts of anthocyanins and the IC_{50} values, regardless of the method used (Table 2). The main anthocyanidins of pomegranate juice are cyanidin, delphinidin, and pelargonidin (di Stefano *et al*., 2019). However, and due to the acid characteristic of anthocyanins, the hydroxyl group at C-7 that is the strongest acid, will be the first to be removed even at pH around 4, giving rise to a neutral quinonoid base stabilized by tautomerization with the hydroxyl group at C5 (Dangles *et al*., 2018; Tena *et al*., 2020). The formation of this neutral quinonoid base may facilitate the disappearance of the catechol group important for quenching free radicals, as aforementioned.

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The results of the twelve juice samples from all tests were used to develop the PCA model (Figure 1). Principal Component 1 (PC1) explained up to 90.3% of the total variance and PC2 explained 9.28%. The two-dimensional graphic presenting the two PCs explained 99.6% of the variance in the data. According to the results, it is possible to distinguish a group constituted by the cultivar *Wonderful* (2), a group constituted by the cultivars *Assaria* (1) and *Mollar* (4), whereas the *Acco* cultivar was in an intermediate position between *Wonderful* and *Assaria*/*Mollar de Elche*.

Biological Activities

Pomegranate juice is an important source of phenolic compounds, such as anthocyanins, especially the 3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin (Miguel *et al*., 2004; Zhao and Yuan, 2021). Nevertheless, other phenols have been detected in pomegranate juices such as ellagitannins (punicalagins and punicalins) and ellagic acid (Kalaycıoğlu and Erim, 2017), with strong *in vitro* antioxidant activity, although multiple polyphenols had higher activity than single purified polyphenols (Yu-qing *et al*., 2017). In the present work, the antioxidant activity of single anthocyanins was evaluated and compared to those of pomegranate juices (Table 3). The antioxidant evaluation was followed through the determination of scavenging ABTS and NO free radicals, as well as through the capacity for inhibiting the lipoxygenase activity. This inhibition activity is also related to the anti-inflammatory activity. Table 3 depicts that single anthocyanins are much better free radicals' scavengers than juice samples. In what concerns the capacity for scavenging ABTS free radicals, all monoglycosylated anthocyanins were better scavengers than the diglycosylated. Such results show that glycosylation at 3-OH is more important on the ability for scavenging ABTS radicals than the number of OH groups in the B-ring of the anthocyanin. With regard to the ability to quench nitric oxide radicals, pelargonidin-3-*O*-glucoside was a better scavenger than the remaining anthocyanins (Table 3), followed by pelargonidin-3,5-*O*-diglucoside. This distinct ability of anthocyanins for scavenging different free radicals is expected, since the effects are very much dependent on the methods used (Kähkönen and Heinonen, 2003). Pelargonidin-3,5-*O*-diglucoside was the best anthocyanin for inhibiting the lipoxygenase activity (Table 3).

The inhibitory α-glucosidase activities by monoglycosylated anthocyanins were higher than those found for the juice samples. The diglycosylated anthocyanins presented the worst activities. In this case, it was not possible to determine the IC_{50} values, that is, the highest concentrations of the anthocyanins that the assay permitted to determine these values were not able to inhibit 50% of the α-glucosidase activity. Anthocyanins have been described as possessing the ability for inhibiting α-glucosidase activity, particularly diacylated anthocyanins (McDougall *et al*., 2008). However, according to the results obtained in the present work (Table 2), there is no significant correlation between the amounts of anthocyanins and the inhibition activity.

The results of the four juice samples and six anthocyanins from all tests were used to develop the PCA model (Figure 2). Principal Component 1 (PC1) explained > 99% of the total variance. The two-dimensional graphic presenting the two PCs was able to explain 99.9% of the variance in the data. According to the results, it is possible to distinguish a group constituted by all individual anthocyanins, which are far away from the pomegranate juices. The pomegranate juice of the cultivar *Wonderful* was the sole in the same side of the PC1 that is closer to those of the individual anthocyanins, in contrast to the remaining juice samples and, particularly, cultivar *Assaria* (Figure 2). The lowest total monomeric anthocyanins observed in this cultivar along with those of

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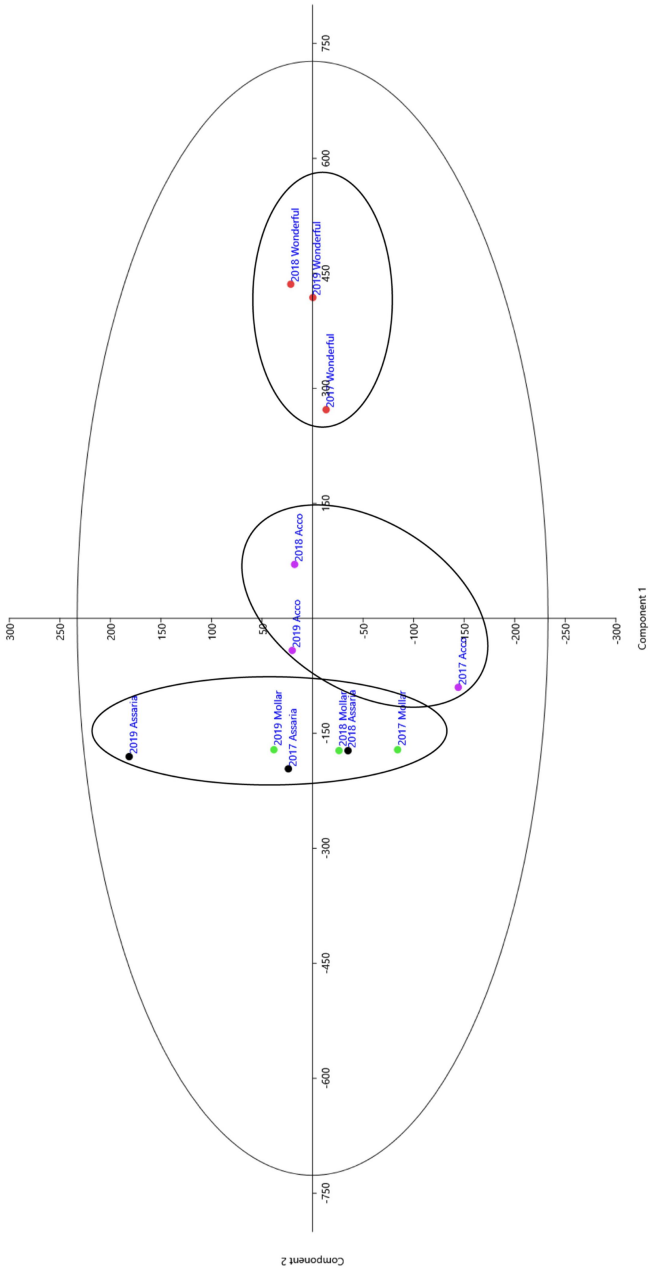

Figure 1. Principal Component analysis (PC1 and PC2) of several parameters of pomegranate juices obtained from *Assaria*, *Wonderful*, *Acco* and *Mollar de Elche* over three years (2017-2019).

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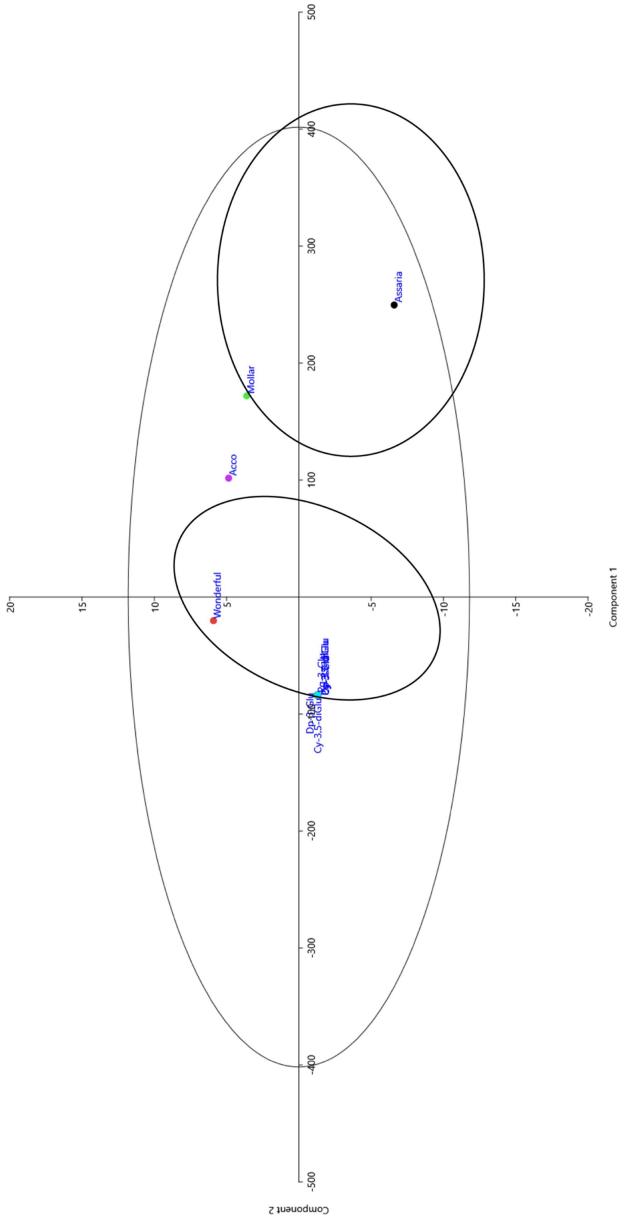

Figure 2. Principal Component analysis (PC1 and PC2) of *in vitro* biological properties of four pomegranate cultivars (*Assaria*; *Wonderful*; *Acco*; *Mollar de Elche*) and individual anthocyanins (cyanidin-3-glucoside; delphinidin-3-glucoside; pelargonidin-3,5-diglucoside; delphinidin-3,5-diglucoside; cyanidin-3,5-diglucoside; pelargonidin-3-glucoside).

Table 3. *In vitro* biological activities [mean and Standard Deviation (STD)] of pomegranate juices from *Assaria*, *Mollar de Elche*, *Acco*, and *Wonderful* cultivars and individual anthocyanins.[a]

	NO (IC_{50}, mg mL^{-1})	ABTS (IC_{50}, mg mL^{-1})	Lipoxygenase (IC_{50}, mg mL^{-1})	Glucosidase (IC_{50}, mg mL^{-1})
	Mean±STD	Mean±STD	Mean±STD	Mean±STD
Assaria	330.31 ± 130.82	3.84 ±1.28	48.36 ± 14.47	3.07 ± 4.89
Wonderful	61.55 ± 23.38	0.91 ± 0.22	17.81 ± 2.64	1.35 ± 1.56
Acco	182.32 ± 67.51	2.34 ± 0.40	33.32 ± 1.71	9.66 ± 7.46
Mollar	251.61 ± 56.11	4.19 ± 0.97	45.74 ± 14.81	4.47 ± 2.97
Cy-3-Glu	0.010 ± 0.001	0.004 ± 0.000	0.071 ± 0.001	0.033 ± 0.001
Dp-3-Glu	0.012 ± 0.000	0.003 ± 0.000	0.033 ±0.001	0.48 ± 0.034
Pg-3,5-diGlu	0.002 ± 0.000	0.024 ± 0.001	0.014 ± 0.000	---
Dp-3,5-diGlu	0.014 ± 0.000	0.011 ± 0.001	0.036 ± 0.004	---
Cy-3,5-diGlu	0.014 ± 0.000	0.009 ± 0.001	0.052 ± 0.006	---
Pg-3-Glu	0.008 ± 0.000	0.004 ± 0.000	0.039 ± 0.001	0.224 ± 0.016

[a] Cy-3-Glu: Cyanidin-3-*O*-Glucoside; Dp-3-Glu: Delphinidin-3-*O*-Glucoside; Pg-3,5-*O*-diGlu: Pelargonidin-3,5-*O*-diGlucoside; Dp-3,5-diGlu: Delphinidin-3,5-*O*-diGlucoside; Cy-3,5-diGlu: Cyanidin-3,5-*O*-diGlucoside; Pg-3-Glu: Pelargonidin-3-*O*-Glucoside. (---): The activities found were very low which did not permit to determine the IC_{50} values.

Acco and *Mollar de Elche* may explain this distance between these cultivars and the individual anthocyanins. Nevertheless, this cannot be the sole reason because cultivar *Wonderful,* although in the same side (PC1) of the individual anthocyanins, is at distant. This result may indicate that other secondary metabolites present in pomegranate, including anthocyanins, may interact among them, resulting in a poorer activity.

CONCLUSIONS

In a study made over three years with pomegranate of four cultivars (Portuguese *Assaria*, *Mollar de Elche*, *Acco*, and *Wonderful*) in the same garden (Algarve, Portugal), we concluded that *Assaria* and *Mollar de Elche* cultivars were closer to each other than cultivar *Wonderful*. The study included evaluation of some physicochemical and *in vitro* biological properties of their juice (pH, titrable acidicity, formol number, total phenols, monomeric anthocyanins, polymeric color, ABTS and NO scavenging activity, and lipoxygenase and α-glucosidase inhibition activity). Moreover, the *in vitro* biological properties of the main individual anthocyanins were higher than the pomegranate juices, with *Wonderful* being much closer to the results obtained for the individual anthocyanins than the remaining samples.

Without genetic studies and only based on chemical and biological properties, this study concluded that *Assaria* and *Mollar de Elche* cultivars are very similar and well distinct from the most popular and widely grown cultivar *Wonderful*. Although the distribution areas of the two Iberian cultivars are hundreds of kilometers apart, one common origin is suggested, possibly from plant material introduced by Arabs during peninsula colonization. The red fruits, such as pomegranate, are considered vital for a healthy life due to their antioxidant and anti-inflammatory activities. Nevertheless, in the present work, it was shown that some of these properties can significantly change depending on the cultivar.

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بررسی مقایسه‌ای برخی خاصیت‌های فیزیکوشیمیایی و بیولوژیکی آزمایشگاهی آب چهار رقم انار

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چکیده

آب، پوست و هسته انار (*Punica granatum* L.) سرشار از متابولیت های ثانویه است که دارای برخی از خواص بیولوژیکی هستند که در تغذیه سالم مهم می‌باشند. در میان آنها، آنتوسیانین موجود در دانه انار(arils) و بنابراین در آب میوه‌ها، رنگدانه هایی هستند با فعالیت آنتی اکسیدانی و ضد التهابی. در منطقه آلگاروه (Algarveجنوب پرتغال)، عمدتاً رقم آساریا با صد سال قدمت کشت می شود، اما در مورد ویژگی های شیمیایی و بیولوژیکی آن اطلاعات کافی وجود ندارد. در این پژوهش، مقایسه آب انار طی سه سال (۱۹-۲۰۱۷) بین این رقم و سه رقم شناخته شده دیگر (Wonderful، Mollar de Elche و Acco) انجام شد. متغیرهای مورد مطالعه pH، اسیدیته قابل تیتراسیون، تعداد فرمول(formol)، محتوای فنل کل، آنتوسیانین مونومرکل و رنگ پلیمری بودند. فعالیت‌های بیولوژیکی آب انار مورد مطالعه در شرایط آزمایشگاهی از طریق ظرفیت حذف رادیکال‌های آزاد(ABTS (scavenging و NO آنتی اکسیدانی بود. و فعالیت‌های مهاری (inhibitory) بر روی فعالیت های لیپوکسیژناز و α-گلوکوزیداز.همزمان، فعالیت بیولوژیکی آنتوسیانین های اصلی آب انار نیز تعیین و با نمونه های آب انار مقایسه شد. نتایج نشان داد که Assaria دارای ویژگی‌های مشابه Mollar de Elche، با مقادیر کمتر فنل کل (به ترتیب ۲.۶۰۹ و ۲.۸۴۹ میلی‌گرم در میلی‌لیتر) و آنتوسیانین‌های مونومر (به ترتیب ۱۰۴.۷۸۵ و ۸۲.۰۴۷ میلی‌گرم در لیتر) و فعالیت آنتی‌اکسیدانی و ضدالتهابی کمتر نسبت به دو رقم دیگر است. رقم Wonderful دارای بالاترین میزان متابولیت ها (به ترتیب ۱۰۴.۷۸۵ و ۵۹۴.۹۴۴) و بهترین فعالیت ها بود. فعالیت های بیولوژیکی آنتوسیانین های فردی در شرایط آزمایشگاهی به گونه قابل توجهی بالاتر از آب میوه ها بود. فعالیت های آب میوه رقم Wonderful نزدیک ترین به فعالیت های آنتوسیانین بود. بهترین فعالیت های آنتوسیانین های جدا شده منجر به این فرضیه می شود که آن اثرات متضاد می تواند در بین اجزای تشکیل دهنده آب میوه ها رخ دهد و فعالیت آنها را کاهش دهد.

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