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The Effects of Different Solvents on Phenolic, Flavonoid, Anthocyanin Contents and Free Radical Scavenging Activity on Pomegranate Juice and Seeds

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Abstract

Our phytochemical investigation defined the polyphenol, anthocyanin and flavonoid content from methanolic, ethanolic 50% and watery extracts from fresh pomegranate juice and seeds with a spectrophotometric method. The antioxidant capacity was defined with the ABTS and DPPH methods. We determined the polyphenols with the Folin-Ciocalteu method, the anthocyanins with the method in Hungarian Pharmacopoeia VIIIth edition at Myrtilli fructus monography and the flavonoids with a modified method of the Romanian Pharmacopeia Xth edition Cynarae folium monography. We expressed the concentration of polyphenols in gallic acids (GA). The values indicated the following: in fruit juice, 47.57 µg GA/ml, in methanol seed extract 7 µg GA/g, the ethanol 50% and in watery seed extracts 8 µg GA/g. The definition of flavonoids indicated the following values expressed in quercetin (QE): in fruit juice 706 µg QE/ml, in methanol seed extract 416 µg QE/g, in ethanol 50% seed extract 46 µg QE/g and in watery seed extract 57 µg QE/g. The anthocyans from juice 3.95 mg/100 ml and seed 6.47 mg/100 g expressed in cyanidin-3-0-glycoside. We determined the following values with the ABTS method: the antioxidant capacity of juice is 25.40 µg/ml, in methanol seed extract 2.87 µg/ml, in ethanol seed extract 2.52 µg/ml, in watery 9.15 µg/ml. With the DPPH method, we obtained the following values: from juice 54.2 µg/ml, from methanolic extract 14.7 µg/ml, from ethanolic extract 10.58 µg/ml and watery extract 18.22 µg/ml. The received data shows the importance of pomegranate, which can be a new phytotherapeutic potential resource.

Keywords

pomegranate fruit, polyphenol, flavonoid, anthocyan, antioxidant activity

1 Introduction

The pomegranate (*Punica granatum L.*) belongs to the Punicaceae family. Throughout history, it was a precious fruit; it was called the fruit of paradise.

The history of pomegranate dates back to 4000 B.C. It was delivered from the territory of Persia in 100 B.C. It appeared for the first time in Jewish culture as a fruit of a land of covenant. Later, it appeared in other cultures as well.

It is mentioned in Karan, Torah, Homeric Hymns, and Mesopotamian records. It is often mentioned in the Bible as the 'love apple' or simply as food which defines the economy of the land of Canaan [1].

Finally, it became a symbol which characterised the power of kings, and it was called the "crowned apple", near the sceptre, became the country apple.

In other cultures, it appeared during migrations. In Spain, the moorsnamed a city after it, Granada. It had passed by some Spanish conquerors in America and in Egypt it appeared on the frescos in the grave of Ramses IV, the Pharaoh. In Italy appeared by the Puns, and it was called "Punicum malum". Nowadays, it is called Punica granatum, suggesting that the fruit contains many seeds inside. It was also the symbol of the Society of Jesus, and pharmacies were named after the pomegranate. In the 15th century, it was used as a decorative

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element in the textile industry and architecture. In Egypt, it was used to decorate sarcophan, and it was also the symbol of wealth and ambition [2, 3].

In ancient times, people discovered that pomegranate has medical significance. It was used to the treatment of diseases like diabetes, parasitic infections, diarrhoea, inflammatory diseases and rheumatism [3].

Pomegranate occurs from Iran to East India, mainly in Asian countries, but also in lands with warmer climates, like the Mediterranean Sea area, is growing, too [4]. The various active substance concentration has been discovered in its composition, which provides a beneficial therapeutic chance of medicine. Diverse, active substance resides in the different parts of the plant. We can talk separately about the composition of the active substance of the leaf, the root, the bloom, the seed, the seed aril, and the peeling crust. These phytochemicals are phenols, anthocyans, tannins, carbohydrates, sterols, fatty acids, vitamins and mineral salts.

The peeling of the fruit contains many essential substrates like phenols, flavonoids, proanthocyanidins and ellagitannins. The representatives of phenols in the peeling are: caffeic acid, p-cumaric acid, chlorogenic acid, gallic acid and ellagic acid. At the same time contains hydrolysable tannins, too or with other expressions ellagitannins like the corilagin, granatin A and B, tellimagrandin, pedunculagin, punicalagin and punicalin. Besides the peeling still has flavonol ingredients such as kaempferol, quercetin, and myricetin. The content of the flavon-3-ols materialised in catechin and epicatechin.

The unique ingredient of the pomegranate is the punicalagin which is a content of the peeling, the seed, the leaf and the juice.

The seed coat contains water, sugar, pectin, and organic acids like citric acid and ascorbic acid. The juice is rich in ellagic acid and contains anthocyanins in the combination of sugar. These are the delfidin-3-glucoside, cyanidine-3-glucoside, pellargonidine-3-glucoside or the same in the -3-5-diglucoside variations. The polyphenol and the lipid content of the fruit seeds are significant. The main active ingredient is the punica acid and besides of this is very rich in fatty acids, too like the oleic acid, linoleic acid, stearic acid and palmitic acid [5, 6].

The seed oil's composition contains triglycerides and glycolipids: cerebroside, and coumestrol. Here we can also mention the ellagic acid derivatives and the triterpenoid's presence. Among the sterols appear stigmasterol, sitosterol and cholesterol. The leaves are rich in ellagitannins like punicalin, punicalagin, punicafoline and corilagin. Other phytochemicals, like the apigenin, tercathaine and flavon glycosides, are present here. The root is rich in alkaloids like pelletierines and piperidines [7].

The punicalin, punicalagin and punicacortein were discovered in the peeling crust. The bloom contains gallic acid, hydroxybenzoic acid and ursolic acid. These active substances were examined for therapeutic purposes on preclinical and clinical examination. Their benefic effects was discovered in many diseases, treatments such as inflammatory disease [8], acute leukaemia [9], breast cancer [10], carcinomas, Alzheimer's disease, multiple sclerosis [11], respiratory disease, cardiovascular disease, metabolic disorder, etc. [12].

The aim of our phytochemical study was to have a contribution to the accumulation of knowledge and to investigate the compounds from the *Punica granatum* fruit which appear like an antioxidant in some nutritional supplements; and to evaluate and compare the juice and seeds polyphenol (flavonoids and anthocyans) content, especially the used extraction solvent influence on it. In scientific literature many organic, polar protic and aprotic solvents are mentioned in context with the extractability and effects of the active ingredients from different *Punica* plants parts. The Punica fruit peel aqueous extract were used *in vitro* for citotoxicity effect [13] and also the peel ethanolic 70% extract for antigenotoxic effects [14]. On the other hand the aqueous and ethanolic extracts were suitable for the determination of the chemical constituents. The polyphenols were extractable with polar solvents (ethanol and water) from *Punica* peel and these extracts had a proven antioxidant effect [15].

To study the different type of extracts is important in at many view of points: extractibility of phenols from plants depends on numerous factors (solubility, temperature, concentration in plants, volume of the used solvent, etc. and cumulative effect of these), so prediction of the extraction rate is not possible based on the solubility only (however this is the most important factor); and the pomegranate contains an appreciable quantity and many kinds of flavonoids, with different phisico-chemical properties. Solubility of the studied phenols depends on their occurrence status in the biological matrix, such free form or linked with various sugars, named glycosides. This latters are soluble in water, and aglycons are soluble in organic solvents (methanol, ethanol, diethyl-ether, etc). [16, 17].

Our goal was to find the best *Punica* extract, with a favorable properties, regarding the antioxidant effect and the amount of phenols in it. Reason of using different type

of solvents, is that the amount of active ingredient in absolut terms and the those which can be extracted by biocompatible solvents differs in some cases [13].

2 Materials and methods

2.1 Chemicals and instrumentation

We used the following standards for the identification of phenolics: chlorogenic acid (Extrasynthese, Genay, France), caffeic acid (Extrasynthese, Genay, France), catechin (Rott GmbH, Karlsruhe, Germany), epicatechin (Rott GmbH, Karlsruhe, Germany), quercetin (Extrasynthese, Genay, France), isoquercetin (Rott GmbH, Karlsruhe, Germany) and kaempferol (Extrasynthese, Genay, France). For the DPPH radical scavenging activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (Germany), and for the ABTS radical scavenging activity 2,2'-Azino-bis-(3-ethylbenzothiazoline)-6 sulfonic acid diammonium salt (VWR Chemicals, France) and potassium persulfate were from Sigma-Aldrich (Germany). The solvents for extraction and those used as mobile phase were HPLC grade, and they were from Merck (Darmstadt, Germany). For the total phenolic determination, Folin-Ciocalteu phenol reagent (VWR Chemicals, France), sodium carbonate (Chimreactiv, Bucuresti, Romania), and gallic acid (Sigma) were used. Aluminium chloride hexahydrate (Chimopar, Romania) and sodium acetate (Carl Roth GmbH, Karlsruhe, Germany) were used for the total flavonoid content. Hydrochloric acid (Carl Roth GmbH, Karlsruhe, Germany) and methanol (ADR Chim, Iasi, Romania) were used for the total anthocyanins content determination. For the plant extracts, ethanol (Chemical, Bucharest, Romania) and water were double distilled and purified with the Direct–Q system (Millipore, Bedford, USA). The extracts were made in Digital ultrasonic bath heating (Nahita, Auxilab, France) and the spectrophotometric determination with the Specord 210 UV-VIS (Analytik Jena GmbH, Germany). The quantitative determination of the active ingredients for the watery extract and juice was made using an HPLC Merck Hitachi L-7000 HPLC system with the following components: interface D-7000, quaternary pump L-7100, solvent degasser L-7612, autosampler L-7200, column oven L-7360 and DAD detector L-7455 (Merck KGaA, Germany).

2.2 Plant materials

For our examinations, the used extracts were obtained from a commercially purchased pomegranate (*Punica granatum L.*) from a supermarket in Târgu Mures, Romania.

2.3 Preparation of the extracts

The seeds and the pulp were dried and minced. During our examinations, we worked with fresh juice and seed powder. We have made three types of extracts from the seed powder: ethanolic (50 %), methanolic and watery extracts. The steps of preparation of extracts: we had measured out 2.5 g ground seed powder on an analytical balance and added 25 ml dissolver in an Erlenmeyer dish. All the three extracts were placed in a digital ultrasonic bath at 25 °C for 30 minutes. We filtered the extracts in 25 ml volumetric flash and supplemented them up to 25 ml with solvent. Until use, we stored it in the freezer at −20 °C. All determinations were performed in triplicate, and the results were expressed as mean ± standard deviation (SD).

2.4 Determination of the flavonoid content

The total flavonoid content of the *Punica granatum L.* juice and extracts was defined with the method described in the Xth edition of Romanian Pharmacopeia, Cynarae folium monography. This assay's principle is that the flavonoids' keto and hydroxyl groups form a stable complex in an acidic medium with aluminium chloride in an acidic medium. Total flavonoids were expressed as µg of quercetin equivalents/g weight seeds and µg flavonoid/ml juice [18].

500 µl from the three seed extracts (ethanol 50%, methanol, water), and juice were, pipetted in the test tubes. Then 1000 µl of 100 g/l of sodium-acetate solution, and 600 µl of 25 g/l of aluminium chloride solution were added. To the received mixture, 1400 µl methanol (*R*) and 1500 µl distilled water were added and homogenised. To prepare the blank solution, 500 µl from the extracts were taken out. Still, we ignored the sodium acetate and the aluminium chloride and added 1600 µl distilled water and stirred it well. In the last step, we added 1400 µl methanol (*R*) and 1500 µl distilled water, then homogenised an it. The extracts were left for 15 minutes then the absorbances were measured at 430 nm with a spectrophotometer in a 1 ml quartz cuvette (Specord 210 UV-VIS) [13].

2.5 Determination of the total polyphenol content

For the determination of the polyphenol content, the procedure developed by V. L. Singleton and his collaborators was used [19]. Its advantage is that this procedure is sensible and simple, requires a low amount of substance, and the interference of different partsis negligible on the given wavelength.

The hydroxy group of phenols with Folin-Ciocalteu reagent constitute a blue complex. Measured on 765 nm,

the light absorption of this, we obtained proportional values with the examined extracts of phenol contents.

The content of polyphenols was expressed as μg of gallic acid equivalents/g weight of seeds and μg polyphenols/ml juice.

Extracts and juice were measured off 20 μl then added to these 1580 μl distilled water and 100 μl Folin-Ciocalteu reagent. After shaking were, let it stand for 6 minutes. The obtained solutions were added 300 μl 200 g/l sodium carbonate then let stand for 2 hours at 20 °C. The absorbances were measured with 1 cm cuvettes by spectrophotometer. The blank solution was made without extract [19].

2.6 Determination of the anthocyanin content

The anthocyanin content was determined using the monograph of Myrtilli fructus from Hungarian Pharmacopoeia VIIIth edition.

The anthocyanin compounds are sensible to pH changes, and they change colour in an acidic medium. This chromatism is followable on 528 nm spectrophotometric. The more intensive the color of the extract the highest the anthocyanin content is.

Five g juice/seed powder was treated with 95 ml methanol (R). The obtained mixtures were shaken for 30 min and at 25 °C. The obtained extracts were diluted up to fifty-fold with 0.1% V/V HCl solution and homogenised. For the absorbance measuring, two-fold dilutions were done. The light-absorption on the wave mentioned above length with 1 cm cuvettes in spectrophotometer were defined. As a blank solution, the HCl (*R*) 0.1% in methanol solution was used [20].

2.7 Definition of the antioxidant capacity

2.7.1 The ABTS-method

The ABTS method is based on the antioxidant of potassium persulfate, therefore creating a bluish bluish-green chromophore group that discolours in contact with antioxidant features compounds. On 734 nm with a spectrophotometer, the reaction is traceable. In the first step, 16.5 mg potassium persulfate was dissolved in 25 ml distilled water. Into 2.6 ml of this solution was dissolved a 10 mg ABTS tablet (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)). The solution was stored in a dark place for 12–16 hours. To do adequate measurements, the absorbance of the solution must be at around 0.9 on 734 nm. The preparation of the formula happened in the following way: 10, 20, 25, 30, and 40 μl solutions were pipetted and added to 2.5 ml ABTS solution. All these solutions were shaken and let stand for 6 minutes, and then the absorbance was measured with a spectrophotometer in 1 cm cuvettes [21].

2.7.2 The DPPH-method

The method was developed by Blois in 1958 [21]. The compound DPPH is made up of 1,1 diphenyl-2-2 picryl-hydrazyl, a stable free radical, a purple solution whose color fades off in the presence of a compound with antioxidant capacity. The changing of colour intensity can be measured with a spectrophotometerat 515 nm.

0.010 g of DPPH was measured by using an analytical balance, and it was dissolved in 25 ml methanol. This solution was stored in a dark place for 10–12 hours. The concentrations were chosen between 20–80 inhibition percentages. Then were taken from this solution 10 ml and diluted with methanol up to 100 ml. For measurements, 2.5 ml DPPH solution pipetted, then different volumes of extracts (10, 20, 25, 30, 40 μl) of the proper sample and absorbances were recorded after 6 minutes against methanol [22].

2.8 Quantitative determination of polyphenols by HPLC

The quantitative determination of polyphenolsis listed at Section 2.1., was performed using the HPLC technique. The juice and the watery extracts of seeds were used because these had the highest polyphenol content and showed the most increased antioxidant activity, using the ABTS and DPPH method.

For the HPLC-DAD method, a Merck Hitachi L-7000 HPLC system was used with a DAD detector and NUCLEODUR C18 Gravity, 3 μm, 150 × 3 mm (Macherey-Nagel) column as stationary phase and a flow rate of 0.750 ml/min. The sample volume was 50 μl using the loop injection mode. The best chromatogram extraction was recorded at 205 nm.

A gradient elution program was applied, according to Table 1.

Table 1 The gradient elution program of the used HPLC method

Time (min)	Phosphate buffer pH = 2.5 (10 mM) (%)	Acetonitril (%)
0	92	8
4	92	8
30	60	40
35	60	40
35.1	92	8
40	92	8

2.9 Statistical analysis

Results were expressed as mean ± standard deviation. Statistical differences were evaluated with a Pearson correlation coefficient calculator online. The results with $p < 0.05$ were considered significant in the case of total polyphenols with flavonoids and each constituent with ABTS or DPPH tests separately.

3 Results

The total flavonoid content was illustrated in Table 2. The results were expressed in μg quercetin equivalent/g seed powder and in the case of juice to μg flavonoid/ml juice. A calibration curve was made to measure the standard solution. With the help of this, we managed to calculate the results with the following calibration equation $y = 0.0043x - 0.0665$.

The flavonoid content is similar in the ethanol 50% (46 μg/g) and water extracts (57 μg/g), and in the methanolic extract, we had a rich content of flavonoids 416 μg/g, and in the juice, the flavonoid content is high 706 μg/ml.

In the case of total polyphenol content, to calculate the results, we made the calibration curve with gallic acid; the equation: $y = 0.0528x + 0.0141$ to this, the solutions were disposed with 0. 50, 100, 150, 250, 500 mg/L gallic acid. We measured the absorbance, and the results were illustrated in Table 2. The results were expressed in μg equivalent gallic acid/ g seed powder and μg polyphenol/ml juice.

The polyphenol content is similar in the ethanol 50% (8.29 μg/g) and water extracts (8.81 μg/g), and in the methanolic extract, we had a lower content of polyphenols 7.01 μg/g. In the juice, the polyphenol content is high: 47.5 μg/ ml.

At the definition of anthocyanin content, we have expressed the results in cyanidin-3-glucoside chloride, which are presented in Table 3. At the definition of antioxidant capacity, as the ABTS, as in the DPPH method case, the obtained values we expressed in IC 50% are illustrated in Table 4.

The anthocyanin content, expressed in cyanidin-3-glucoside, is higher in seed extract (6.47 mg/100 g) than in juice (3.95 mg/100 ml).

Table 2 The total phenolic and flavonoid content of pomegranate juice and seeds with methanolic, ethanolic (50%) and watery extracts

Sample	Juice (μg/ml)	Seeds (μg/g)		
		Methanolic	Ethanolic (50 %)	Watery
Flavonoids	706 ± 71.5	416.3 ± 1.7	46.1 ± 3.19	57.7 ± 6.1
Polyphenols	47.5 ± 9.0	7.0 ± 0.89	8.2 ± 0.29	8.8±1.91

The antioxidant activity increased in the extracts as follows: juice < water < methanol < ethanol 50% (Table 4) which is not correlated with the total polyphenol content (juice: 47.5 μg/ml; water extracts: 8.8 μg/g; ethanolic 50% extracts: 8.9 μg/g; methanolic extracts: 7.0 μg/g) and total anthocyanin content (juice: 3.95 mg %; seeds: 6.47 mg %) measured with the spectrophotometric methods. The measured polypenols amount using the HPLC method (Table 5) shows higher values in the case of the watery extracts, which correlates with the IC50 (is the concentration required to result in a 50% antioxidant activity, to scavenge 50% of the initial ABTS/DPPH radicals) values obtained with the ABTS method.

A comparison of the major constituents in the seed extracts and the commercially available pomegranate juice was positively evaluated. The link between the antioxidant activity of juice polyphenols, flavonoids, and anthocyanins has been confirmed partially.

The HPLC-UV-VIS analysis confirmed the presence of the following polyphenols in the pomegranate juice (given in μg/ml) and seed (given in μg/mg): chlorogenic acid (43.13 μg/ml and 53.59 μg/g), caffeic acid (1.23 μg/ml and 12.95 μg/g), catechin (0.08 μg/ml and 42.57 μg/g), epicatechin (3.06 μg/ml and 30.55 μg/g), quercetin (2.33 μg/ml and 31.05 μg/g), isoquercitrin (0.49 μg/ml and 10.20 μg/g) and kaempferol (2.26 μg/ml and 42.26 μg/g), respectively (Table 5). The corresponding chromatograms are presented in Figs. 1 and 2.

4 Discussion

In our experiments, we worked with pomegranate juice and pomegranate-seed with seed-coat because many articles focused on its possible use in cancer therapy and other illnesses.

At the determination of flavonoid content, we can observe that in the case of the three seed extract, the methanolic one the highest flavonoid content, the highest flavonoid

Table 3 The anthocyanin content of pomegranate juice and seeds

Sample	Juice (mg/100 ml)	Seeds (mg/100 g)
Anthocyanin (cyanidin -3-glucoside)	3.95 ± 0.13	6.47 ± 18.5

Table 4 In vitro antioxidant activity of pomegranate juice and pomegranate seeds in the three types of extracts

Sample method	Juice (IC50 μg/mL)	Seeds (IC50 μg/mL)		
		Methanol	Ethanol (50%)	Water
ABTS	25.40 ± 2.3	2.87 ± 0.25	2.52 ± 0.37	9.15 ± 1.9
DPPH	54.24 ± 9.8	14.70 ± 3.5	10.58 ± 442	18.27 ± 2.9

Table 5 The amount of polyphenols in the tested samples

Compound	Pomegranate juice (µg/ml)	Watery extract of the pomegranate seeds (µg/g)
Chlorogenic acid	43.13	53.59
Caffeic acid	1.23	12.95
Catechin	0.08	42.57
Eepicatechin	3.06	30.55
Quercetin	2.33	31.05
Isoquercitrin	0.49	10.20
Kaempferol	2.26	42.26

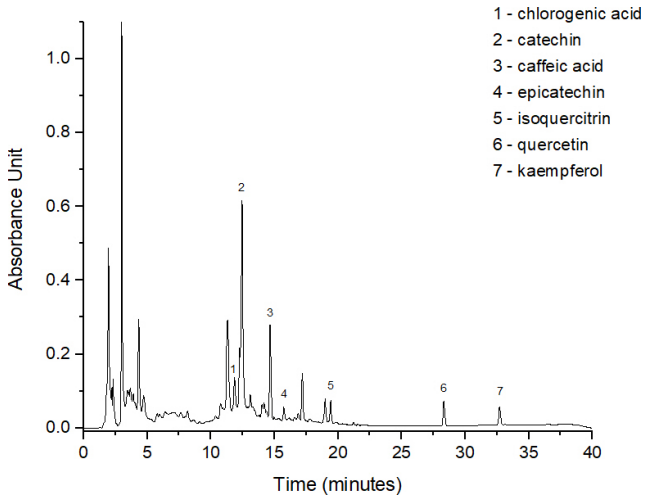

Fig. 1 HPLC chromatogram recorded at 205 nm with pomegranate juice

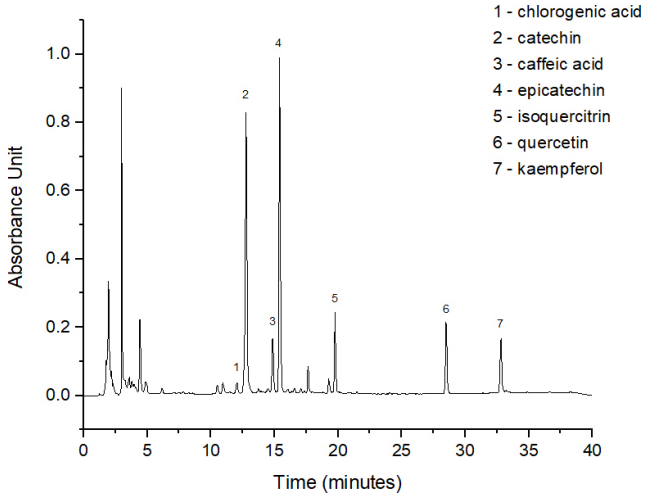

Fig. 2 HPLC chromatogram recorded at 205 nm from watery extracts with pomegranate seeds

content, 416 ± 1.76 µg QE/g seed powder. The measured flavonoid content from the juice was 706 ± 71.5 µg/ml. According to this determination, in any case of well ripe pomegranate, the flavonoid composition correlates with the total juice quantity. In our case, the concentration of flavonoids was more minor compared to literature data. For example, the pomegranate lyophilised fruits from Algeria have 12.95 mg/g QE [23].

At the determination of total polyphenol content from all the three types of seed extracts, the highest polyphenol value in the watery (8.8 ± 1.91 µg GA/g seed powder) and ethanolic 50 % extracts (8.29 ± 0.29 µg GA/g seed powder) was observed. The mean of the polyphenol content of juice was 47.5 ± 9 µg/ml, the highest concentration at this determination. In a study by Zeghad et al., higher concentrations of polyphenols from lyophilised fruits, with 15.39 mg GAE/ were obtained g [23].

To measure anthocyanin content, we worked with juiced and a hydrochloric acid seed extract. The seed extract showed a higher anthocyanin value (6.47 mg/100 g ± 18.5) than the juice (3.95mg/100 ml ± 0.13).

At measuring the antioxidant capacity, expressed as IC50 value, there is an inverse correlation with the polyphenol content. The lower measured value shows a higher antioxidant capacity.

The ABTS and DPPH radical scavenging activities of the tested extracts are shown in Table 4. According to both methods, the ethanolic extract of the seeds has the highest antioxidant capacity.

The results from the literature are varied. For example, the value of DPPH antioxidant activity from fermented juice can not be compared with our results [23]. Gullon and Pintado, in 2015, the DPPH and ABTS values expressed as mg Trolox equivalents/g, and they worked from peel flour. With DPPH, they obtained 52.36 mg Trolox/g and for ABTS, 41.24 mg Trolox/g. [24]. The Algerian scientists, Zeghad and Ahmed, from slyophilised fruits, obtained ABTS 0.58 mg/ml and DPPH 0.6 mg/ml values [23].

Mayasankaravalli and Deepika in 2020 for fruit peels with the DPPH method from watery extracts obtained 87% and from ethanolic extracts 78%, which are lower antioxidant capacity than our results [15].

5 Conclusion

At determining flavonoids and total polyphenolics, we can conclude that the seed extracts contain these compounds in significant amounts.

With regard to both the watery seed extract with high polyphenol content and the methanol seed extract with high flavonoid content, the juice contains a lot of active ingredients in both situations. Attending to methanolic, ethanolic 50% and watery seed extract with polyphenol content, have a small amount of these constituents. However, the juice disposed with high active agent content in the cases.

The anthocyanin content values show that the seed extract contains anthocyanin in a larger, more significant concentration than the juice.

In both examination methods (ABTS and DPPH), the juice showed a great antioxidant capacity.

Finally, during these examinations, we can express that the juice disposes with a rich active agent content. Owing to these active agents' synergistic effect, these have a helpful impact on the human body, can help us get over some conditions and improve our quality of life.

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