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Research Article

EFFECTIVE EXTRACTION OF POLYPHENOLS AND PROANTHOCYANIDINS FROM POMEGRANATE'S PEEL

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ABSTRACT

Pomegranate peels (Punica granatum) are usually discarded as waste even a significant portion of polyphenols are often present in high concentrations in the outer parts of fruits. The objective of this study was to establish an extraction procedure for polyphenols and proanthocyanidins from pomegranate's peels (PMP), and to assess their potential radical scavenging activity. Water extraction efficiency at extraction temperature of 20 to 90 °C and extraction times of 5 to 60 min were studied. The recovery of polyphenols and proanthocyanidins was the highest at 50°C for 20 min. Water gave the highest extract yield of polyphenols and proanthocyanidins (17.78%, 1.22%) respectively, followed by 50% aqueous ethanol while ethyl acetate gave the lowest extract yield (0.75%, 0.049%) respectively. The DPPH (2,2-Diphenyl-1- picrylhydrazyl) radical scavenging activity of extracts had a linear relationship with the polyphenols yield in the extracts. The study of the effect of different pH medium revealed that polyphenols were more extractable by water and proanthocyanidins recovery was better in a buffer at pH 3.5. The extracts were stable in a freezer for about two weeks. This study revealed that two sequential water extractions has the economic and safety merits, and can be used as an environmentally friendly method for producing antioxidants from the PMP.

Keywords: Pomegranate peel, Polyphenols, Proanthocyanidins, Extraction, Temperature, Time, Solvents, DPPH, pH

INTRODUCTION

Pomegranate peel (Punica granatum) is a nutritive-rich byproduct. It attracted attention due to its apparent anti-bacterial activity[1], wound-healing properties[2], anti-cancer activity[3], anti-atherosclerotic[4], anti-inflammatory[4] and anti-oxidative capacities[5]. This antioxidant capacity has been mainly attributed to the water-soluble polyphenols, proanthocyanidins, and hydrolysable tannins.[5]

Polyphenols are broadly distributed in the plant kingdom and are the most abundant secondary metabolites found in plants.[6] These phenolic substances or polyphenols include more than 8,000 compounds with great structural diversity (although each has at least one aromatic ring with one or more hydroxyl groups). They can be divided into 10 different classes depending on their basic chemical structure.

The most abundant polyphenols in the diet are phenolic acids (benzoic and cinnamic acids), and flavonoids (30 and 60% of the total, respectively).[7] In addition to this diversity, polyphenols may be associated with various carbohydrates and organic acids and with one another.

Proanthocyanidins (PAs) are oligomeric and polymeric end products of the flavonoid biosynthetic pathway.[8] The existence of PAs in common foods including cereals, fruits, nuts and spices affects their texture, color, and taste.[9] They are increasingly recognized as having beneficial effects on human health[8] because of their potent antioxidant capacity and possible protective effects on human health in reducing the risk of chronic diseases such as cardiovascular diseases and cancers.[10]

On the one hand, phenolic acids occur in different forms in plants, including aglycones (free phenolic acids), esters, glycosides, and/or bound complexes. These different forms of phenolic acids show variable suitability for different extraction conditions and vary in their susceptibility to degradation. On the other hand, the common structure of flavonoids consists of two aromatic rings linked by three carbons that usually form an oxygenated heterocycle. In plants, flavonoids can be found as aglycones, although they are usually found as glycosides.[11]

The extraction of compounds from plant materials is the first step in the utilization of phytochemicals in the preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceutical, and cosmetic products.12 Solvent extractions are the most commonly used procedures to extract phenolic compounds from their plant sources and to liberate theme from the vacuolar structures where they are found due to their ease of use, efficiency, and wide applicability.13 It is generally known that the yield of chemical extraction depends on the type of solvents with varying polarities, extraction time and temperature, as well as on the chemical composition and physical characteristics of the samples.

However, efficient methods for extraction of PAs from pomegranate peel for designing efficient extraction process for their production from peels have not been widely studied.

Accordingly, the objective of this research was to find the most suitable and nontoxic solid-solvent extraction of PAs from the pomegranate peel which enables the use of the extract in food industry. Further, to elucidate how different temperature conditions, extraction time, solvents, pH and number of extraction steps affect the quantitative extraction of PAs.[14] This research also evaluated how the temperature of storage affected the stability of polyphenols and proanthocyanidins in the final extract.

MATERIALS AND METHODS

Chemical and reagents

Folin-Ciocalteu reagent 2N (Sigma-Aldrich, Switzerland), gallic acid (Sigma-Aldrich, China), vanillin (Sigma-Aldrich, Belgium) and 2,2-Diphenyl-1- picrylhydrazyl (DPPH) (Sigma-Aldrich, USA), sodium carbonate anhydrous (Surechem, England), ferric ammonium sulfate (Carl Roth, Germany), sodium bicarbonate (Himedia, India), citric acid (Titan Biotech, India), malic acid (Qualikems, India), tri-sodium citrate (Riedel-de Haen, Germany), sodium phosphate dibasic (Merck, Germany), methanol and ethanol were obtained from Sharlau (Spain), ethyl acetate, 1-butanol, hydrochloric acid and glacial acetic acid were obtained from Surechem (England).

Equipment

Micropipette 100-1000 µl (Iso lab, Germany), sensitive balance (Sartorius, Germany), ultra sonic bath, electric stirrer and heater, moisture analyzer balance (Precisa, Switzerland), centrifuge (Shanghai surgical instruments factory, China), pH-meter (Crison, spain), spectrophotometer (Jasco V-530, USA).

METHODS

Sample preparation

Fresh pomegranates were cleaned with water and dried with a cloth. The peels were manually separated, dried for a few days in an open air shade. The dried samples were then powdered in a blender. They were stored at -18°C until analysis.

The moisture content was determined by using a moisture analyzer balance.

Extraction procedure

200 mg of dried and ground peel were placed in a thermostatic water bath shaker with 10 ml of DI water at 50°C for 20 min. The liquid extract was separated from solids by centrifugation at 2000 rpm for 10 min. The supernatant was transferred to a 10 ml flask, and DI water was added to make the final volume 10 ml. All samples were extracted in duplicates and the total polyphenols and proanthocyanidins concentrations as well as the radical scavenging activity were measured.[15]

Total Polyphenol Content

The total polyphenol content in the extract was determined by the Folin-Ciocalteu method according to the method described by the International Organization for Standardization (ISO). [16] 250 µl of the extract was diluted with distilled water to 10 ml. Aliquots of 1 ml of samples were mixed with 5 ml of 10-fold-diluted Folin-Ciocalteu reagent. After 3 min, 4 ml of 7.5% sodium carbonate was added.[17] The mixtures were allowed to stand for 30 min at 40°C temperature (water bath) before the absorbance was measured at 734 nm. The total polyphenol content in the extract was calculated and expressed as gallic acid equivalents (GAE; g/100 g dry mass) using a gallic acid (0–120 mg/l) standard curve. All samples were prepared in duplicates. [18]

Proanthocyanidins Content

The proanthocyanidin content in the extract was determined by the Acid Butanol assay according to the method of Porter et al. [19]. A sample of 200 µl extract diluted with 300 µl of acetone 70% was pipetted into a 100 x 12 mm test tube. 3.0 ml of butanol –HCL reagent (95:5) and 0.1 ml of 2% ferric acid prepared in HCl 2N were added. The tube was vortexed and then the mouth of the tube was covered with a glass marble and put in the heating block at 97 to 100 °C for 60 minutes. The tube was then allowed to cool and absorbance was recorded at 550 nm. The formula for calculating percentage of condensed tannins as leucoanthocyanidin equivalent is (absorbance 550 nm x 78.26 x dilution factor)/(%dry matter) . All samples were prepared in duplicates. [19]

DPPH radical–scavenging activity

The antioxidant activity of different extracts was measured in term of hydrogen donating or radical scavenging ability using the stable DPPH method according to the method proposed by Brand-Williams et al. [20] 250 µl of the extract was diluted with distilled water to 10 ml. Aliquots of 200µl of samples were mixed with 2 ml of 100 µM DPPH methanolic solution. The mixture was placed in the dark at room temperature for 60 min. The absorbance of the resulting solution was then read at 520 nm. The antiradical activity was expressed in terms of the percentage reduction of the DPPH. The ability to scavenge the DPPH radical was calculated using the following equation

DPPH scavenging effect (%) = $[(A_0-A_1)/A_0]*100$

Where A_0 is the absorbance of the control at 60 min, and A_1 is the absorbance of the sample at 60 min. All samples were analyzed in duplicates. [21]

Relative degree of polymerization

The extracts were assayed with the acid butanol assay and the modified vanillin assay, the ratio of the absorbance describes the relative degree of polymerization according to the method proposed by Butler. [22,23]

Modified vanillin assay

400 µl of the extract was diluted with 600 µl glacial acetic acid. 2.5 ml of 1% vanillin in glacial acetic acid and 2.5 ml of 8%HCl in glacial acetic acid were added. The mixtures were allowed to stand for 20 min at 30°C temperature (water bath) before the absorbance was measured at 510 nm. [24]

Effect of extraction procedures and different parameters

Extraction Temperature

To study the effect of extraction temperature, samples were extracted with water at 20, 30, 40, 50, 60, 70, 80 and 90 °C for 20 min.

Extraction Time

To study the effect of extraction time, samples were extracted with water at 50°C for (5, 10, 15, 20, 30, 40, 50, 60) min.

Solvents

Three different solvents and their mixtures were used to identify the most suitable one for the recovery of polyphenols and proanthocyanidins. The solvents used in this experiment were: deionized (DI) water, ethanol, aqueous ethanol 75%, aqueous ethanol 50%, aqueous ethanol 25%, and ethyl acetate. In addition to the quantitative analysis of polyphenols and proanthocyanidins, the relative degree of polymerization was measured.

pH

Eight solutions with different pH were used to identify the most suitable one for the extraction of polyphenols and proanthocyanidins. The solutions used in this experiment were: citric acid 5 mM, malic acid 5 mM, citric acid/sodium phosphate dibasic buffer at pH 2.5, citric acid/tri-sodium citrate buffers at pH 3.5, 4.5 and 5.5, sodium carbonate/sodium bicarbonate buffers at pH 9 and 10.

Number of Extraction

200 mg of dried and ground peel was extracted in a thermostatic water bath shaker with 10 ml of water in a thermostatic water bath shaker at 50°C for 20 min. The aqueous extract was separated from solids by centrifugation at 2000 rpm for 10 min. The supernatant was transferred to a 10 ml flask, and water was added to make the final volume 10 ml. Then, 10 ml of DI water was added to the solid residue and extracted for 20 min in a thermostatic water bath shaker at 50°C. Five sequential extractions were done and the total polyphenols, proanthocyanidins concentration and the radical scavenging activity were measured.

Storage Temperature

Three different storage temperatures were used to identify the rate of degradation of polyphenols and proanthocyanidins in the aqueous extracts in order to choose the most suitable one for the storage of extracts. The storage temperatures used in this experiment were: laboratory temperature (+20ºC), refrigerator temperature (+4ºC), and freezer temperature (-18ºC).

RESULTS AND DISCUSSION

Influence of Extraction Temperature

Conventional extraction and concentration of polyphenols is typically conducted at temperatures ranging from 20 to 50°C [25], because temperatures above 70°C have been shown to cause rapid polyphenol degradation [26]. For example, Careri et al [27], in order to extract flavanones from orange juice, added methanol and heats the mixture to 55°C for 15 min. Also, Spigno et al [28] extracted phenolics from grape marc at 60°C and Pinelo et al [29] extracted antioxidant phenolics from pine sawdust at 50 °C for 90 min.

We studied the effect of extraction temperature on the total polyphenols and proanthocyanidins yield in PMP extract and results are shown in **Fig (1)**. An increase in temperature increases the efficiency of the extraction since heat render the cell walls

permeable, increase solubility and diffusion coefficients of the compounds to be extracted and decreases the viscosity of the solvent, thus facilitating its passage through the solid substrate mass. However, the use of temperatures higher than 50°C decreases the total polyphenols and proanthocyanidins yield which is probably due to their degradation.

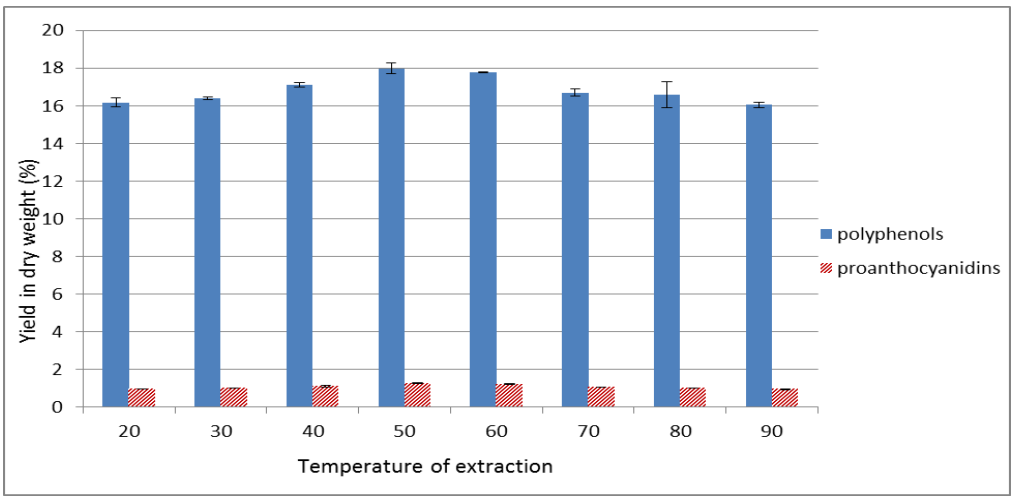

Fig. 1: Effect of extraction temperatures on total polyphenols and proanthocyanidins yield

As presented in **Fig (2)** the DPPH radical scavenging activity of extracts has a linear relationship with the polyphenols yield in the extracts (R^2=0.9462).

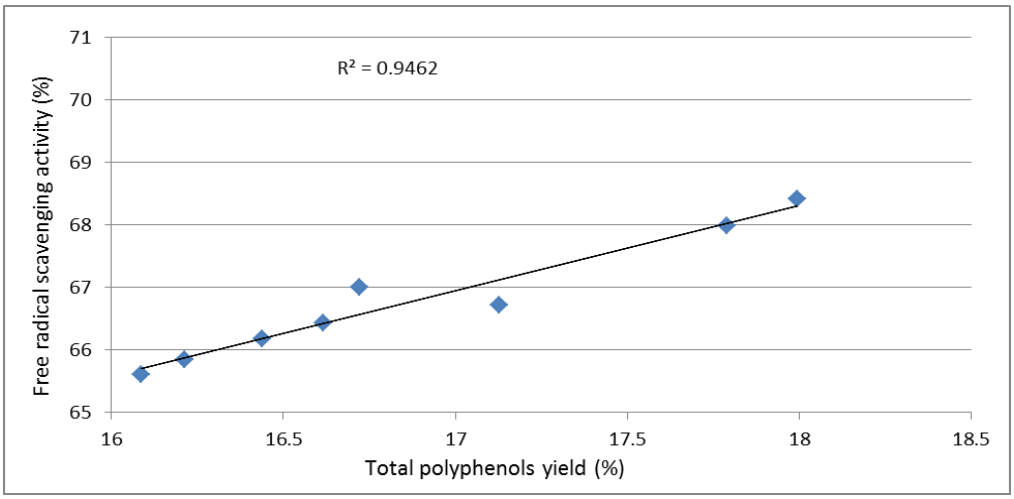

Fig. 2: The relationship between total polyphenols yield and DPPH radical scavenging activity

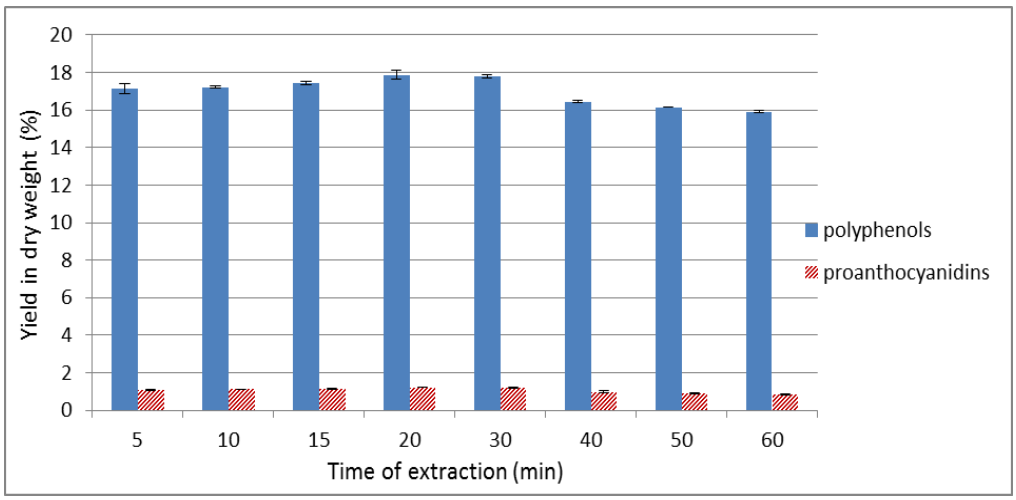

Fig. 3: Effect of extraction time on total polyphenols and proanthocyanidins yield

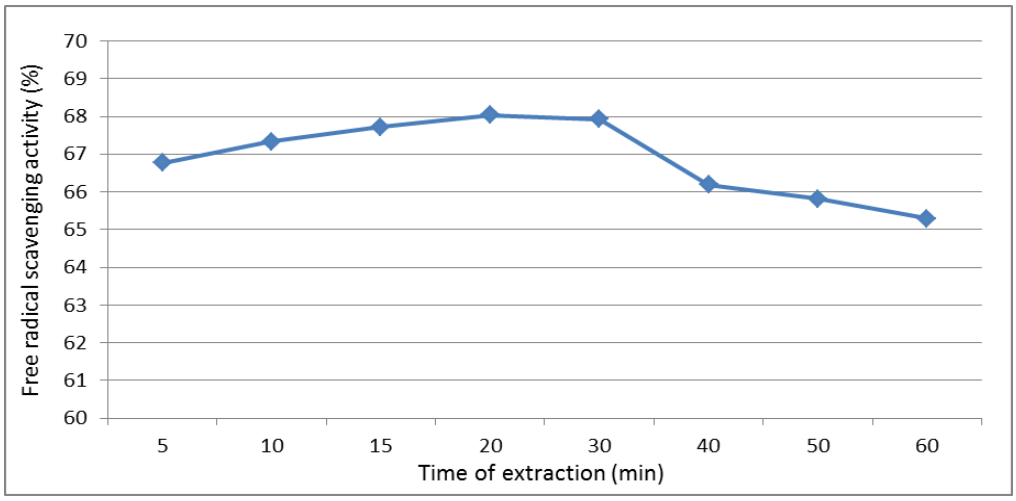

Fig. 4: Effect of extraction time on the DPPH radical scavenging activity

Influence of Extraction Time

As shown in **Fig (3)** as extraction time increased (5 to 20 minutes), the yield of polyphenols and proanthocyanidins also increased. Times longer than 30 min have been shown to cause rapid polyphenols and proanthocyanidins degradation.

The degradation of polyphenols and proanthocyanidins is accompanied with a significant decrease in DPPH radical scavenging activity as shown in **Fig (4)**.

Influence of Solvents

Solvents, such as methanol, ethanol, propanol, acetone, ethyl acetate, and their combinations have been used for the extraction of polyphenols, often with different proportions of water.[7]

In general, phenolic compounds in plants are polar compounds, which usually are extracted with polar solvents such as aqueous acetone and methanol. The phenolic profiles also differed when different solvents were used. Ethanol extracted more polar compounds, while ethyl acetate extracted more nonpolar flavonols.[30]

The total polyphenols and proanthocyanidins yield in PMP extracts obtained using different solvents show significant differences. As seen in **Fig (5)**, water extracts show the highest total polyphenols and proanthocyanidins yield, followed by 50% ethanol, and there is no significant difference between 75% and 25% ethanol extracts. While ethyl acetate extracts show the lowest total polyphenols and proanthocyanidins yield.

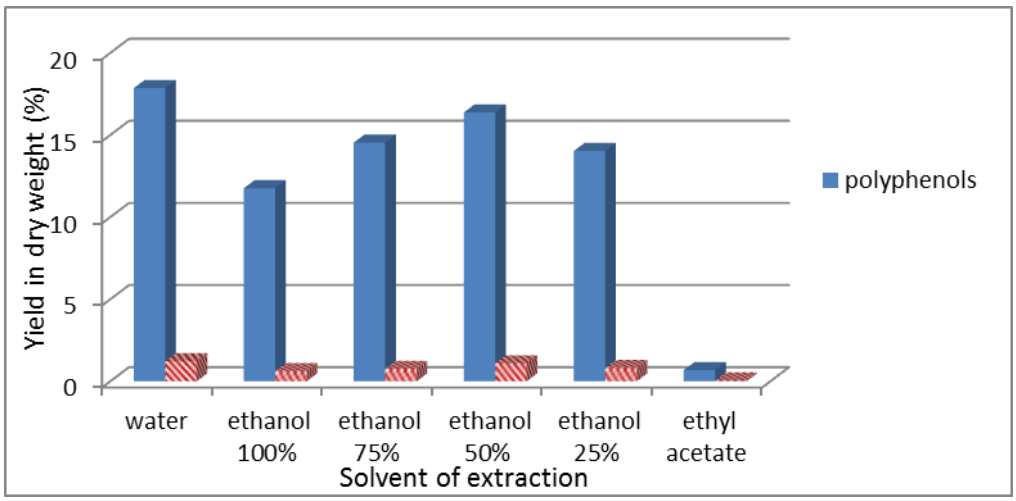

Fig. 5: Effect of different solvents on total polyphenols and proanthocyanidins yield

As shown in **Fig (6)** the DPPH radical scavenging activity of extracts has a linear relationship with the polyphenols yield in the extracts (R^2=0.9695).

The phenolic compounds degradation can be triggered both by external and internal factors. Oxygen is one of the most important external factors that facilitate degradation reactions.[31] As presented in **Table (1)** oxygen is more soluble in pure ethanol, followed by ethanol 75% then ethanol 50%. This may explain the low total yield of polyphenols in pure ethanol extracts compared to water which has the lowest oxygen's solubility.

Also, the low solubility of the polyphenols in absolute organic solvents may be due to strengthening of the hydrogen bonds between polyphenols and protein in these solvents. On the other hand the increase in solubility upon the addition of water to organic solvents could be due to the weakening of the hydrogen bonds in aqueous solutions. It could also be due to the increase of ionization of the polyphenols in such solutions.[33]

The solubility of polyphenols is mainly affected by the polarity of solvent used. Therefore, the phenolic extracts from plant materials are always a diversified mixture of plant polyphenols

soluble in the solvent system used. For that reason the relative degree of proanthocyanidins' polymerization extracted in each solvent was determined. As shown in **Table (2)** there is a relationship between the dielectric constant of the solvent and the relative degree of proanthocyanidins' polymerization extracted.

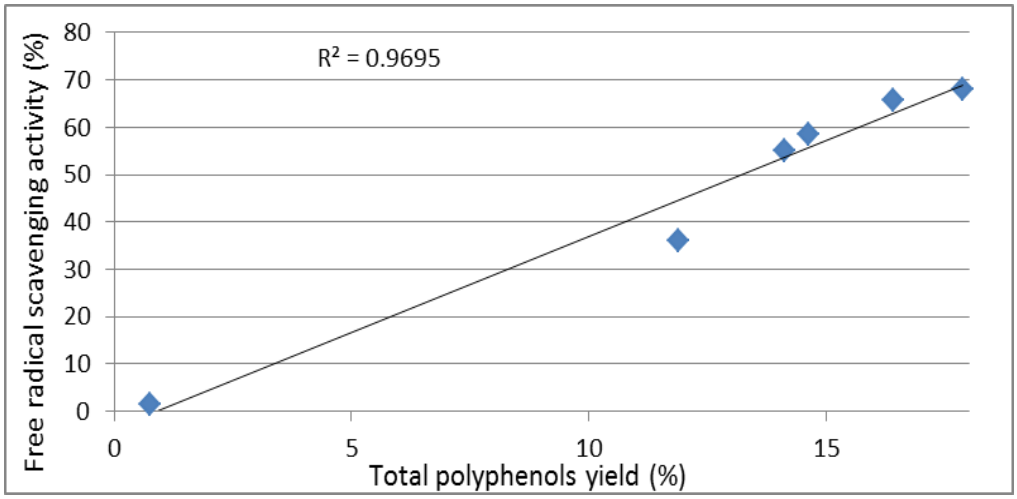

Fig. 6: The relationship between total polyphenols yield and DPPH radical scavenging activity

Table 1: Mole fraction solubilities of oxygen in different mixtures of ethanol: water[32]

Ethanol	Water	Mole fraction solubility of oxygen
100	0	5.593
75	25	3.521
50	50	1.567
25	75	0.2696
0	100	0.2492

Table 2: The relation between the dielectric constant of solvent and the relative degree of proanthocyanidins' polymerization[34]

Dielectric constant of solvent at 25 °C	Relative degree of proanthocyanidins' polymerization
Ethanol 100% (ε=25)	1.88
Ethanol 75% (ε =36)	1.77
Ethanol 50% (ε =52)	1.64
Ethanol 25% (ε =66)	1.42
Water 100% (ε =78)	0.62

Therefore, water is selected as the optimal solvent for extraction of total polyphenols and PAs.

Influence of pH

In different aqueous buffer, maximum yield of total phenolic compounds was obtained in DI water, while the maximum yield of proanthocyanidins was obtained at pH 3.5 as shown in Figure (7) and (8).

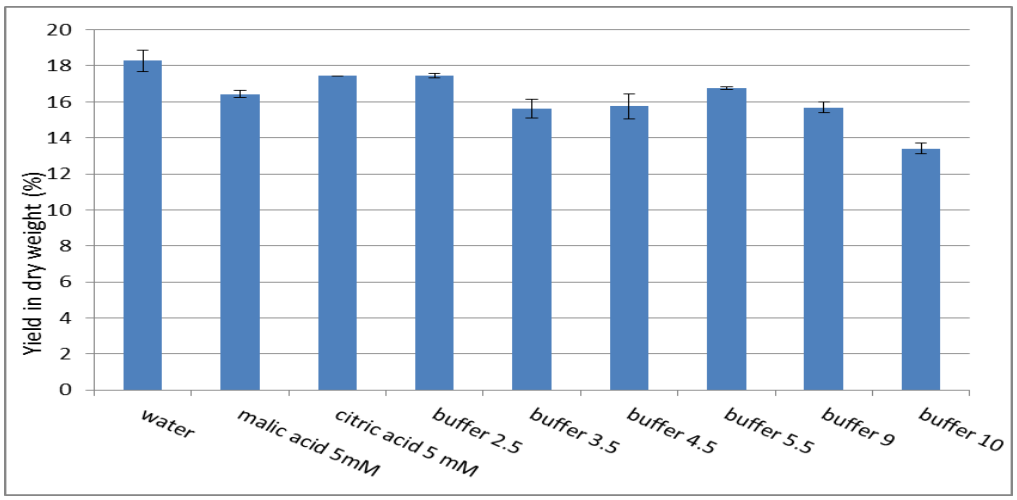

Fig. 7: Effect of different solvents and buffers on total polyphenols yield

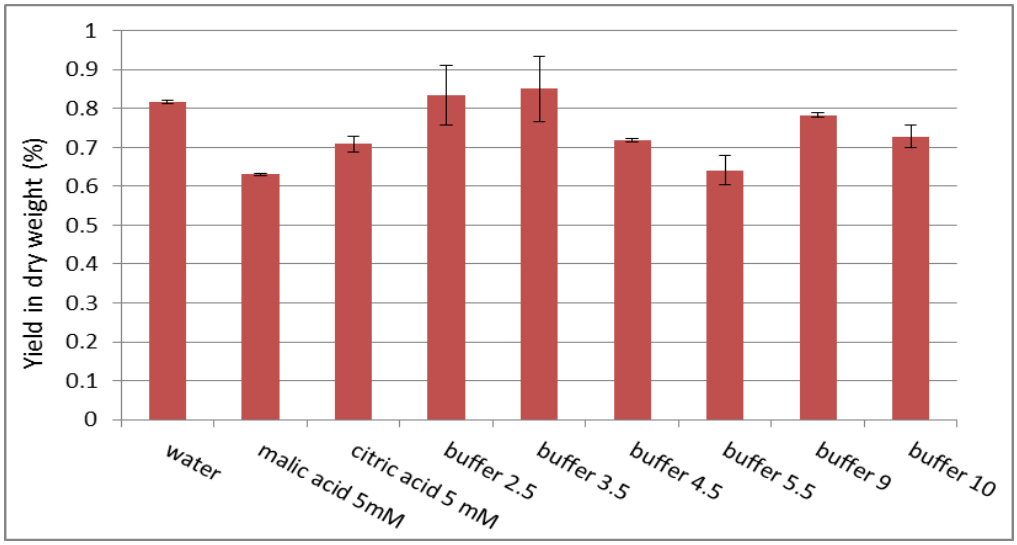

Fig.8: Effect of different solvents and buffers on proanthocyanidins yield

Phenolic compounds are known to undergo oxidation to the corresponding quinones at alkaline pH [35]. That explain the decrease in total polyphenols at pH>9, whereas more than 25% of polyphenols degrade when extracted with sodium carbonate/sodium bicarbonate buffer at pH 10.

The term "proanthocyanidins" is derived from the fact that they are cleaved into anthocyanidins upon heat treatment at acidic pH.[36] Proanthocyanidins are stable in alkaline pH and the use of acidified solvents induce degradation of proanthocyanidins during extraction, therefor more than 20% of proanthocyanidins degrade when extracted with Malic acid 5mM.

Polyphenol oxidase (PPO) is the main enzyme involved in the oxidation of phenolic compounds and its activity is pH dependent.[37] This reaction is called enzymatic browning and occurs readily when the pH is between 5 and 7, while the activity of PPO is irreversibly inhibited at pH less than 3.5.[38] this may explain the decrease in PAs' yield when extracted with tri-sodium citrate/citric acid buffer at pH 5.5.

Influence of Number of Extraction

Two sequential extractions appear sufficient; the first extract contain more than 80% of total extractable polyphenols and proanthocyanidins and the second one contain about 10%. Only small amount of these compounds are found in the third and the fourth extract as shown in **Fig (9)**. While proanthocyanidins are not detectable in the fifth extract.

Influence of Storage Temperature

The total polyphenols degrade quickly during the initial period of storage and then it will slow down as shown in **Fig (10)**.

Proanthocyanidins degrade quickly when stored at laboratory temperature (+20ºC) and are more stable when stored at refrigerator temperature (+4ºC), and at freezer temperature (-18ºC). As shown in **Fig (11)** 4% of proanthocyanidins was lost within one day at laboratory temperature and within 7 days at refrigerator temperature, while only the same percentage was lost within 14 days at freezer temperature.

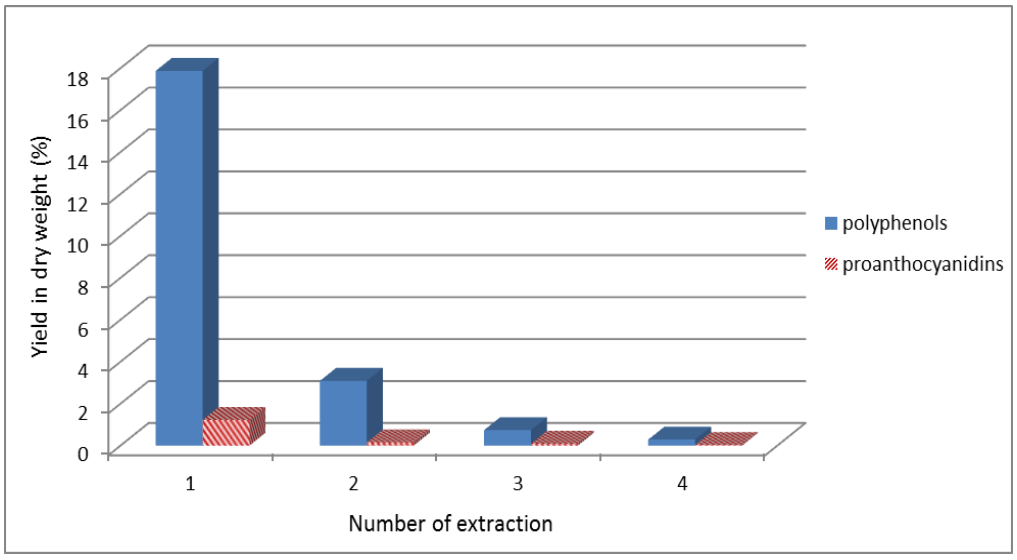

Fig. 9: Total extractable polyphenols and proanthocyanidins in four sequential extractions

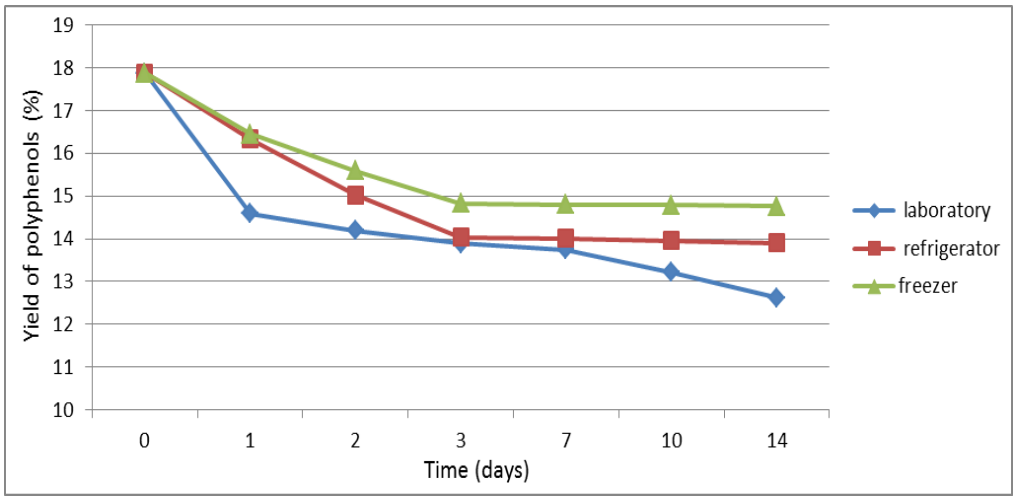

Fig.10: Yield of total polyphenols in different storage temperatures for 14 days.

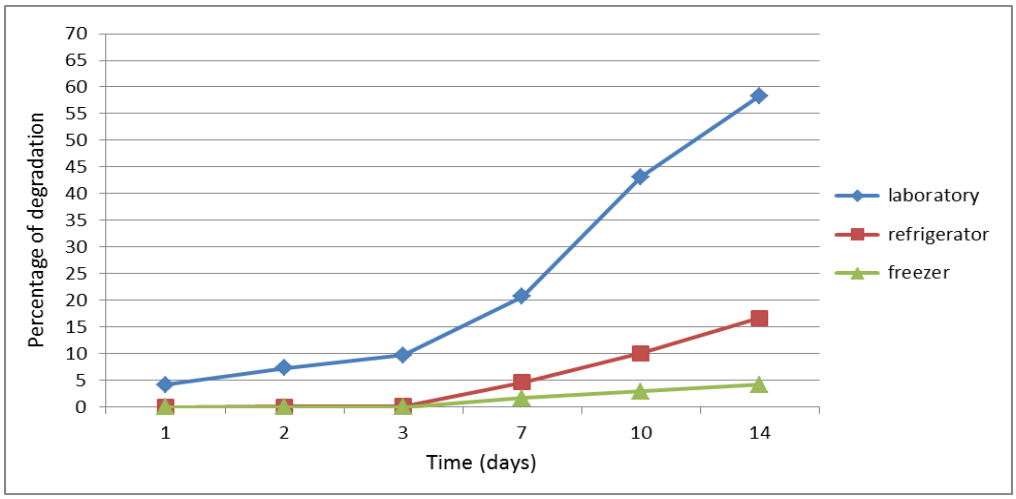

Fig. 11: Proanthocyanidins degradation in different storage temperatures for 14 days.

CONCLUSION

The recovery of phenolic compounds from plant materials is influenced by the extraction time and temperature, which reflects the conflicting actions of solubilization and analyte degradation by oxidation. Therefore, it is of critical importance to select efficient extraction procedure and to maintain the stability of phenolic compounds.

Long extraction times and high temperature increase the chance of oxidation of phenolic compounds which decrease the yield of total polyphenols in the extracts. Water is an interesting solvent for preparation of phenolic compounds for food and pharmaceutical industry because it is non-toxic and gives an acceptable yield of those compounds.

The stability of proanthocyanidins in the extract can be maintained for over then two weeks by storing the extract in freezer.

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