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Research Paper / Araştırma Makalesi

Effects of Extraction Solvents on Polyphenol Contents, Antioxidant and Antibacterial Activities of Pomegranate Parts

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ABSTRACT

Polyphenols in rinds, membranes and seeds of pomegranates were extracted with acetone, acetone:water (70:30, v:v), methanol, ethanol and water for 12 h at 4°C. Gallic acid, punicalin, α-punicalagin, β-punicalagin and ellagic acid derivatives (EAD) were identified in rind and membrane, while only EAD were identified in seed by HPLC. The rind and membrane contained exceptionally high amounts of polyphenols. Strong correlations were found between antioxidant activity (AOA) and total polyphenol content (TPC) for the extracts of rind (r=0.967), membrane (r=0.976) and seed (r=0.972). Moreover, the highest TPC and AOA were determined in acetone:water (70:30, v:v) extracts. Punicalagins at high amounts in the pomegranate rind and membrane provided the highest AOA and antibacterial activity (ABA). The extracts of rind and membrane showed ABA against *Bacillus megaterium* and *Staphylococcus aureus.* Water extracts of rind and membrane may be used as a food additive and preservative due to their high AOA and ABA.

Keywords: Pomegranate rind, Membrane, Seed, Antioxidant activity, Antibacterial activity

Narın Farklı Bölümlerinin Polifenol İçeriği, Antioksidan ve Antibakteriyel Aktivitesi Üzerine Ekstraksiyon Çözgenlerinin Etkisi

ÖZ

Nar kabuğu, dilim zarı ve çekirdeklerinde bulunan polifenoller; aseton, aseton:su (70:30, v:v), metanol, etanol ve su ile 4°C'de 24 saat süresince ekstrakte edilmiştir. Nar kabuğu ve dilim zarında; gallik asit, punikalin, α-punikalajin, β-punikalajin ve ellajik asit türevleri HPLC ile tanımlanırken, çekirdekte sadece elajik asit türevleri tanımlanmıştır. Nar kabuğu ve dilim zarının çok yüksek miktarda polifenol içerdiği belirlenmiştir. Nar kabuğu (r=0.967), dilim zarı (r=0.976) ve çekirdeklerden (r=0.972) elde edilen ekstraktların toplam polifenol miktarı ve antioksidan aktiviteleri arasında güçlü korelasyonlar bulunmuştur. Ayrıca, en yüksek toplam polifenol madde miktarı ve antioksidan aktivite aseton:su (70:30, v:v) çözeltisiyle elde edilmiştir. Nar kabuğu ve dilim zarının yüksek antioksidan ve antibakteriyel aktivitesi punikalajinlerden kaynaklanmaktadır. Nar kabuğu ve dilim zarı *Bacillus megaterium* and *Staphylococcus aureus'a* karşı antibakteriyel aktivite göstermiştir. Yüksek antioksidan ve antibakteriyel aktiviteleri nedeniyle, nar kabuğu ve dilim zarının sulu ekstraktları gıda katkısı ve koruyucusu olarak kullanılabilir.

Anahtar Kelimeler: Nar kabuğu, Dilim zarı, Çekirdek, Antioksidan aktivite, Antibakteriyel aktivite

INTRODUCTION

There was an intense interest in pomegranate products because of their nutritional and health benefits, including reduced oxidative stress, atherogenic modifications to LDL [1], as well as anticancer, antibacterial, and antiviral activities [2]. Due to high demand, the production of pomegranates was increased by 74% during the last four years in Turkey [3]. In 2010, 22% of the pomegranates produced in Turkey (78 700 metric tons) were processed into juice [4]. Among over 100 local and registered pomegranate varieties grown in Turkey, Hicaznar variety investigated in the present study is the mostly preferred pomegranate variety by the Turkish fruit juice industry. The juice yield of pomegranates ranges from 35 to 50%, depending on the processing method used. Considering the yield values, the amount of pomegranate marc created in 2010 was very high (about 53 000–57 000 metric tons). The marc contains about 78% rind and 22% seeds based on wet weight [5]. The studies showed that pomegranate rind and seed had high polyphenol contents and thus showing high antioxidant and antimicrobial activities (AMA) [2, 5, 6].

Due to high polyphenol contents, thus AOA and AMA, instead of using as animal feeds, the marc could also be used as the source of food preservative and additive. To use the marc as the source of food preservatives and additives, the most important step is to find the appropriate extraction method. Since the polarities of phenolic compounds which contain multiple hydroxyl group that can be conjugated to sugars, acids or alkyl groups vary significantly, to date, a single method for optimum extraction of all polyphenols could not have been developed [7]. Currently, the methods used for polyphenol extraction are solid-liquid extraction, liquid-liquid extraction, supercritical fluid extraction, pressurized liquid extraction, microwave-assisted extraction and ultrasound-assisted extraction [7]. Among all these extraction methods, the most widely used one is the solid-liquid extraction for solid samples [4]. The solvents such as methanol, ethanol, propanol, acetone, ethyl acetate, and their combinations with each other and water have been used to extract the polyphenols from plant materials [7].

The selection of appropriate solvent is very important to make an effective extraction of polyphenols from plant material. For example, methanol provided more effective extraction of total polyphenols (39 mg gallic acid/g dry weight) and flavonoids (7 mg quercetin/g dry weight) from ginger leaves (*Zingiber officinale Roscoe*) than acetone (35 mg gallic acid/g dry weight, 6 mg quercetin/g dry weight, respectively) and chloroform (34 mg gallic acid/g dry weight, 6 mg quercetin/g dry weight, respectively) extracts [8]. Addition to total polyphenol content, extraction solvent also affects the profile of polyphenol extracted from plant material. In a research conducted by Wu and Prior [9], 9 polyphenols from beans (*Phaseolus vulgaris* L.) were extracted with methanol, while Espinosa-Alonso et al. [10] extracted 17 polyphenols with aqueous methanol (methanol:water, 80:20, v:v) in the same material.

To date, TPCs, AOAs and AMAs of rinds and seeds of some pomegranate varieties (Poost Syah variety [6], Wonderful variety [11], Asinar, Lefan, Katirbasi, and Cekirdeksiz-IV [12]) were investigated. However, the effects of extraction solvents on these compounds and properties in rind, membrane and seed of Hicaznar variety have not been investigated, although the highest amount of pomegranate marc is obtained from this variety in Turkey. As known, antioxidative and antibacterial activities of plants including pomegranates varied with variety and depended on the profile and content of polyphenols. Therefore, the most suitable solvent for the extraction of polyphenols from pomegranate varieties should also be determined.

The main objective of the present study was to determine the effects of five different extraction solvents [methanol, ethanol, acetone, water and aqueous acetone (acetone:water, 70:30, v:v)] on TPCs, AOA and ABA in the parts (rinds, membranes and seeds) of Hicaznar pomegranate variety and to compare TPCs, AOA and ABA values of the pomegranate parts. Moreover, the correlations between TPCs and AOA were also determined.

MATERIALS and METHODS

Chemicals and Reagents

Standards of gallic acid and punicalagin were purchased from Sigma (St. Louis, MO, USA). Standard of punicalin was purchased from Biopurify Phytochemicals Ltd. (Sichuan, China). Standard of ellagic acid was purchased from Fluka (Seelze, Germany). In all analyses, the ultra pure water was used (Millipore Simplicity UV, Molsheim, France). The reagents used for liquid chromatography were HPLC grade and purchased from Merck (Darmstad, Germany). All other reagents were analytical grade and obtained from Merck.

Sampling

Pomegranates (*Punica granatum* L. var. Hicaznar) were obtained from Alata Horticultural Research Institute (Erdemli, Mersin). Hicaznar variety is native to Turkey and highly cultivated in Mediterranean region. Rinds, membranes and seeds of the pomegranates were manually separated.

Preparation of Samples to Analyses

The rind, membrane and seed samples were first frozen at –25°C (Caravell 614-107, Viborg, Denmark). Then, the rinds and membranes were cut into cubes and finally all of the pomegranate parts were frozen again in an ultra-cold freezer at –86°C (Hettich AG HS 4486, Tuttlingen, Germany). The frozen cubic samples were lyophilized at −55°C and 0.021 mbar for 3 days (Labconco FreeZone6L, Kansas City, MO, USA) and then powdered in a porcelain mortar using a pestle. To minimize light damage, the powdered samples were

transferred to amber colored vials. All analyses were carried out with these powdered samples.

Extraction

Methanol, ethanol, acetone, water and aqueous acetone (acetone:water, 70:30, v:v) were used as extraction solvents to determine the effects of different solvents on polyphenols, AOA and AMA in the samples. One gram (±0.01 g) of powdered samples was mixed with 40 mL of each extraction solvent and the mixtures were kept at 4°C for 12 h. Then, they were sonicated using an ultrasonic bath (Bandelin Sonorex, Berlin, Germany) at 20°C for 15 min. The resulting extract was filtered on a Buchner funnel using Whatman No. 1 filter paper. The filter cake was also extracted with 15 mL of each extraction solvent. After the filtrates were combined, they were transferred to a rotary evaporator (Heidolph Laborota 4003, Schwabach, Germany) to remove residual extraction solvent at 40°C. The extract was dissolved in purified water (containing 0.01% HCl, v:v) and the final volume was brought to 10 mL with purified water. The resulting extract was filtered through a 0.45 µm PVDF (polyvinylidene fluoride) filter (Millipore, Bedford, MA, U.S.A.) directly to an amber colored bottle. Two extracts were prepared from each sample.

Total Polyphenol Contents (TPCs)

Total polyphenol content of the samples was determined by the Folin-Ciocalteu method. Gallic acid was used as a standard for the preparation of calibration curve. 1 mL of 1:10 dilutions of the rind and membrane extracts, and 1 mL of seed extracts were added to 75 mL purified water in 100 mL volumetric flask. Then, 5 mL of Folin-Ciocalteu reagent was added to the mixture which was held at room temperature for 3 min. After the addition of 10 mL of saturated Na_2CO_3 solution to the volumetric flask, the absorbance values were determined at 720 nm at the end of 60 min incubation. Results were calculated and expressed as “milligrams of gallic acid equivalent per kg dry matter (dm).” Total polyphenol measurements were replicated two times.

HPLC Separation of Polyphenols

Polyphenol purification: The prepared extracts of pomegranate parts were purified on a C-18 cartridge (Waters Co., Milford, MA, U.S.A) using a vacuum manifold system (Waters Co.). Prior to sample load, the cartridge was activated with 5 mL ethyl-acetate followed by 5 mL methanol (containing 0.01% HCl, v/v) and 2 mL aqueous 0.01% HCl (v/v). Upon loading of 1 mL extract, the cartridge was washed with 2 mL aqueous 0.01% HCl to remove the compounds not adsorbed by the column such as sugars and organic acids. The cartridge was then dried under a stream of nitrogen for 10 min. Polyphenols were removed from the cartridge by rinsing with 5 mL ethyl-acetate. The extract containing polyphenols was then evaporated to dryness under a stream of nitrogen at 40°C (Caliper TurboVap LV, Hopkinton, MA, U.S.A.). The residue containing polyphenols was dissolved in aqueous 0.01% HCl. The resulting extract was filtered through a 0.45 µm PVDF filter (Sartorious AG, Goettingen, Germany) directly to an amber colored auto sampler vial, and the filtered extract was immediately injected to HPLC without further delay.

Instrumentation and chromatography: Polyphenols were determined using HPLC (Agilent 1200 series, Waldbronn, Germany) with a binary pump, a photo diode array (PDA) detector, a thermostatted auto sampler, a degasser and a thermostatted column compartment. Chromatographic data were recorded and processed on an Agilent 1200 series ChemStation rev.B.02.01 software. Polyphenols were separated on a C_{18} (5 µm) column (250x4.6 mm) (Phenomenex Inc., Los Angeles, CA, U.S.A.) with a C_{18} (5 µm) guard column (4x3 mm, 5 µm) (Phenomenex Inc.). The mobile phase consisted of acetonitrile (100%, eluent A) and formic acid (1%, eluent B).

Separation was performed with gradient elution. The linear gradient program for the separation of polyphenols from the samples was as follows: from 97.5% to 75% B in 45 min, from 75% to 50% B in 5 min, from 50% to 0% B in 5 min, holding at 0% B isocratic for 13 min and from 0% to 97.5% B in 2 min. The sample injection volume was 100 µL and the column temperature was set at 25°C. Monitoring was performed at 280 nm at a flow rate of 0.7 mL/min. Gallic acid, punicalin, punicalagins and ellagic acid derivatives (EAD) in samples were identified by comparing absorption spectra and retention time of unknown peaks with external reference standard by HPLC. Quantification of the polyphenols was carried out using calibration curves of the following external reference standards; punicalin (R^2 = 0.998), α-punicalagin (R^2 = 0.999), β-punicalagin (R^2 = 0.999) and ellagic acid (R^2 = 0.999). The calibration curves for each polyphenol standard contained 6 data points. The quantification of total polyphenols was based on gallic acid.

Antioxidant Activity (AOA)

AOA was measured according to ABTS method described by Miller & Rice-Evans [13]. ABTS, a chromogen and a colorless substance, is changed into its colored monocationic radical form ($ABTS^{\cdot+}$) by an oxidative agent. The absorption peak of $ABTS^{\cdot+}$ is at 734 nm. The addition of antioxidants, such as Trolox which is a water soluble analog of vitamin E, reduces $ABTS^{\cdot+}$ into its colorless form. The extent of decolorization as percent inhibition of $ABTS^{\cdot+}$ was determined as a function of concentration. AOA was expressed as “m*M* of Trolox equivalents per g dm.”

Antibacterial Activity (ABA)

Preparation of samples: Before ABA analysis, the samples were sterilized by passing the samples from 0.45-µm sterile filter (Millipore, Bedford, MA, U.S.A.). Purified water (containing 0.01% HCl, v:v) was used as a control in the determination of ABA.

Test microorganisms: ABA of pomegranate parts was tested against Gram-positive bacteria [*Bacillus*

megaterium, (*B. megaterium*) (Ankara University, Food Engineering Department's culture collection), *Staphyococcus aureus, Cowan* strain (*S. aureus*)] and Gram-negative bacteria [*E. coli* O157:H7 (Ministry of Health National culture collection, RSKK 232)] strains.

Preparation of bacterial inoculum: All the bacterial strains were frozen-preserved in 20% (w/v) glycerol for long term storage. They were also preserved at 4°C on an agar slant in a capped tube as stock culture. Tryptic soy broth (TSB) (Merck Co.) was used to activate each bacterium strain and determine the ABA. Bacteria culture from agar slant was inoculated into 10 mL of TSB medium by using an inoculating loop with a diameter of 2 mm. After incubation at 35°C for 20 h, a second bacterial passage was prepared from TSB culture by inoculating bacterial culture in 10 mL of TSB by a loop and incubated at 35°C for 20 h. A 0.5 mL of the second bacterial passage was diluted in 2 mL of sterile physiological saline (SS, 0.85% NaCl, w/v) and then 80 µL of the dilution was inoculated into 4 mL of TSB medium. That was incubated at 35°C until the cell culture turbidity reached to 0.2 OD (optical density) at 600 nm by spectrophotometer (Spectronic 20, Bausch and Lomb, Germany). Before ABA test, the culture was diluted (1:10, v:v) with SS and the bacterial suspension was used as a standardized inoculum (1 x 10^8 CFU/mL). Total bacteria count in the standard inoculum was determined from appropriate dilutions by the spread plate technique on TSA agar which was incubated for 37°C for 24−48 h.

Agar well diffusion method: The extracts were subjected to antimicrobial assay using agar well-diffusion method [14]. A 250 µL of standardized bacterial inoculum was added to 25 mL TSA, cooled to 42°–45°C, and mixed gently, and then the mixture was poured into a sterile 12-mm diameter petri plate. After the solidification of the medium, a cork borer with a diameter of 9.0 mm was flame sterilized and used to make six uniform cups/wells in each plate. The cups/wells were filled with 100 µL of extracts and the plates were incubated at 35°C for 18−20 h. The zones of inhibition seen as full clear zone around each well were measured with a caliper in "mm." The experiment was carried out in triplicates.

Statistical Analyses

The results were analyzed using the Minitab statistical software, version 14 (Minitab Inc., State College, PA, U.S.A.). Different extraction solvents and pomegranate parts were considered as the main effects. Statistical differences among means were determined by the Duncan's multiple range test at the 5% significance level.

RESULTS and DISCUSSION

Polyphenol Profiles and TPCs of Rind, Membrane and Seed Extracts

Significant differences in the polyphenol profiles of pomegranate parts were found (Figure 1). Gallic acid, punicalin, α-punicalagin, β-punicalagin and ellagic acid derivatives (EAD) were identified in rind and membrane, while only EAD were identified in seed (Figure 1). Similarly, Wang et al. [15] also identified the derivatives of ellagic acid such as 3,3'-di-*O*-methylellagic acid and 4,4'-di-*O*-methylellagic acid in pomegranate seed. Similar to our findings, the pomegranate rinds were characterized by the presence of hydrolysable tannins (e.g. punicalagin, punicalin [16]), phenolic acids (gallic acid [16], ellagic acid [15]), flavonoids (e.g. catechin, epicatechin, kaempferol, luteolin, rutin, quercetin and naringin [17]) and the absence of typical pomegranate anthocyanins [16]. β-Punicalagin was the major polyphenol in rind (274−296 g/kg dm) and membrane (265−301 g/kg dm). Although α-punicalagin and β-punicalagin contents in rind and membrane were close, rind (108−130 g/kg dm) had higher EAD contents than membrane (72−103 g/kg dm). Among pomegranate parts, seed had the lowest EAD content (1.98−2.20 g/kg dm).

TPCs of pomegranate rind, membrane and seed were determined by both HPLC and spectrophotometer to compare TPCs of these parts, and the results were presented in Figure 2. As shown in Figure 2, TPCs determined by spectrophotometer were lower than those by HPLC. However, there was strong correlation (r=0.834) between TPCs determined by HPLC and spectrophotometer. Among pomegranate parts, pomegranate rind (120−152 g/kg dm) had the highest TPC, followed by membrane (102−129 g/kg dm) and seed (0.430−0.582 g/kg dm), respectively (Figure 2).

These results are in an agreement with the previous studies [12, 18] showing that pomegranate seed extracts had the lowest TPC in comparison with juice, pulp, aril, membrane, rind leaf and flower. The low content of polyphenols extracted from seed may also be attributed to the low extraction yield of its polyphenols. The yield of polyphenol extraction from seed (2%) was lower than that from rind (10%) [5]. This may be also due to the inherent physical and chemical differences between the rind and seed [5]. Since the seed contains much more cellulosic compounds and has a firm physical structure than the rind, the extraction of polyphenols from seed is much more difficult [5].

The amount of polyphenols extracted also changes not only depending on the parts of pomegranate but also depending on fruit variety, extraction solvent, solvent/sample ratio, particle size and extraction temperature [5]. For example, the TPC of the rind from Poost Syrah variety was 8.4 g/kg dm [6], and lower in comparison with those of Hicaznar variety (120−152 g/kg dm) in the present study. The difference between TPCs of Hicaznar and Poost Syrah varieties might have not only resulted from the difference in variety but also resulted from the differences in solvent and solvent/sample ratio. As a matter of fact, the solvent (ethanol:water, 50:50, v:v) and solvent/sample ratio (16:1) used in their study were quite different from the present study (methanol, ethanol, acetone, water, aqueous acetone; 55:1).

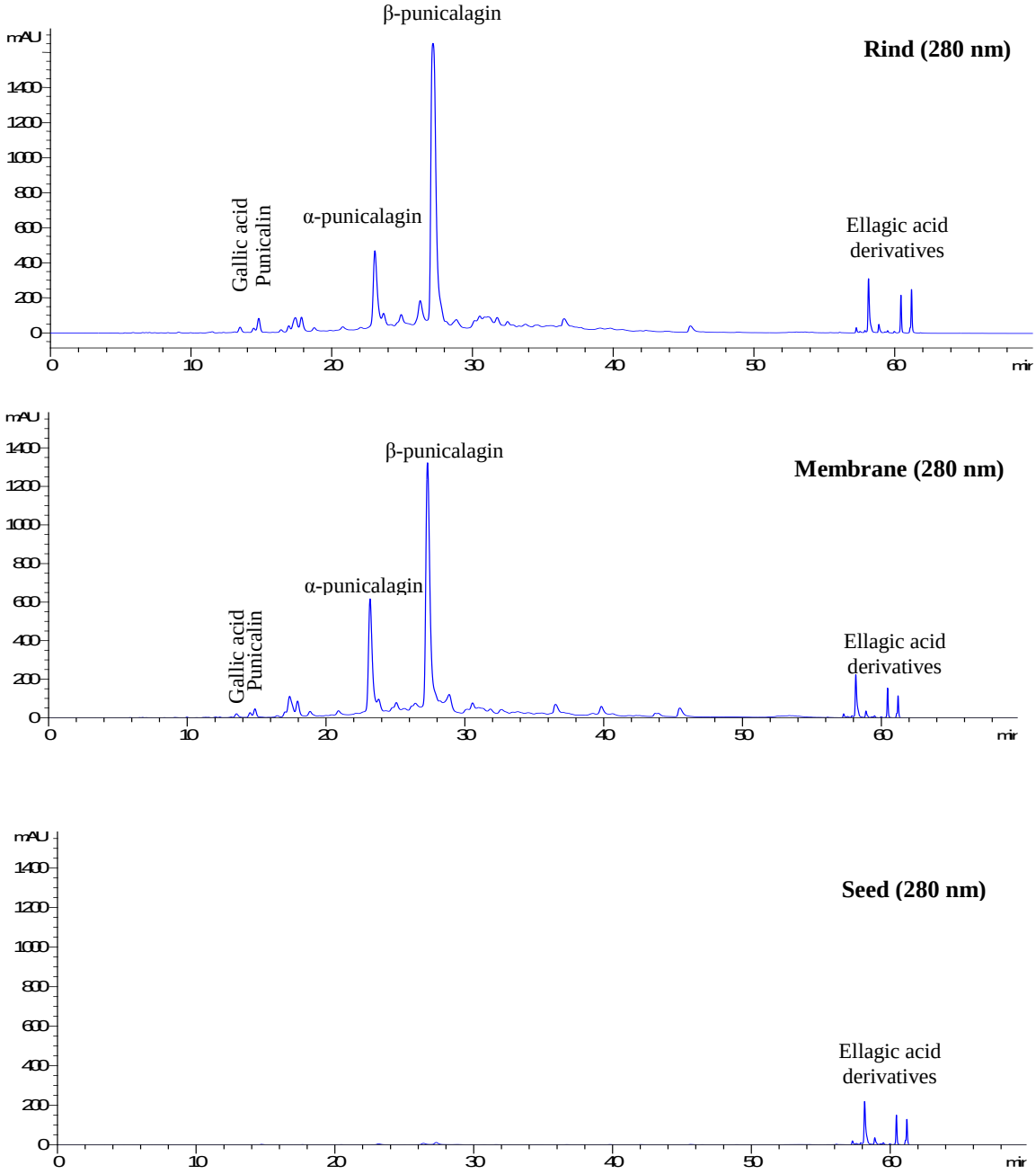
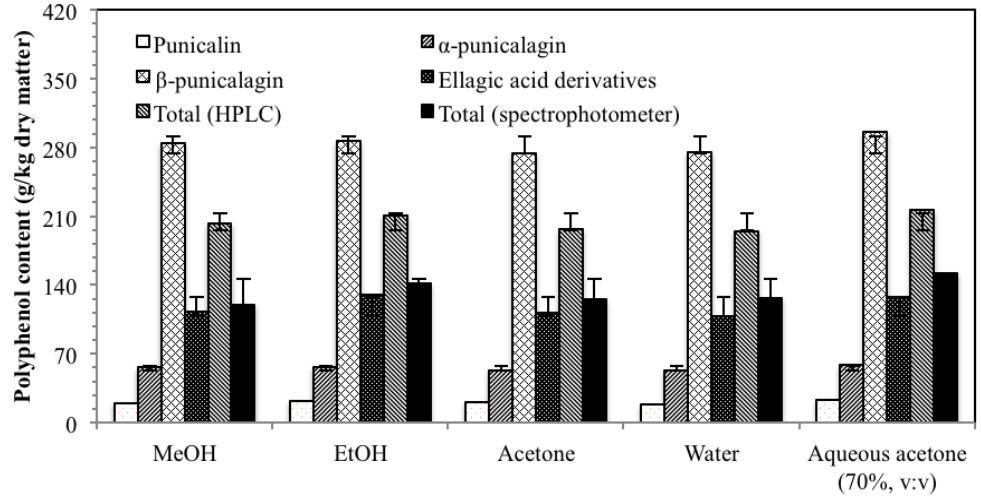


Figure 1. Polyphenol profiles of pomegranate parts

In the present study, the effect of different solvents on TPCs of pomegranate parts was also investigated. For this purpose, the polyphenols of pomegranate rind, membrane and seed were extracted with methanol, ethanol, acetone, water and aqueous acetone (acetone:water, 70:30, v:v). Significant differences (P<0.05) in the amounts of polyphenols extracted with different solvents were found. The highest TPCs in pomegranate rind (152±0.83 g/kg dm) and membrane (129±0.17 g/kg dm) were determined in aqueous acetone extract. This was attributable to better extraction of higher molecular weight polyphenols (punicalin and punicalagins) in rind and membrane with aqueous acetone [19]. The second highest TPCs in rind (142±0.50 g/kg dm) and membrane (119±0.17 g/kg dm) were determined in ethanol extracts, while those (0.565±0.007 g/kg dm) in seed was determined in methanol extraction. While aqueous acetone was the solvent for extracting the high molecular weight polyphenols, methanol was generally found to be more efficient in the extraction of lower molecular weight polyphenols [19]. And, since pomegranate seeds particularly contain polyphenols with lower molecular weight such as EAD, TPC in methanol extract of pomegranate seed is slightly higher than that in ethanol extract. However, due to the safety measurements, water and ethanol are the good solvents for polyphenol extraction from plant materials [20].



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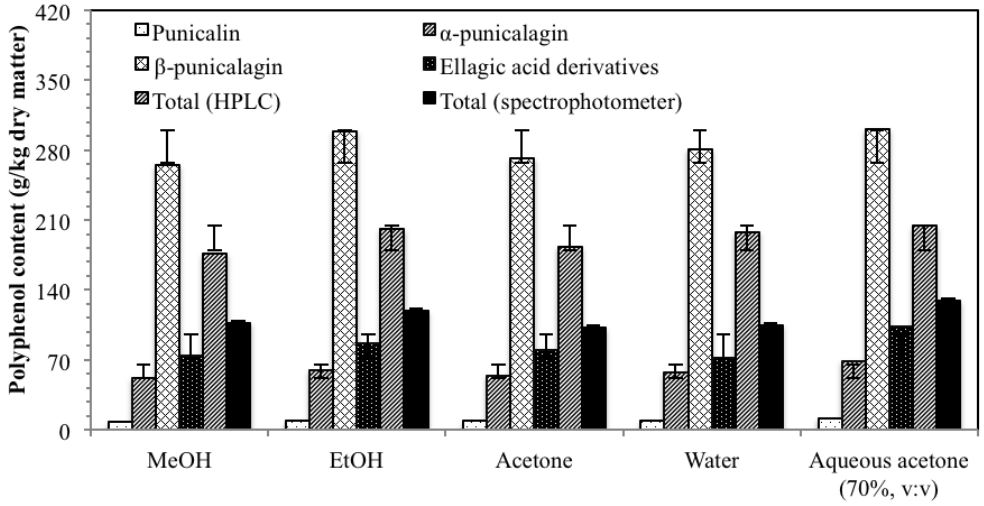

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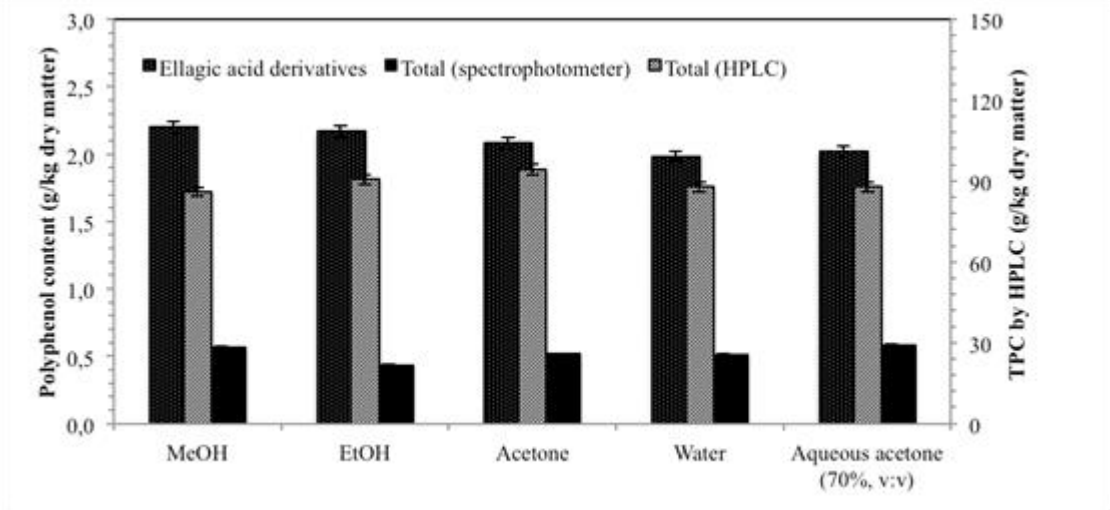

c

Figure 2. Effects of extraction solvents on individual polyphenol contents and TPCs of pomegranate parts, a: rind, b: membrane, c: seed, ETOH: ethanol, MEOH: methanol

A suitable solvent should be determined for the efficient extraction of polyphenols from different foods [7]. In fact, Pinelo et al. [21] reported that methanol was the best extraction solvent for polyphenols from pine sawdust, while ethanol was the best extraction solvent in almond hulls. Similarly, in wild ginseng leaves, the ethanol extracts contained higher amounts of total polyphenols and flavonoids than water and methanol extracts [22].

On the contrary, no difference was found for the extraction of total polyphenols of grapes seeds (*Vitis vinifera*) using acetone, methanol and water [23].

AOAs in Extracts of Rind, Membrane and Seed

AOAs of the extracts of various pomegranate parts were analyzed by ABTS free radical assay. This assay was chosen because it can measure the AOAs of both aqueous radicals and lipid peroxyl radicals [24]. The AOA values of sample extracts showed significant differences, ranging from 48.3 to 2989 m*M*/g dm (Figure 3). Among the parts studied, the highest AOA was determined in the extracts of pomegranate rind (2519−2989 m*M*/g dm). The AOA values of membrane (2421−2871 m*M*/g dm) extracts were very similar to those of rinds, while seed extracts showed significantly lower AOA (48.3−65.2 m*M*/g dm) than the others (P<0.05). Similar to these results, Elfalleh et al. [18] also found that pomegranate (Gabsi variety) peel extracts had stronger AOAs (3.80–7.50 mmol TEAC/100 g dm) than seed extracts (1.10–0.76 mmol TEAC/100 g dm).

The antioxidant compounds of pomegranates mainly comprise the compounds with phenolic hydroxyl groups and double bonds, such as tannins and flavonoids [15]. Polyphenols possess ideal structure chemistry for free radical scavenging activities because they have: (1) phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to a free radical; (2) extended conjugated aromatic system to delocalize an unpaired electron [25]. The present study also showed that there were strong correlations between TPCs and AOA values (r=0.994). Pomegranate rind, which had the highest TPCs, showed the highest AOA (r=0.967) as pomegranate seed, which had the lowest TPC, showed the lowest AOA (r=0.972). Similarly, strong correlation between TPCs and AOAs of pomegranate membrane was also determined (r=0.976).

Polyphenol profiles as well as TPCs of pomegranate parts had significant effects on AOA values. Therefore, the correlations between individual phenolic contents and AOA values of pomegranate parts were determined. There were good correlations between AOA values and the contents of punicalagin (r=0.735) and EAD (r=0.759). These results agree with the results reported by Gil et al. [16] who clearly showed that the AOA of pomegranates was mainly due to the hydrolysable tannins including punicalagins and EAD. Since the extracts of rind and membrane also contained hydrolysable tannins including punicalagins and had 33–66 times higher EAD than that of seed, the rind showed higher AOA.

The AOA values of the extracts were also strongly dependent on the type of the solvent as well as on polyphenol profiles (P<0.05) due to solvent polarity. Aqueous acetone extracts of all pomegranate parts showed significantly higher AOA than the others, which is parallel to the highest TPCs in the aqueous acetone extracts of pomegranate parts. The AOA value in the extracts of aqueous acetone was found about 1.2 times higher than that in acetone extracts. This finding is in an agreement with the previous study which showed that the aqueous solutions of ethanol, methanol or acetone were good solvents for the extraction of antioxidative compounds in grape seeds [26].

ABA in Extracts of Rind, Membrane and Seed

The ABAs of pomegranate rind, membrane and seed in methanol, ethanol, acetone, water and aqueous acetone extracts are presented in Table 1. These activities of the sample extracts were tested against *B. megaterium, S. aureus* and *E. coli* O157:H7 (Table 1). No significant effect of extraction solvents on ABA was found in the pomegranate parts studied (P>0.05). While pomegranate rind and membrane showed ABA on *B. megaterium* and *S. aureus*, no activity on *E. coli* O157:H7 was detected. The results showed that Gram-positive bacteria are more sensitive than Gram-negative bacteria to the different extracts of pomegranate parts due to the differences in the structure of their cell walls. The highest antibacterial activity (18.42−20.50 mm) was recorded on *S. aureus* among Gram-positive bacteria by rind (19.77−20.50 mm) and membrane (18.42−19.14 mm) extracts. Similar to our results, the antibacterial and antifungal activities in extracts of rind, seed, juice and whole fruit on *Bacillus cereus*, *Bacillus coagulans, Bacillus subtilis*, *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *S. aureus* were determined [27]. The highest ABA was recorded against *S. aureus* by methanolic extracts (methanol:water, 80%, v:v) of rind (25 mm) and seed (19 mm). Moreover, other studies also reported that the extracts of pomegranate exhibit significant inhibiting effect against the common pathogenic bacteria, especially to Gram-positive pathogens such as *S. aureus, S. hemolyticus, B. paratyphosus, B. subtilis, Mycobacterium tuberculosis* and *Listeria monocytogenes* [28, 29].

The maximum ABA (12.42−20.50 mm) on both Gram-positive bacteria was recorded by rind extracts, followed by membrane extracts (12.12−19.14 mm). On the contrary, pomegranate seed extracts did not show any ABA on the tested bacteria. The ABAs of pomegranate parts highly depended on the type and content of polyphenols and their specific targets such as cell membrane, cell wall, metabolic enzymes, protein synthesis and genetic systems of microorganisms [30]. Since the site and the number of hydroxyl groups on the polyphenols affect the toxicity against the microorganisms, the type of polyphenols in pomegranate parts was very important. Reddy et al. [11] evaluated AMAs of punicalagin, punicalin, ellagic acid and gallagic acid, which were isolated from a pomegranate rind extract, against pathogenic fungi (*Candida albicans, Cryptococcus neoformans* and *Aspergillus fumigatus*), a non-pathogenic strain of *E. coli,* and pathogenic bacteria (*P. aeruginosa* and *Mycobacterium intracellulare*). Punicalin and ellagic acid did not show any AMA at the highest concentration tested (20 µg/mL). However, gallagic acid and punicalagin inhibited the growth of *E. coli, P. aeruginosa* and *C. neoformans* with IC_{50} values lower than 15 µg/mL [11]. Similarly, the results of the present study also showed that pomegranate rind and membrane, which

contained punicalagin at high concentration, had the highest ABAs, and the seeds, which contained EAD, did not show ABA on the bacteria tested (Table 1). Another reason for this phenomenon may be that the amount of antimicrobial compounds in seeds was lower than the threshold of substrate concentration which is necessary for inhibition. As mentioned before, pomegranate seed (0.430−0.582 g/kg dm) had the lowest TPC among the pomegranate parts investigated in the present study (Table 1).

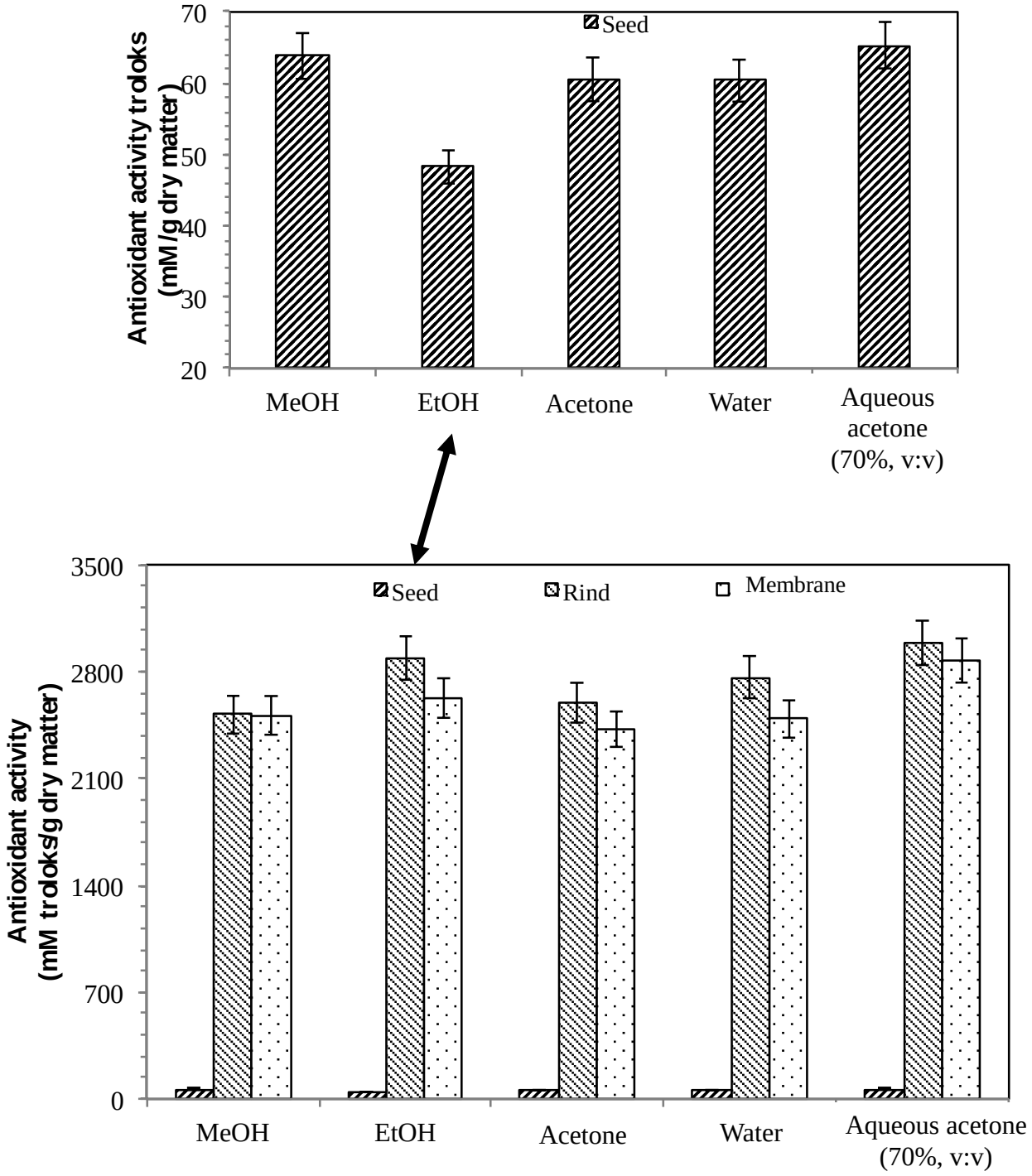

Figure 3. Effects of extraction solvents on AOAs of pomegranate parts (EtOH: ethanol, MeOH: methanol)

Table 1. ABAs of pomegranate parts in various extraction solvents against *B. megaterium* and *S. aureus*

Solvents	Diameter of inhibition zone (mm)[a]		
	Seed	Rind	Membrane
B. megaterium			
Methanol	–	13.32 ± 0.32B[b]	13.31 ± 0.35B
Ethanol	–	14.06 ± 0.21C	12.12 ± 0.22A
Acetone	–	14.40 ± 0.29C	12.40 ± 0.18A
Water	–	12.42 ± 0.25A	12.20 ± 0.31A
Aqueous acetone (acetone:water, 70:30, v:v)	–	13.36 ± 0.36B	13.84 ± 0.33B
S. aureus			
Methanol	–	20.19 ± 0.33A	18.46 ± 0.40A
Ethanol	–	19.77 ± 0.36A	18.71 ± 0.32A
Acetone	–	20.40 ± 0.30A	18.42 ± 0.19A
Water	–	20.43 ± 0.55AB	19.14 ± 0.46A
Aqueous acetone (acetone:water, 70:30, v:v)	–	20.50 ± 0.27B	18.68 ± 0.30A

There was no ABA of purified water containing 0.01% HCl (v:v) against *B. megaterium* and *S. aureus*. [a]Diameter of the zone was measured with the diameter of the wells. [b]Significant differences at $p<0.05$ were indicated with different letters.

CONCLUSION

Extraction solvents showed significant effect on AOA and TPCs due to the differences in the polarity of solvents ($P<0.05$). Aqueous acetone (acetone:water, 70:30, v:v) provided the highest TPC and thus AOA, since the pomegranate parts (rind and membrane) had polyphenols with high molecular weight such as hydrolysable tannin at high contents and aqueous acetone was especially effective on the extraction of high molecular weight polyphenols. Among pomegranate parts, the highest TPCs and AOA values were determined in pomegranate rind followed by membrane and seed. Due to their high AOA values, pomegranate rind and membrane could be used as natural antioxidant sources for the prevention of lipid oxidation that generates undesirable flavor and odor compounds and renders the foods unacceptable to consumers. Other than AOAs, the extracts of rinds and membranes had also ABAs on *S. aureus* and *B. megaterium,* while the seed extracts showed no ABA on the bacteria tested. Since choosing the suitable solvent affects the amount of polyphenol extracted, a solvent which is the most suitable for the effective extraction of polyphenols from any plant sources should be determined.

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ABBREVIATIONS

Total polyphenol (TP), antioxidant activity (AOA), antimicrobial activity (AMA), antibacterial activity (ABA).

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