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Polyphenols isolated from pomegranate juice (*Punica granatum L.*): Evaluation of physical-chemical properties by FTIR and quantification of total polyphenols and anthocyanins content

Isolamento de polifenóis do suco da romã (*Punica granatum L.*): Avaliação das propriedades físico-química por FTIR e quantificação do teor total de polifenóis e antocianinas

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ABSTRACT

Pomegranate (*Punica granatum L.*) is a polyphenols source, such as anthocyanins, punicalagin, ellagitannins and tannins. Polyphenols are antioxidant compounds present in foods as cereals, fruits (peels, seeds and juice), vegetables, wine, and among others. Polyphenols are responsible to protect cells and to limit the risks of degenerative and tumoral diseases, as well as, to prevent cardiovascular diseases, neuronal diseases, and present important anti-inflammatory effects. The purpose of this study was to isolate polyphenols from pomegranate juice using solvents without heating. The solvents used were ultrapure water, ethanol, ethanol 70% and methanol. The samples were characterized by FTIR to evaluate the physical-chemical properties, the total polyphenols content was quantified by Folin-Ciocalteau method, using gallic acid as standard equivalent, and the total anthocyanins content was quantified by pH-differential method, using anthocyanins (cyanidin-3-glucoside) as standard equivalent. FTIR spectra showed the main characteristic groups of polyphenols, as hydroxyl group and stretching vibration of benzene rings. And the characteristic groups of solvents were CH_2 ou CH_3, hydroxyl, carboxyl e carbonyl group. For the quantification of total polyphenols and anthocyanins content, the best results were found to the pomegranate juice: ultrapure water samples. According to the data obtained it was possible to conclude, who the process employed was effective to isolate the polyphenols from pomegranate juice and the use of different types of solvent influenced in the achievement of these results.

Keywords: Pomegranate juice; Polyphenols; Anthocyanins.

RESUMO

Romã (*Punica granatum L.*) é uma fonte de polifenóis como as antocianinas, punicalagina, elagitaninos e taninos. Os polifenóis são compostos antioxidantes presentes em comidas como cereais, frutas (casca, sementes e suco), vegetais, vinho e entre outros. Polifenóis são responsáveis por proteger as células e limitar os riscos de doenças degenerativa e tumoral, assim como, prevenir doenças cardiovasculares, doenças neuronais e apresentam importantes efeitos anti-inflamatórios. O objetivo desse estudo foi isolar polifenóis do suco da romã usando solventes sem aquecimento. Os solventes usados foram água ultrapura, etanol, etanol 70% e metanol. As amostras foram caracterizadas por FTIR para avaliar as propriedades físico-química, o teor total de polifenóis foi quantificado pelo método de Folin-Ciocalteau, usando o ácido gálico como equivalente padrão e o teor total de antocianinas foi quantificado pelo método de pH-diferencial, usando antocianinas (cianidina-3-glucosídeo) como equivalente padrão. Os espectros de FTIR mostraram os principais grupos característicos dos polifenóis, como grupo hidroxil e estiramento de vibrações de anéis de benzeno. E os grupos característicos para os solventes foram os grupos CH_2 ou CH_3, hidroxil, carboxil e carbonil. Para quantificação do teor total de polifenóis e antocianinas, os melhores resultados foram encontrados para as amostras de suco de romã: água. De acordo com os dados obtidos foi possível concluir, que o processo empregado foi efetivo para isolamento dos polifenóis do suco da romã e que o uso de diferentes tipos de solvente influenciou na obtenção desses resultados.

Palavras-chave: Suco da romã; Polifenóis; Antocianinas.

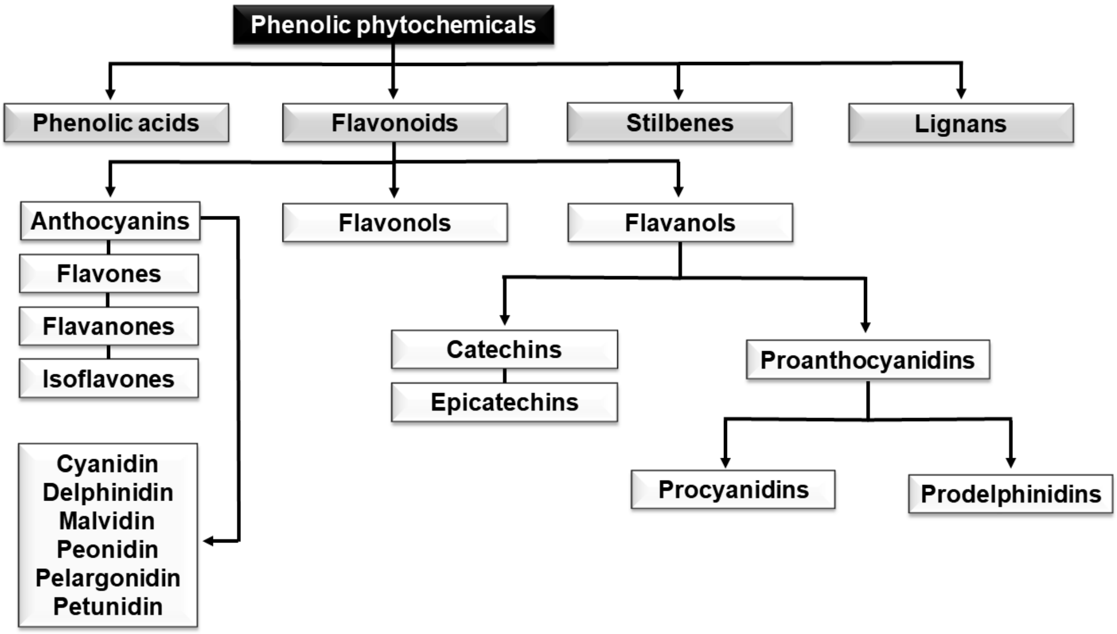
1 INTRODUTION

Pomegranate (*Punica granatum L.*) belongs to family Punicaceae, being cultivated in subtropical and tropical region, the fruit has been used in many countries and cultures of the worldwide (PUTNIK et al., 2018; BASSIRI-JAHROMI; DOOSTKAM, 2019). The pomegranate fruit contains many arils (red pulp and seeds) separated by a membrane called of pericarp. Pomegranate (peel, seeds and juice) has a valuable source of polyphenols, such as punicalagin, ellagitannins, tannins, flavonoids, anthocyanins and among others (AMBIGAIPALAN; CAMARGO; SHAHIDI, 2016; BASSIRI-JAHROMI; DOOSTKAM, 2019).

Polyphenols are originated from metabolism secondary of plants acting as an antipathogenic agent and contributing to pigmentation, and they are responsible for colour, astringency, aroma and oxidative stability (NACZK; SHAHIDI, 2004; MARTÍN et al., 2017). In our diets, the polyphenols are the most abundant antioxidants present in vegetables, cereals, teas, wines, and fruits such as orange, tangerine, cherry, grapes, blueberries and pomegranate (D'ARCHIVIO et al., 2007; FREITAS, 2019).

Chemically, the polyphenols present several hydroxyl groups on aromatic rings. Thus, polyphenols are classified by classes, according to number of phenol rings and structural elements to bind these rings. Thereby, the main polyphenols groups are phenolic acids, flavonoids, stilbenes and lignans (MARTÍN et al., 2017). Figure 1 describe the flowchart of classes and sub-classes of polyphenols.

Figure 1. Flowchart of classification of polyphenols based in the types of phenolic phytochemicals.



Anthocyanins belong to flavonoids class, being the largest group of water-soluble pigments (SHAIDI; NACZK, 2005). Basically, the structural chemical of anthocyanins is formed by benzene rings bonds (C6-C3-C6) who differ by number, position and hydroxylation and methoxylation degree of rings. In literature, there are six main anthocyanins described, being cyanidin, delphinidin, malvidin, peonidin, pelargonidin and petunidin (DELGADO-VARGAS; JIMENEZ; PAREDES-LOPEZ, 2010).

Anthocyanins from pomegranate has bioactive properties, although these properties may be varying according to cultivation type, growing, location, climate, and the maturity at harvest. In literature, the pomegranate is considered the healthy food, due its benefits and high antioxidants content. The mains medicinal properties related are antioxidant activity, wound healing, antidiabetic, antitumoral, anti-inflammatory, antimicrobial, and cardiovascular protective effect against low-density lipoprotein (LDL), high-density lipoprotein (HDL), and atherosclerosis (AVIRAM; ROSENBLAT, 2013; NASCIMENTO JÚNIOR et al., 2016; PUTNIK et al., 2018).

The purposes of this study was to isolate polyphenols from pomegranate juice using solvents applied mechanical agitation followed by centrifugation without heating. The solvents used were ultrapure water, ethanol, ethanol 70%, and methanol. The samples were characterized by FTIR to evaluate the physical-chemical properties. Total polyphenols content was quantified by folin-ciocalteau method, using gallic acid as standard equivalent and the total anthocyanins content was analysed by pH-differential method, using anthocyanins (cyanidin-3-glucoside) as standard equivalent. Total polyphenols and anthocyanins content were evaluate using spectrophotometric methods.

2 MATERIALS AND METHODS

2.1 MATERIALS

Pomegranate (*Punica granatum L.*) (Harvest in Sorocaba, São Paulo, Brazil), ethanol (Synth, São Paulo, Brazil), ultrapure water (18,2 $M\Omega.cm^{-1}$), and methanol (Chemco, São Paulo, Brazil) were used to obtain the samples. Gallic acid (Dinâmica, São Paulo, Brazil), folin-ciocalteau reagent (Dinâmica, São Paulo, Brazil) and sodium carbonate (Na_2CO_3) were used to total polyphenols content. Potassium chloride (Dinâmica, São Paulo, Brazil) and sodium acetate (Dinâmica, São Paulo, Brazil) were used to quantified total anthocyanins content. All reagents used was analytical grade.

2.2 OBTAINING OF POMEGRANATE JUICE (*PUNICA GRANATUM L.*)

Pomegranate was harvested from tree located in Sorocaba (São Paulo, Brazil) before complete maturation. After manual harvesting, the fruit were transported to Laboratory of Biomaterials and Nanotechnology (LaBNUS). Figure 2 showed the pomegranate fruit portions. In this study was used the arils (red pulp and seeds), to obtain the pomegranate juice. Briefly, the fruits were selected and washed with water to remove impurities. And thus, manually were cut to separate the peels and arils. The pomegranate juice was obtained by compressed of arils, and after this process, the juice was stored in freezer (-18 °C).

Figure 2. Whole pomegranate and pomegranate portions (peels and arils).

2.3 POLYPHENOLS ISOLATED FROM POMEGRANATE JUICE (*PUNICA GRANATUM L.*)

Polyphenols isolated from pomegranate juice was performed using four types different of solvents. Briefly, the samples were prepared in the proportion 1:10 (sample: solvent), and the solvents used were ultrapure water, ethanol (99.5%), ethanol 70% and methanol. The sample: solvent (v/v) were agitated by orbital shaker (Tecnal, TE-4200, Piracicaba, Brazil) in 100 rpm at 25 °C during 60 minutes, and in sequence, the samples were centrifuged (Celm, Combate, Barueri, Brazil) in 3.400 rpm (2.232 g-force) during 30 minutes. After this process, the supernatant was collected and stored in freezer (-18 °C).

2.4 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

FTIR (IRAffinity-1S. Shimadzu, Kyoto, Japan) analysis was used to determine the chemical interaction between pomegranate juice and solvents. The characterization of specific chemical groups was evaluated by FTIR using the attenuated reflectance technique (FTIR-ATR) in the transmittance mode. Spectra were obtained in the wavelength range from 4000 to 600 cm^{-1}, with a resolution of 4 cm^{-1} and 128 scans. The results were collect using Labsolutions Software. The spectra were normalized, and the vibration bands were associated with the main chemical groups of each component of the samples.

2.5 ANALYTICAL CURVE TO DETERMINE TOTAL POLYPHENOLS CONTENT

Total polyphenols content was determined by folin-ciocalteau method, described by Çam; İçyer (2013) and Fawole; Opara (2016) with modifications. For the quantification, the gallic acid was used as standard equivalent. The stock solution was prepared by dissolution aqueous of gallic acid (5 wt.%). Further dilutions were also performed in ultrapure water to obtain the calibration curve, in concentration range of 50-400 µg/mL. After this preparation, in each dilution was collect 500 µL and transferred in a glass test tube and added 2.5 mL of folin-ciocalteau reagent and 2.0 mL of sodium carbonate. The mixture was incubated for 15 min. in a thermostatic bath (Brookfield, TC 550, Middleborough, USA) at 50 °C and fast cooled in a freezer at -18 °C for 5 min.

The calibration curve had obtained by interpolation method, which unequivocally related to the analyte concentration, with the corresponding analytical signal at 760 nm (λ), resulting from a linear relationship. The coordinates between at least two points on the straight line was defined as the set of points, whose coordinates satisfied the linear equation. The calibration curve had carried out in triplicate on seven points. To interpolation of signal with analyte concentration was used spectrophotometer (Femto 800XI, São Paulo, Brazil).

2.6 TOTAL POLYPHENOLS CONTENT

Total polyphenols content was evaluated according to the folin-ciocalteau method. Briefly, the samples of 50 µL were dispersed in 2.5 mL of folin-ciocalteau reagent (2:10) and 2.0 mL of sodium carbonate (7.5%). The samples were maintained at 50 ºC in thermostatic bath, and in sequence, cooled in freezer (-18 ºC) for 5 min. The samples were analyzed in triplicate and the total polyphenols content was determined at wavelength (λ) of 760 nm by spectrophotometer, using FemtoScan Software. The results were expressed as µg of gallic acid equivalents (GAE) per mL of samples.

2.7 TOTAL ANTHOCYANINS CONTENT

Total anthocyanins content was evaluated according by the pH-differential method described by Lee; Durst; Wrolstad (2005) and Ambigaipalan; Camargo; Shahidi (2016) with slights modifications. The samples of 0.5 mL were dispersed in 9.5 mL of 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5), separately. After 20 min, the samples were analysed in triplicate at 520 and 700 nm by spectrophotometer, using FemtoScan Software. The results of anthocyanins (cyanidin-3-glucoside) content were expressed in µg per mL of sample, being calculated according to Eq. (1).

$$C\ (mg/L) = \frac{[(A520 - A700)\ pH\ 1.0 - (A520 - A700)\ pH\ 4.5].MM.DF.10^3}{\varepsilon\ .\ 1} \tag{1}$$

Where C (mg/L) represents total anthocyanins (cyanidin-3-glucoside) content, A is absorbance, MM is molecular weight of cyanidin-3-glucoside (449.2 g/mol), DF is dilution factor, 10^3 is used to conversion from gram to milligram, ε is molar absorptivity (26.900), and 1 is de path length (1 cm).

2.8 STATISTICAL ANALYSIS

All data were presented with the mean values ± standard deviation (SD). The data obtained were evaluated by one-way analysis of variance (ANOVA), followed by Tukey analysis. The test was performed using 95% of confidence interval. A two-sided P value of <0.05 was considered significant.

3 RESULTS AND DISCUSSION

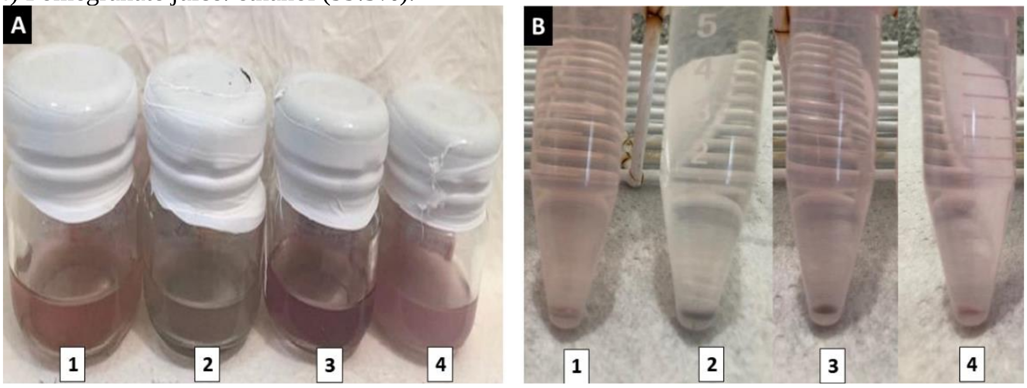
3.1 POLYPHENOLS ISOLATED FROM POMEGRANATE JUICE (*PUNICA GRANATUM L.*)

Polyphenols isolated from pomegranate juice was carried out with liquid-liquid samples (1:10). The liquid-liquid is a process to separate the components, due its distribution between two liquid phases. This process can be occurs, when the transfer of mass from one liquid phase into second liquid phase, this method may be performed in many different ways (ROBBINS; CUSACK, 1999; MARTÍN et al., 2017).

Therefore, in this study the method applied was the liquid-liquid process without heating, associating mechanical agitation, followed by centrifugation. Thereby, the centrifugation method act as sorting out of mixture, causing the suspended particles to tend to sedimentary (sedimentation process), and this is due to the difference in density, influenced by the centrifuge acceleration (g-force) that is employed in samples (ANLAUF, 2007). This sedimentation process that occurs can be seen in Figure 3 (B).

Figure 3 shown the samples of pomegranate juice (A) before and (B) after the polyphenols isolate process. Polyphenols isolate from pomegranate juice was performed using different solvents, being ultrapure water, methanol, ethanol 70%, and ethanol (99.5%).

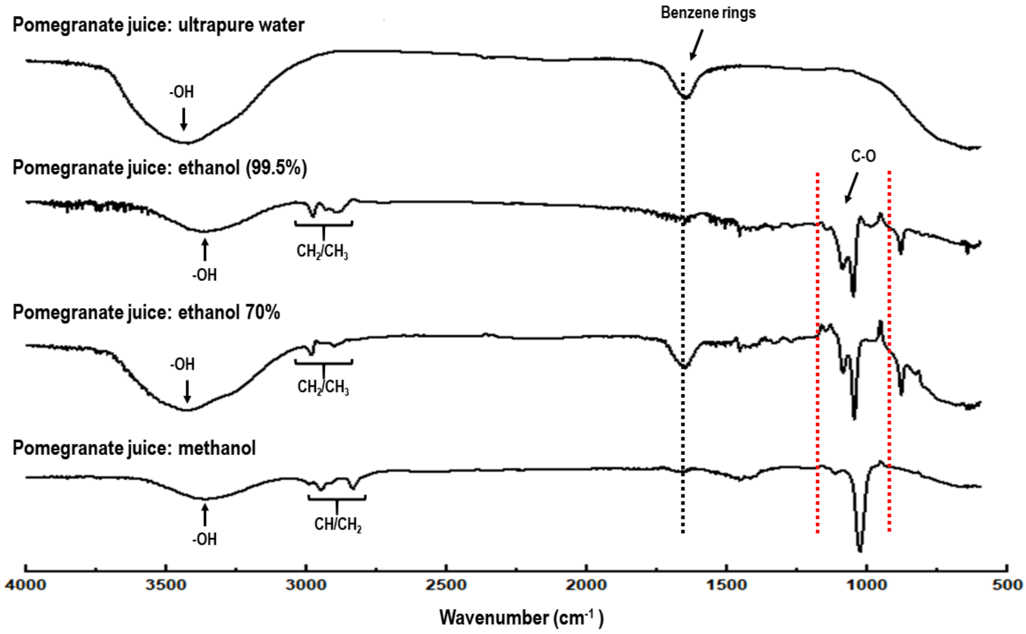
Figure 3. Samples of pomegranate juice (A) before and (B) after polyphenols isolate process. The number identify the samples, being (1) Pomegranate juice: ultrapure water; (2) Pomegranate juice: methanol; (3) Pomegranate juice: ethanol 70%, and (4) Pomegranate juice: ethanol (99.5%).



3.2 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

The mid-infrared spectrometer was used to analyse the fundamental vibration energy linked rotational-vibrational that attached to interaction of infrared radiation between pomegranate juice samples. Infrared spectra were analysed in graph with FTIR in transmittance mode. The Figure 4 shows the samples of pomegranate juice with ultrapure water, ethanol (99.5%), ethanol 70%, and methanol.

Figure 4. FTIR spectra in samples of polyphenols isolated pomegranate juice. All spectra were obtained at 23±1ºC (room temperature).



The two characteristic peaks of pomegranate shown in the FTIR spectra (Figure 4) in pomegranate juice: ultrapure water. The 3421 cm^{-1} peak was corresponding hydroxyl group (-OH) and 1651 cm^{-1} peak was related to C=C stretching vibration of benzene rings (black dashed line) (EDISON; SETHURAMAN, 2013; OLIVEIRA et al., 2016), these peaks indicate the presence of polyphenols in pomegranate juice: ultrapure water samples.

Pomegranate juice: ethanol (99.5%) sample was observed characteristics peaks of ethanol and an intensity reduction between 1650-1600 cm^{-1} of C=C group (black dashed line). The region between 3371-3419 cm^{-1} was related to -OH group, the peak in 3000-2980 cm^{-1} were related to stretching vibration of CH_2 or CH_3 groups (ÖMEROĜLU AY et al., 2012; OLIVEIRA et al., 2016), and the 1200-900 cm^{-1} bands were observed frequencies specific of C-O bonds (red dashed line) (COLDEA et al., 2013).

While in pomegranate juice: ethanol 70% sample in 3419 cm^{-1}, 2982 cm^{-1} and 1649 cm^{-1} corresponding, respectively, stretching vibration of hydroxyl group, CH_2 or CH_3 and stretching vibration of C=C (black dashed line) was similar with pomegranate juice: ultrapure water sample (ÖMEROĜLU AY et al., 2012; OLIVEIRA et al., 2016). And peaks observed between 1200-900 cm^{-1} bands were specific stretching of C-O bonds (red dashed line) (COLDEA et al., 2013).

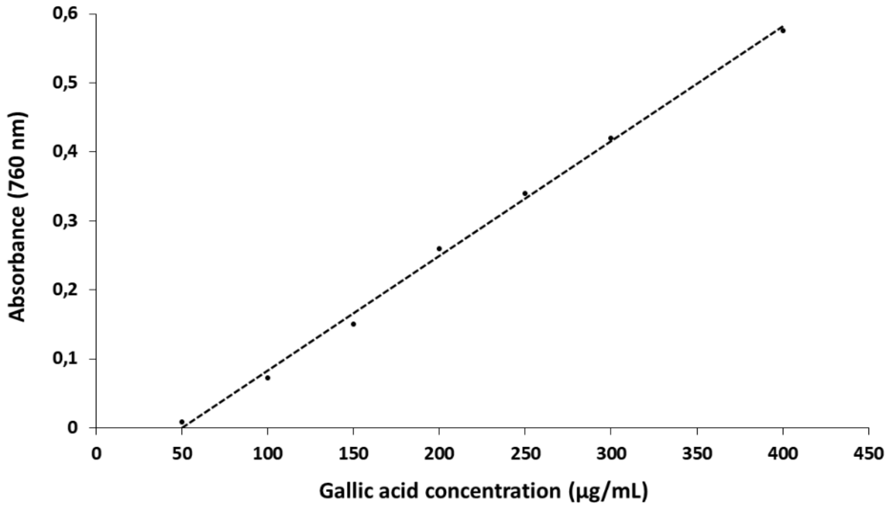
Pomegranate juice: methanol sample was observed characteristics peaks of methanol and an intensity reduction between 1650-1600 cm^{-1} of C=C group (black dashed line). The peak found were resemble with methanol, as described by Coldea et al., (2013) and Rizwana; Alwhibi; Soliman

(2016). Peak obtained at 3358 cm^{-1} represents hydroxyl group, the CH and CH_2 stretching were observed at 2943 cm^{-1} and 2833 cm^{-1}, and the peak at 1028 cm^{-1} represents C-O stretch (red dashed line).

3.3 ANALYTICAL CURVE TO DETERMINE TOTAL POLYPHENOLS CONTENT

Analytical curve (Figure 5) was developed to determine total polyphenols content. The linearity was obtained between 50-400 µg/mL, corresponding to absorbance values between 0.01 and 0.6. The correlation coefficient was of 0,998 and linear equation obtained was $y=0{,}0017x - 0{,}0832$. The calibration curve is an empirical equation that relates the response of a specific instrument to the concentration of specific sample analyse. The analytical method obtained by spectrophotometer to determine polyphenols content was simple, reliable, easy to perform, reproducible and low-cost (BLAINSKI; LOPES; MELLO, 2013).

Figure 5. Analytical curve to determine total polyphenols content in samples of polyphenols isolated from pomegranate juice.

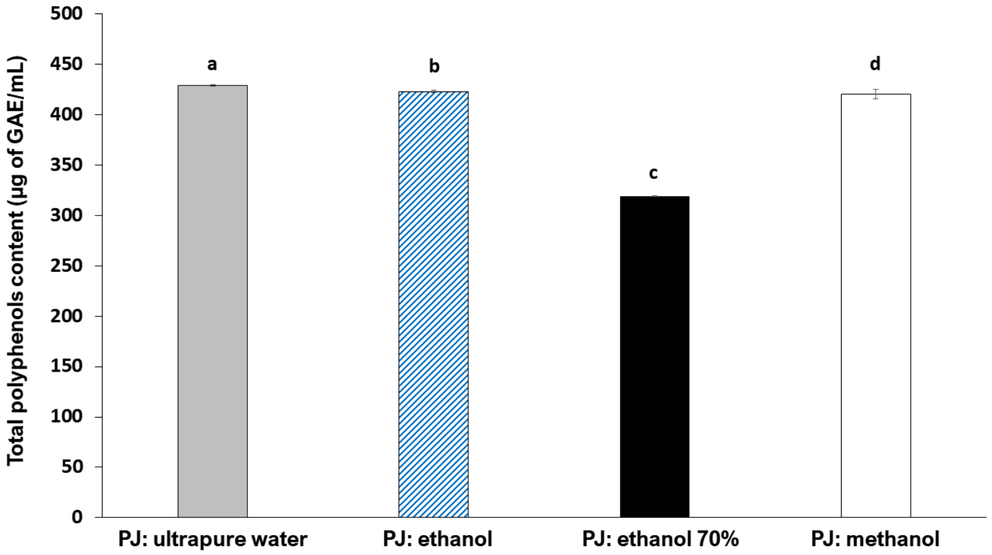


3.4 TOTAL POLYPHENOLS CONTENT

Total polyphenols content of samples were evaluated by folin-ciocalteau method. This colorimetric method relies in the reduction of phosphomolybdic-phosphotungstic acid reagent, due the transfer of electrons in alcaline medium of polyphenols to obtain blue complexes able to read by spectrophotometer (NACZK; SHAHIDI, 2004).

Total polyphenols content (Figure 6) was performed to analyze the polyphenols isolate from pomegranate juice using different types of solvents. The results were expressed as µg of gallic acid equivalents (GAE) per mL of samples. The results of total polyphenols content in pomegranate juice samples were different statistically ($p>0.05$). The results of samples showed who higher value was found to pomegranate juice: ultrapure water, followed by pomegranate juice: ethanol, pomegranate juice: methanol and pomegranate juice: ethanol 70%.

Figure 6. Total polyphenols content in samples of polyphenols isolated from pomegranate juice.



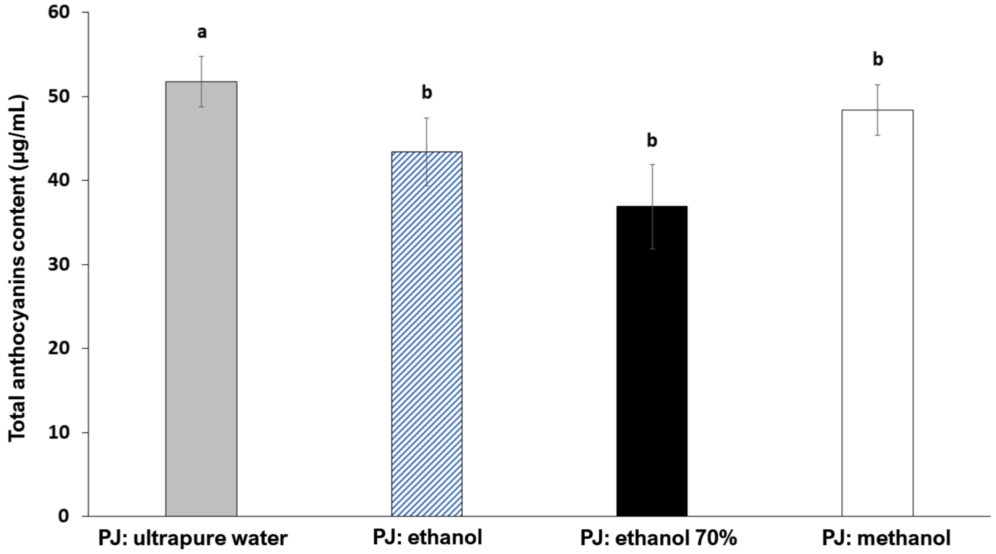
Note: PJ - Pomegranate juice. Equal letters (for the same analysis) indicate that there is no significant difference between the mean values ($p>0.05$) (n=3).

3.5 TOTAL ANTHOCYANINS CONTENT

Total anthocyanins content of samples were evaluated by pH-differential method. The pH is crucial factor that influence in the colouring of anthocyanins, due the anthocyanins may be different colour and structural form. The difference of absorbance obtained, in this method to promote infer the real anthocyanins fraction present in samples (TEIXEIRA; STRINGHETA; OLIVEIRA, 2008; BORDIGNON JR. et al., 2009).

Total anthocyanins content (Figure 7) was performed to determine the polyphenols isolate from pomegranate juice using different types of solvents. The results of anthocyanins (cyanidin-3-glucoside) content were expressed in µg per mL of sample. The higher result was found to pomegranate juice: ultrapure water, followed by pomegranate juice: methanol, pomegranate juice: ethanol 99.5% and pomegranate juice: ethanol 70%. The samples of pomegranate juice with methanol, ethanol (99.5%), and ethanol 70% were similar statistically ($p<0.05$), being different statistically ($p>0.05$) to pomegranate juice: ultrapure water.

Figure 7. Total anthocyanins content in samples of polyphenols isolated from pomegranate juice.



Note: PJ - Pomegranate juice. Equal letters (for the same analysis) indicate that there is no significant difference between the mean values (p>0.05) (n=3).

Table 1 present values (mean ± SD) of total polyphenols content *versus* total anthocyanins content in samples of polyphenols isolate from pomegranate juice. When correlated the results of total polyphenols and anthocyanins content, in the samples of pomegranate juice: ultrapure water was found 12% of total anthocyanins content, 11.5% to ethanol 70%, and 10% to ethanol (99.5%) and methanol. Therefore, the sample of pomegranate juice: ultrapure water presented higher result to total anthocyanins content.

Table 1. Results of total polyphenols content *versus* total anthocyanin content in samples of polyphenols isolated from pomegranate juice.

Samples	**Total polyphenols content (µg/mL)**	**Total anthocyanins content (µg/mL)**
PJ: ultrapure water	429,05±0,75 ^{a}	51,77±2,99 ^{a}
PJ: ethanol	423,01±1,29 ^{b}	43,42±4,98 ^{b}
PJ: ethanol 70%	319,16±0,75 ^{c}	36,92±4,99 ^{b}
PJ: methanol	420,43±4,66 ^{d}	48,43±2,97 ^{b}

Note: PJ - Pomegranate juice. Equal letters (for the same analysis) indicate that there is no significant difference between the mean values (p>0.05) (n=3).

The different results found in the analysis to FTIR (Figure 4), total polyphenols content (Figure 6) and total anthocyanins content (Figure 7), may be attributed to type of solvents used to promote the process of polyphenols isolated from pomegranate juice, due the solubility of polyphenols vary according to, the polarity of the solvents, the degree of polymerization of polyphenols and their interactions with other constituents of pomegranate juice (*Punica granatum L.*). The solvents most used, described in literature, were water, methanol or acidified methanol, acetone, ethanol and their combination (NACZK; SHAHIDI, 2004; ANGELO; JORGE, 2006; WANG, 2011).

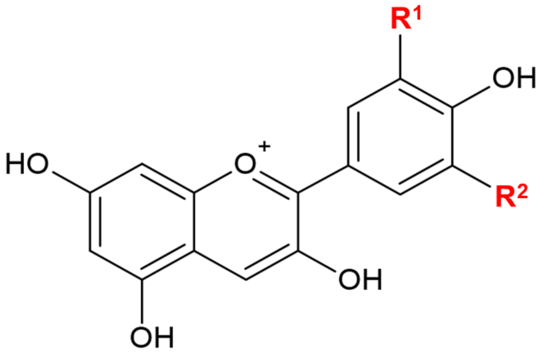
The compound solubility in solvent system depends on of structural characteristic of each molecule polar and apolar portion present in structure. Thus, molecules with a higher apolar ratio or poorly soluble in water decrease water solubility, thereby, its do not easily form hydrogen bonds. However, the polarity and dielectric constant is a crucial factors to improve the solubility, due its be a parameter that influences the dissolution process (GREMIÃO; CASTRO, 1999; MEDEIROS; KANIS, 2010).

According to Gremião; Castro (1999), in our study, the dielectric constant at 20 ºC were 25, 33.6, 41.6 and 80.4, respectively, to ethanol, methanol, ethanol 70% and water. Therefore, the better results of total polyphenols and anthocyanins content was found to pomegranate juice: ultrapure water, being the water the solvent with higher value of dielectric constant. Other factors that also influenced the process of polyphenols isolated from pomegranate juice in food are chemical nature, isolation method, sample particle size, storage time and conditions, thereby, the presence of interfering substances. The chemical nature of polyphenols vary from simple to highly polymerized substances, that include varying proportions of phenolic acids, phenylpropanoids, anthocyanins, tannins and among others substances (GREMIÃO; CASTRO, 1999; SHAIDI; NACZK, 2005).

Anthocyanins belong to a class of natural compounds known as flavonoids who also include flavones and isoflavones, who are formed via condensation of phenylpropane (C6-C3) compound. The anthocyanins constitute the largest group of water-soluble pigments in the plant kingdom, being present in tissues of higher plants, from leaves, stems, roots, flowers and fruits (peel, seeds and juice) (SHAIDI; NACZK, 2005; KHOO et al., 2017; FREITAS, 2019).

The chemical structural basic of anthocyanins (aglycone) is formed by C6-C3-C6. There are described, in literature, 17 types of anthocyanins who differ in the number, position and hydroxylation and methoxylation degree of rings, but six main of them are the most commonly described, being cyanidin, delphinidin, malvidin, peonidin, pelargonidin and petunidin (DELGADO-VARGAS; JIMENEZ; PAREDES-LOPEZ, 2010), these six main anthocyanins are described in Figure 8.

Figure 8. Chemical structure of the six main anthocyanins (cyanidin, delphinidin, malvidin, peonidin, pelargonidin and petunidin).



Cyanidin: **R**^{1}= -OH; **R**^{2}= H

Delphinidin: **R**^{1}= -OH; **R**^{2}= -OH

Malvidin: **R**^{1}= -OCH_3; **R**^{2} = -OCH_3

Peonidin:R^1= -OCH_3; **R**^{2}= H

Pelargonidin:R^1= H; **R**^{2}= H

Petunidin:R^1= -OH **R**^{2}= -OCH_3

According to Freitas (2019) the composition of anthocyanins are present in plants, foods and fruit (peel, seeds and juice), in general, beside this are described some specifically anthocyanins to some fruits. The cyanidin was found isolated in apples, figs and peach. Delphinidin in eggplant and pomegranate and some fruits have two or more types of anthocyanins as cherry, grapes and blueberries.

Anthocyanins-rich food are higher consumed due the potential antioxidant, therefore, it is having been widely studied and used in medicine for treat several diseases. Anthocyanins possess antidiabetic, anticancer, anti-inflammatory and antimicrobial effect. In the cardiovascular disease, the pomegranate are considered a healthy fruit, because have antioxidants agents to protecting low-density lipoprotein (LDL) and high-density lipoprotein (HDL) from oxidation, attenuates atherosclerosis development and decrease the blood pressure (AVIRAM; ROSENBLAT, 2013; KHOO et al., 2017).

Nascimento Jr. et al., (2016) described the use of pomegranate (*Punica granatum L.*) to promote the healing action of stomatitis induced by burns on the tongue of rats. The rats were treated with pomegranate juice by gavage (G1), pomegranate juice by gavage associated with local application of pomegranate peel tea (G2), and only local application of pomegranate peel tea (G3). And it was found that after 14 days, the G2 group showed the better results. Thus, confirming the findings who the pomegranate (*Punica granatum L.*), also has a healing action on the lingual mucosa of rats.

On the other hand, structurally the anthocyanins (Figure 8) present interest due its composition. Specifically, in the hydroxyl (-OH) terminal, for being attractive to acting as cross-linking agent. However, in literature, there are no studies who related anthocyanins as crosslinker effect in extracellular matrix (e.g. collagen) or biological devices, but there are some structural characteristics who evidence this potential. Cross-linking agent may be classified as chemical cross-linking agent (e.g. epoxy compounds, diphenylphosphoryl azide (DPPA) and glutaraldehyde) or natural cross-linking agent (e.g. genipin and proanthocyanidins). The cross-linking process can have chemical, physical, or biological approaches, and both are able to connect the group functional of polymers chain to another one through covalent, ionic or hydrogen bond (ORYAN et al., 2018; ALVES et al., 2019).

Although of use both cross-linking (chemical or natural), the natural cross-linking agent have more advantage, because its biocompatible and present low cytotoxic effect to clinical applicability (CHOI; KIM; MIN, 2016; ALVES et al., 2019). Thereby, some studies presented results using a natural cross-linking agent (ZHAI et al., 2013; PINHEIRO et al., 2015; VIDAL et al., 2016; WEI et al., 2018). Song et al., (2018) described the use of genipin as crosslinker to *in situ* formation of injectable hydrogel of chitosan-gelatine for sustained intraocular drug delivery. The use of proanthocyanidins were described by Choi; Kim; Min, (2016) and Alves et al., (2019) to promote crosslinker effect in scaffold to application in medicine regenerative.

4 CONCLUSION

According to set of results findings it concludes who, the technique employed to isolate polyphenols from pomegranate juice (*Punica granatum L.*) by liquid-liquid process using solvent method, and the quantification of total polyphenols and anthocyanins content were effective and satisfactory. In the study, it may conclude that the solvents used influenced in directly in process, being confirmed in the results of FTIR, total polyphenols content and total anthocyanins content. Thus, in the results were observed who sample obtained with ultrapure water (pomegranate juice: ultrapure water) showed the better results. Although the method applied is satisfactory and effective to isolation, the results may be optimized to future applications. The use of anthocyanins have been widely studied, due the significant results in disease treatment, presented the potential to antioxidants effect, anti-inflammatory effect, preventing cardiovascular diseases and neurodegenerative diseases.

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