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Phenolic Compounds of Pomegranate Byproducts (Outer Skin, Mesocarp, Divider Membrane) and Their Antioxidant Activities

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Supporting Information

ABSTRACT: Pomegranate peel was separated into outer leathery skin (PS), mesocarp (PM), and divider membrane (PD), and its phenolic compounds were extracted as free (F), esterified (E), and insoluble-bound (B) forms for the first time. The total phenolic content followed the order PD > PM > PS. $ABTS^{\bullet+}$, DPPH, and hydroxyl radical scavenging activities and metal chelation were evaluated. In addition, pomegranate peel extracts showed inhibitory effects against α-glucosidase activity, lipase activity, and cupric ion-induced LDL-cholesterol oxidation as well as peroxyl and hydroxyl radical-induced DNA scission. Seventy-nine phenolic compounds were identified using HPLC-DAD-ESI-MS^n mainly in the form of insoluble-bound. Thirty compounds were identified for the first time. Gallic acid was the major phenolic compound in pomegranate peel, whereas kaempferol 3-*O*-glucoside was the major flavonoid. Moreover, ellagic acid and monogalloyl-hexoside were the major hydrolyzable tannins, whereas the dominant proanthocyanidin was procyanidin dimers. Proanthocyanidins were detected for the first time.

KEYWORDS: *pomegranate, outer skin, mesocarp, divider membrane, antioxidant activity, phenolic compounds, insoluble-bound*

INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the family Punicaceae and is widely grown in tropical and subtropical regions of the world. Pomegranate is native to Iran and grown up to the Himalayas in northern India.[1] Global production of pomegranate was approximately 1,500,000 tons in 2009 with Iran being the largest producer (47%) and exporter.[2] Pomegranate is consumed as a fresh fruit or processed into juice or processed to syrup and sauce, where the rind or peel and seeds of the fruits are discarded.[3] We have previously identified the phenolic compounds from pomegranate seeds and juice using HPLC-DAD-MS^n.[3]

The pomegranate fruit contains many seeds (arils) separated by a white membrane called the pericarp. Pomegranate pericarp is a rich source of tannins, flavonoids, other polyphenols, and anthocyanins.[4] Duke and Ayensu[5] reported that the pericarp, containing up to 30% tannins, is used in tanning of leather. Several studies have shown the therapeutic effect of pomegranate peels, arils, juice, and flower, such as antiatherogenic activity,[6] antiangiogenic activity,[7] inhibition of formation of advanced glycation end products,[8] protection against hepatic oxidative stress,[9] and anti-hyperglycemic and anti-hyperlipidemic activities as well as protection against damage to kidneys[4] and anticarcinogenic activity.[10] A recent study revealed that pomegranate husk extracts, punicalagin and ellagic acid, inhibited fatty acid synthase and adipogenesis of 3T3-L1 adipocyte and could have potential effect in the prevention and treatment of obesity.[11] Anticarcinogenic activities include reduction in cell proliferation and induction of apoptosis in breast cancer,[10] suppression of inflammatory cell signaling in colon cancer,[12] down-regulation of expression of androgen-synthesizing genes in human prostate cancer cells, and overexpressing the androgen receptor.[13] The health beneficial potential of pomegranate fruit has been mainly attributed to the presence of ellagitannins, which are the predominant class of phenolics in pomegranate fruit and contain galloyl esters, punicalagin and punicalin, as the major compounds.[14]

Furthermore, several studies have focused on the phenolics and antioxidant activity of pomegranate peel.[10,15−22] Recently, Çam et al.[23] found that fortifying ice cream with pomegranate byproducts improved the functional properties of ice cream. In addition, Mosele et al.[24] have shown pomegranate peel extract to be the best source of antimicrobial substrates at colonic level compared to the pulp and juice. Moreover, pomegranate rind powder extract has been reported to exhibit the highest inhibition against oxidation of raw ground pork meat stored at 4 °C for 12 days compared to pomegranate juice and pomegranate seed powder extract.[25] Although many studies have been conducted on pomegranate peel, there is no detailed identification of soluble and insoluble-bound phenolic compounds from pomegranate peel components. Thus, in this study, we have isolated and identified free, esterified, and insoluble-bound phenolics from pomegranate leathery outer skin (PS), mesocarp (PM) (spongy part of peel), and divider membrane (PD). The in vitro antioxidant and biological activities of the isolated fractions were also determined.

MATERIALS AND METHODS

Materials. Pomegranate fruits, grown in California, were purchased from a local grocery store (St. John's, NL, Canada). α-Glucosidase from *Saccharomyces cerevisiae* (≥10 units/mg of protein), lipase from

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porcine pancreas (100−500 units/mg protein), *p*-nitrophenyl glucopyranoside, and 4-nitrophenyl octanoate were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). All chemicals used were obtained from Thermo Scientific Ltd. (Ottawa, ON, Canada) or Sigma-Aldrich Canada Ltd. The solvents used were of ACS grade, pesticide grade, or HPLC grade and were used without any further purification.

Sample Preparation and Defatting. Pomegranate peels were manually separated from the flesh, and the peel was segregated into outer skin (PS), mesocarp (PM), and divider membrane (PD). All samples were freeze-dried for 72 h and ground using a coffee bean grinder (model CBG5 series, Black & Decker, Canada Inc., Brockville, ON, Canada) to obtain a fine powder (mesh 16, sieve opening = 1 mm, Tylor test sieve, Mentor, OH, USA). Ground samples were defatted with hexane (1:5, w/v) for 5 min in a Waring blender (model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT, USA) at room temperature. The above procedure was repeated twice. The defatted samples were then air-dried and stored at −20 °C prior to extraction of phenolics, usually within 2 days.

Extraction of Phenolic Compounds. *Free Phenolics.* The defatted samples (PS, PM, and PD; 10 g) were mixed with 400 mL of 70% acetone and placed in an ultrasonicated water bath (300 Ultrasonik, Whittemore Enterprises, Inc., Rancho Cucamonga, CA, USA) for 20 min at 30 °C. The extracts were then centrifuged at 4000*g* (IEC Centra MP4, International Equipment Co., Needham Heights, MA, USA) for 5 min, and the supernatant was collected in a round-bottom flask. The residue obtained from centrifugation was oven-dried at 40 °C for 48 h and subsequently used for the extraction of insoluble-bound phenolics. The above procedure was repeated twice, and the combined supernatants were evaporated at 40 °C using a rotary evaporator until all of the acetone (∼280 mL) was removed from the supernatant. After evaporation, the water phase (∼100 mL) was acidified (pH 2.0) with 6 M HCl. Free phenolics were extracted five times with equal volumes of diethyl ether and ethyl acetate (1:1, v/v; ∼100 mL) in a separatory funnel. The organic phase was filtered through anhydrous sodium sulfate and evaporated to dryness under vacuum at 40 °C. Extracts were reconstituted in methanol (5 mL) and stored at −20 °C for subsequent analyses.

Esterified Phenolics. The water phase after the extraction of free phenolic was treated with an equal volume of 4 M NaOH to release the esterified phenolics. The hydrolysis was carried out under nitrogen for 4 h at room temperature with continuous stirring. After acidification (pH 2.0) with 6 M HCl, the contents were centrifuged at 4000*g* for 5 min. Phenolics released from soluble esters were extracted five times with equal volumes of diethyl ether and ethyl acetate (1:1, v/v), as described above, followed by evaporation to dryness under vacuum and reconstitution in 5 mL of methanol. Samples were stored at −20 °C for subsequent analyses.

Insoluble-Bound Phenolics. The solid residue obtained after free and esterified phenolic extraction centrifugation was oven-dried at 40 °C for 48 h and used for the extraction of insoluble-bound phenolics. The samples were hydrolyzed with 4 M NaOH (for 1 g sample, 20 mL of alkali) at room temperature under nitrogen for 4 h with continuous stirring. The resulting slurry was acidified (pH 2.0) with 6 M HCl and centrifuged at 4000*g* for 5 min. Then the phenolics released from soluble esters were extracted five times with equal volumes of diethyl ether and ethyl acetate (1:1, v/v), as described above, followed by evaporation to dryness under vacuum and reconstitution in 5 mL of methanol. Samples were stored at −20 °C for subsequent analyses.

Total Phenolic Content. The total phenolic contents of each pomegranate extract obtained from PS, PM, and PD were determined using the method described by Singleton and Rossi[26] with minor modifications. Samples dissolved in methanol (0.5 mL) were mixed with Folin−Ciocalteu's reagent (0.5 mL) and distilled water (4 mL), and the contents were thoroughly vortexed. After 3 min, the reaction mixture was subsequently neutralized by the addition of a solution of saturated sodium carbonate (0.5 mL) and mixed thoroughly. The final mixture was allowed to stand at room temperature in the dark for 2 h. The absorbance of the supernatant (blue color) was read at 760 nm using a UV−visible spectrophotometer. The total phenolic content of pomegranate extract was determined using a standard curve prepared for gallic acid, and the results were expressed as micromoles of gallic acid equivalents (GAE) per gram of defatted sample.

Total Anthocyanin Content. The total monomeric anthocyanin content of pomegranate byproduct extracts was determined by the pH-differential method described by Giusti and Wrolstad.[27] Pomegranate sample extracts dissolved in methanol (0.5 mL) were diluted with 2.5 mL of 0.025 M potassium chloride (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5), separately. The diluted solutions were then allowed to stand at room temperature for 15 min and centrifuged at 4000*g* for 5 min. The absorbance of each dilution was read at 520 and 700 nm. A blank was prepared with methanol. The monomeric anthocyanin content was calculated using the equation

$$\text{monomeric anthocyanin content (mg)} = (A \times \text{MW} \times \text{DF} \times 1000)/(\varepsilon \times l)$$

where A = absorbance $(A_{520\ \text{nm}} - A_{700\ \text{nm}})_{\text{pH1.0}} - (A_{520\ \text{nm}} - A_{700\ \text{nm}})_{\text{pH4.5}}$, MW = molecular weight of cyanidin-3-*O*-glucoside (CGE; $C_{15}H_{11}O_6$, 449.2 amu), DF = dilution factor, ε = molar absorptivity (26900), and l = path length (1 cm).

The results of the monomeric anthocyanin content of pomegranate samples were expressed as milligrams per 100 g of defatted sample.

Antioxidant Assays. *2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate) Radical Cation ($ABTS^{\bullet+}$) Radical Cation Scavenging Assay.* The $ABTS^{\bullet+}$ scavenging activity of pomegranate byproduct extracts was determined according to the method described by Chandrasekara and Shahidi[28] with slight modifications. Phosphate buffer (0.1 M, pH 7.4) containing 0.15 M sodium chloride was used to prepare AAPH (2.5 mM) and ABTS (2.5 mM) solutions. The $ABTS^{\bullet+}$ solution was prepared by mixing 2.5 mM AAPH with 2.5 mM ABTS stock solution (1:1, v/v), and the resultant solution was heated for 20 min at 60 °C, protected from light, and stored at room temperature. Samples (40 μL) were mixed with the $ABTS^{\bullet+}$ solution (1.96 mL), and the absorbance of the above mixture was read after 6 min (completion of the reaction time) at 734 nm. A standard curve was constructed by measuring the reduction in absorbance of the $ABTS^{\bullet+}$ solution at different concentrations of Trolox (0−1000 μM). The decrease in the absorbance at 734 nm after 6 min of addition of a test compound was used for calculating the results. Blank measurements of $ABTS^{\bullet+}$ stock solution were also made. ABTS radical cation scavenging activity was expressed as micromoles of Trolox equivalents per gram of defatted sample.

$$\text{ABTS radical cation scavenging activity (\%)} = \left(\frac{\text{absorbance of blank} - \text{absorbance of sample after 6 min}}{\text{absorbance of blank}}\right) \times 100$$

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay. The DPPH radical scavenging activity of phenolic extracts from pomegranate samples was determined.[29] Samples (250 μL) in methanol were added to a methanolic solution of DPPH (0.30 mM, 1 mL), vortexed, and allowed to stand at room temperature in the dark for 10 min. The mixture (1 mL) was injected to the sample cavity of a Bruker E-scan electron paramagnetic resonance (EPR) spectrometer (Bruker E-scan, Bruker Biospin Co., Billercia, MA, USA), and the spectrum was recorded. A standard curve was prepared using Trolox (50−500 μM in methanol). The parameters of the Bruker E-scan EPR spectrometer were set as follows: 5.02×10^2 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.00 G sweep width, 3495.53 G center field, 5.12 ms time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency, and 1.86 G modulation amplitude. DPPH radical scavenging capacities of the pomegranate samples were calculated using the following equation.

$$\text{DPPH radical scavenging capacity (\%)} = \left(\frac{\text{EPR signal intensity for control} - \text{EPR signal intensity for sample}}{\text{EPR signal intensity for control}}\right) \times 100$$

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The DPPH radical scavenging activity was expressed as micromoles of Trolox equivalents per gram of defatted sample.

Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging capacity of pomegranate extracts was determined according to the method of Chandrasekara and Shahidi[28] with slight modifications. For this assay, methanol was removed from pomegranate extracts using nitrogen, and the contents were redissolved in 75 mM phosphate buffer (pH 7.2). Pomegranate samples (200 μL) in 75 mM phosphate buffer (pH 7.2) were mixed with H_2O_2 (10 mM, 200 μL), DMPO (17.6 mM, 400 μL), and $FeSO_4$ (10 mM, 200 μL). Samples were injected into the sample cavity of an EPR spectrometer after 3 min and their spectra recorded. Gallic acid dissolved in 75 mM phosphate buffer (pH 7.2) was used to prepare a standard curve (200−1000 ppm). Hydroxyl radical scavenging capacities were calculated using the following equation:

$$\text{hydroxyl radical scavenging capacity (\%)} = \left(\frac{\text{EPR signal intensity for control} - \text{EPR signal intensity for sample}}{\text{EPR signal intensity for control}}\right) \times 100$$

The hydroxyl radical scavenging activity of the samples was expressed as micromoles of gallic acid equivalents per gram of defatted sample.

Ferrous Ion Chelating Activity. Metal chelation of pomegranate extracts was measured according to the method described by Ambigaipalan et al.[30] with slight modification. Samples in methanol (0.4 mL) were mixed with 0.2 mM $FeCl_2$ (0.5 mL) and 5 mM ferrozine (0.2 mL). Then 2.9 mL of distilled water was added and vortexed. The mixtures were allowed to stand at room temperature for 10 min, and the absorbance of the resulting solution was read at 562 nm. A standard curve was constructed using trisodium salt of ethylenediaminetetraacetic acid (Na_3EDTA), and the inhibition of ferrozine−ferrous ion complex formation (%) was calculated using the following equation:

$$\text{metal chelating activity (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100$$

The results of ferrous ion chelating ability of the pomegranate samples were expressed as micromoles of EDTA equivalents per gram of defatted sample.

In Vitro Biological Assays. *α-Glucosidase Inhibitory Activity.* The α-glucosidase inhibitory activity of pomegranate extracts was measured according to the method of Eom et al.[31] with slight modifications. For this assay, extracted samples in methanol were used without any dilution. Each pomegranate test sample dissolved in methanol (10 μL) was mixed with 620 μL of potassium phosphate buffer (0.1 M, pH 6.8) in an Eppendorf tube. α-Glucosidase (10 U/mL, 5 μL) dissolved in potassium phosphate buffer (0.1 M, pH 6.8) was added to the sample solution. After incubation at 37 °C for 20 min, substrate *p*-nitrophenyl glucopyranoside dissolved in distilled water (10 mM, 10 μL) was added to initiate the reaction. The reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 650 μL of 1 M Na_2CO_3. The amount of released product *p*-nitrophenol (yellow color) was measured at 410 nm using a UV−visible spectrophotometer. Sample blanks without enzyme and a control without sample were also measured. α-Glucosidase inhibition percentage was calculated using the equation

$$\text{α-glucosidase inhibitory activity (\%)} = \left(1 - \frac{\text{absorbance of sample} - \text{absorbance of sample blank}}{\text{absorbance of control} - \text{absorbance of control blank}}\right) \times 100$$

The corresponding IC_{50} value, defined as the concentration of inhibitor required to inhibit 50% of the α-glucosidase activity, was also calculated.

Lipase Inhibitory Activity. The lipase inhibitory activity of pomegranate extracts was determined according to the method described by Marrelli et al.[32] For this assay, extracted samples in methanol were used without any dilution. Lipase from porcine pancreas was dissolved in 1 M Tris-HCl buffer (pH 8.5) to yield a concentration of 5 mg/mL. Tris-HCl (1 M, pH 8.5, 4 mL) was added to sample dissolved in methanol (100 μL). Then 100 μL of pancreatic lipase was added and incubated at 37 °C for 25 min. A substrate solution of 4-nitrophenyl octanoate (10 mM) was prepared in dimethyl sulfoxide (DMSO) and diluted with ethanol to reach a final concentration of 5 mM. To initiate the reaction, 100 μL of 4-nitrophenyl octanoate (5 mM) was added and incubated at 37 °C for 25 min. A control with methanol instead of sample and sample blanks without enzyme were prepared. The absorbance (yellow color) was measured at 412 nm using a UV−visible spectrophotometer. The lipase inhibition percentage was calculated using the equation

$$\text{lipase inhibitory activity (\%)} = \left(1 - \frac{\text{absorbance of sample} - \text{absorbance of sample blank}}{\text{absorbance of control} - \text{absorbance of control blank}}\right) \times 100$$

The corresponding IC_{50} value was also calculated for the pomegranate peel extracts.

Inhibition of Cupric Ion-Induced Human Low-Density Lipoprotein (LDL) Peroxidation. The inhibitory effect of pomegranate peel extracts on cupric ion-induced human LDL peroxidation was determined according to the method described by Ambigaipalan and Shahidi.[33] Initially, 5 mg/mL LDL was dialyzed against 100 volumes of freshly prepared 10 mM phosphate buffer (pH 7.4, 0.15 M NaCl). A dialysis tube with a molecular weight cutoff of 12−14 kDa (Fischer, Carle and Kammerer Scientific, Nepean, ON, Canada) was used to dialyze at 4 °C under a nitrogen blanket in the dark for 12 h. Diluted LDL cholesterol (0.04 mg LDL/mL) was mixed with the pomegranate peel extracts dissolved in phosphate buffer (0.1 mg/mL). The samples were pre-incubated at 37 °C for 15 min, and the reaction was initiated by adding a solution of cupric sulfate (0.1 mL, 100 μM). Then the samples were incubated at 37 °C for 22 h. The formation of conjugated dienes (CD) was recorded at 234 nm using a diode array spectrophotometer (Agilent, Palo Alto, CA, USA). Catechin (100 ppm) was used as a positive control. The appropriate blanks were run for each sample by replacing LDL cholesterol and $CuSO_4$ with distilled water for background correction.

Inhibition of Peroxyl and Hydroxyl Radical-Induced Supercoiled DNA Strand Scission. Peroxyl and hydroxyl radical-induced supercoiled plasmid DNA strand scission inhibitory activity of pomegranate extracts was determined.[33] Supercoiled plasmid DNA (pBR 322) (50 μg/mL) was dissolved in freshly prepared 10 mM phosphate buffer (PBS, pH 7.4). In a 0.5 mL eppendorf tube pomegranate extracts dissolved in PBS (2 μL), PBS (2 μL), pBR 322 (50 μg/mL, 2 μL), and 4 μL of 17.5 mM AAPH [2,2′-azobis(2-methylpropanimidamide dihydrochloride, prepared in PBS] were added to generate peroxyl radicals, whereas pomegranate extracts (2 μL), PBS (2 μL), pBR 322 (50 μg/mL, 2 μL), $FeSO_4$ (0.5 mM, 2 μL), and H_2O_2 (1 mM, 2 μL) were added to produce hydroxyl radicals. The mixture was incubated at 37 °C for 1 h in the dark. A control with DNA alone and a blank devoid of pomegranate extracts were also prepared. After incubation, 1 μL of the loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol) was added to the reaction mixture. The mixture (10 μL) was loaded onto 0.7% agarose gel prepared in Tris−acetic acid−EDTA (TAE) buffer (40 mM Tris−acetate containing 1 mM EDTA, pH 8.5). SYBR safe (5 μL) was added into the agarose gel solution (50 mL) as a gel stain. Electrophoresis was conducted at 80 V for 60 min using a model B1A horizontal mini gel electrophoresis system (Owl Separation Systems Inc., Portsmonth, NH, USA) and a model 300 V power supply (VWR International Inc., West Chester, PA, USA) in TAE buffer. The DNA bands were visualized under transillumination of UV light using an Alpha-Imager gel documentation system (Cell Biosciences, Santa Clara, CA, USA). The intensity (area %) of bands was quantified with Chemi-Imager 4400 software (Cell Biosciences). The retention of supercoiled DNA strand (%) was calculated using following equation:

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$$\text{DNA retention (\%)} = \frac{\text{area of supercoiled DNA with oxidative radical and extract}}{\text{area of supercoiled DNA in control}} \times 100$$

Identification of Phenolic Compounds by HPLC-DAD-ESI-MS^n Analysis. Phenolic compounds in the free (F), esterified (E), and insoluble-bound (B) fractions of PS, PM, and PD were identified using a RP-HPLC Agilent 1100 system equipped with a quaternary pump (G1311A), a degasser (G1379A), an ALS automatic sampler (G1329A), an ALS Therm (G1130B), a Colcom column compartment (G1316), a diode array detector (DAD, G1315B), and a system controller linked to a Chem Station Data handling system (Agilent Technologies, Palo Alto, CA, USA). Separations were conducted with a SUPERLCOSILTM LC-18 column (4.6 × 250 mm × 5 μm, Merck, Darmstadt, Germany). The binary mobile phase consisted of 0.1% formic acid (eluent A) and 0.1% formic acid in acetonitrile (eluent B). The flow rate was maintained at 0.5 mL/min, and the elution gradient used was according to the method described by de Camargo et al.[34] as follows; 0 min, 100% A; 5 min, 90% A; 35 min, 85% A; 45 min, 60% A; held at 60% A from 45 to 50 min; afterward mobile phase A was increased to 100% at 55 min, followed by column equilibration from 55 to 65 min. The phenolic acids and flavonoids were detected at 280 nm, whereas anthocyanins were identified at 520 nm, and the samples were filtered using a 0.45 μm PTFE membrane syringe filter (Thermo Scientific, Rockwood, TN, USA) before injection (50 μL).

HPLC-ESI-MS^n analysis was carried out under the same conditions as described above using an Agilent 1100 series capillary liquid chromatography−mass selective detector (LC-MSD) ion trap system in electrospray ionization (ESI) in the negative mode for phenolic acids and flavonoids as well as positive mode for anthocyanin detection. The data were acquired and analyzed with Agilent LC-MSD software (Agilent Technologies). The scan range set was from m/z 50 to 2000, using smart parameter setting, drying nitrogen gas at 350 °C, flow of 12 L/min, and nebulizer gas pressure of 70 psi. Phenolic acids, namely, protocatechuic, *p*-coumaric, vanillic, gallic, caffeic, ferulic, and ellagic acids, and flavonoids (+)-catechin, (−)-epicatechin, kaempferol, and quercetin were identified by comparing their retention times and ion fragmentation pattern with coded and authentic standards under the same conditions as the samples. Other compounds were tentatively identified using tandem mass spectrometry (MS^n), UV spectral data, and literature data.

Statistical Analysis. All assays were replicated three times, and mean values and standard deviations were reported. One-way ANOVA was performed, and the mean separations were performed by Tukey's HSD test ($p < 0.05$) using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Total Phenolic and Anthocyanin Content of Pomegranate Peel Extracts. The total phenolic content of 70% acetone extracted free (F), esterified (E), and insoluble-bound (B) fractions of PS, PM, and PD were determined by Folin−Ciocalteu's method. Polyphenolic compounds of pomegranate extracts could reduce the Folin−Ciocalteu reagent and form a blue complex that could be measured spectrophotometrically at 760 nm. The total phenolic content of PS (F, E, B), PM (F, E, B), and PD (F, E, B) ranged from 0.48 ± 0.01 to 12.95 ± 1.02 mg GAE/g of sample (Figure 1A). Among all extracts, PD-E made the highest contribution to the total phenolic content (12.95 ± 1.02 mg GAE/g of sample), whereas PS-B and PD-B had the lowest gallic acid equivalents of 0.54 ± 0.03 and 0.48 ± 0.01 mg/g of sample, respectively. In all pomegranate byproduct parts, soluble phenolic content (free and esterified) was higher than that of the insoluble-bound fraction. The total phenolic content of pomegranate peel fractions followed the decreasing order PD (21.18 mg GAE/g of sample) > PM (17.74 mg GAE/g of sample) > PS (9.38 mg GAE/g of sample). This study shows that most of the phenolics of pomegranate peel were concentrated in the divider membrane (~44%). According to Fischer et al.,[35] the total phenolic content of pomegranate mesocarp (198.17 mg GAE/g dry matter) was higher than that of the outer skin extract (101.85 mg GAE/g dry matter) when using aqueous methanol (80%, v/v; 0.1% HCl) extraction. Although a similar distribution trend of phenolics was observed in pomegranate outer skin and mesocarp, the values were significantly higher compared to our data. This could be due to the variation in the solvent used for extraction as well as varietal differences. On the basis of our study, the total phenolic content of pomegranate peel was 48.3 mg GAE/g of sample, which is the sum of total phenolics from PS, PM, and PD. Aviram et al.[36] determined the total phenolic content of peel, membrane, and aril residues of pomegranate extracted using water after enzymatic pretreatment and found 930 mg GAE/g of sample for total phenolics. In another study, Viuda-Martos et al.[37] reported 4.6−10 mg GAE/g of pomegranate bagasse obtained as a coproduct in the juice extraction for total phenolics. Variation in the total phenolic content compared to the reported literature data suggests that this assay could be influenced by the solvent system used for phenolics extraction from pomegranate peel. Wang et al.[20] studied the effect of various solvent systems on the extraction of pomegranate peel and reported that methanol afforded the highest extract yield of the total phenolics (8.26%), followed by water (5.90%), ethanol (1.55%), acetone (0.37%), and ethyl acetate (0.18%), respectively. Later, Elfalleh et al.[38] also showed that the total phenolic content of pomegranate peel was increased from 54 to 86 mg GAE/mg when using methanol

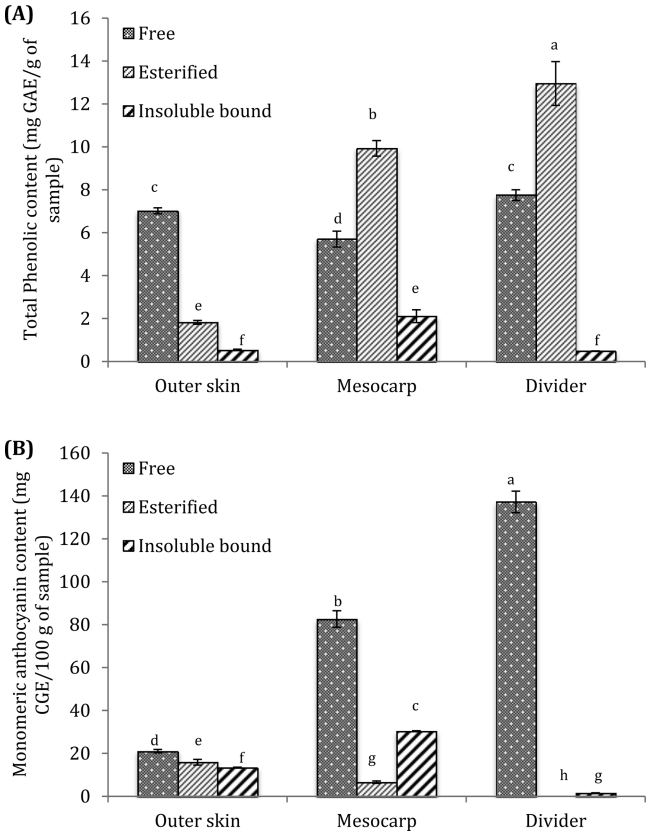

Figure 1. Total phenolic content (mg GAE/g of sample) and monomeric anthocyanin content (mg/100 g of sample) of pomegranate outer skin, mesocarp, and divider membrane.

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instead of water for extraction. As mentioned before, Folin−Ciocalteu's method could also react with nonphenolic reducing substances such as certain sugars, amino acids, vitamin C, and other organic acids. This may explain certain discrepancies with the data reported in the literature.

The total monomeric anthocyanin content of pomegranate peel extracts was determined by a rapid and simple pH-differential method and reported as cyanidin-3-*O*-glucoside (CGE) equivalents. This method is based on the structural transformation of anthocyanins that occurs with a change in pH (colored at pH 1.0 and colorless at pH 4.5).[39] The total anthocyanin content of pomegranate peel ranged from 0 to 137.17 ± 5.02 mg CGE/100 g of sample (Figure 1B). Free phenolic fraction of PS, PM, and PD had the highest monomeric anthocyanin content in comparison with soluble esters and insoluble-bound fractions. Total monomeric anthocyanin content followed the order PD > PM > PS with cyanidin-3-*O*-glucoside equivalents of 139, 120, and 50 mg/100 g, respectively. Moreover, significantly ($p < 0.05$) higher proportions of anthocyanins (137.17 ± 5.02 mg CGE/100 g) were detected in the PD-F that surrounds the pulp and aril parts of the pomegranate fruit. Our study showed that total monomeric anthocyanin content of pomegranate peel was ∼309 mg CGE/100 g. Elfalleh et al.[38] showed that the content of total anthocyanins in pomegranate peel ranged between 5100 and 10200 mg CGE/100 g of dry weight. However, Fischer et al.[35] determined the total anthocyanins of pomegranate peel using HPLC and found 44.7 mg/100 g dry matter from peel, whereas no anthocyanins were detected in mesocarp. However, our study showed the presence of anthocyanins in mesocarp at ∼120 mg CGE/100 g. This indicates that 70% acetone could extract most of the anthocyanins from pomegranate peel compared to methanol extraction and, hence, contributes to the higher values in our study. Discrepancies in the total anthocyanin content could be due to the varietal difference, variation in the solvent system used for extraction, and contamination of pomegranate byproducts with juice as well as various procedures used for the determination.

Antioxidant Activities of Pomegranate Outer Skin, Mesocarp, and Divider Membrane. In vitro antioxidant activities such as radical scavenging activities ($ABTS^{\bullet+}$, DPPH, and hydroxyl radicals) and metal chelation of free, esterified, and insoluble-bound phenolic fractions from PS, PM, and PD were determined and are shown in Table 1. $ABTS^{\bullet+}$ is an artificial radical cation that could be reduced in the presence of hydrogen-donating antioxidants and forms a blue-green chromophore (colored $ABTS^{\bullet+}$) with characteristic absorption at 734 nm.[30] The ABTS radical scavenging activity of PS (F, E, B), PM (F, E, B), and PD (F, E, B) were well correlated with their total phenolic contents. PM-E and PD-E exhibited the highest ABTS radical scavenging activity with Trolox equivalents of 208.73 ± 15.28 and 206.87 ± 2.39 μmol/g of sample, respectively (Table 1). Meanwhile, PS-B and PD-B had the lowest Trolox equivalent values (Table 1). Elfalleh et al.[38] reported that ABTS radical scavenging of the methanol extract from peels of Chinese pomegranate was 75 μmol Trolox equiv/g dry weight, which is lower than our data. However, according to Fischer et al.[35] ABTS radical scavenging activities of pomegranate peel and mesocarp were 1361.9 ± 13.7 and 2887.1 ± 6.5 μmol Trolox equiv/g sample, respectively, which is higher than values in this work. Variation in ABTS experimental procedure as well as the presence of higher

Table 1. Antioxidant Activities of Free, Esterified, and Insoluble-Bound Phenolic Fractions from Pomegranate Outer Skin, Mesocarp, and Divider Membrane[a]

sample		ABTS radical scavenging activity (μmol of Trolox equiv/g sample)	DPPH radical scavenging activity (mmol of Trolox equiv/g sample)	hydroxyl radical scavenging (μmol of gallic acid equiv/g sample)	metal chelation (μmol of EDTA equiv/g sample)
outer skin	free	122.62 ± 6.01b	3.36 ± 0.02d	59.02 ± 4.50d	0.07 ± 0.03e
	esterified	52.58 ± 0.77d	0.89 ± 0.00f	47.14 ± 2.99d	0.00 ± 0.00e
	insoluble-bound	11.72 ± 0.40f	0.23 ± 0.03g	29.31 ± 5.16e	0.86 ± 0.06e
mesocarp	free	104.55 ± 2.26c	2.56 ± 0.07e	84.18 ± 5.62c	3.44 ± 0.31d
	esterified	206.87 ± 2.39a	5.79 ± 0.16b	166.50 ± 5.96a	14.32 ± 0.14a
	insoluble-bound	32.63 ± 1.99e	0.95 ± 0.02f	50.72 ± 3.81d	7.93 ± 0.18c
divider	free	115.89 ± 1.76bc	3.58 ± 0.01c	143.22 ± 2.16b	4.01 ± 0.23d
	esterified	208.73 ± 15.28a	6.71 ± 0.06a	171.97 ± 6.97a	11.97 ± 1.41b
	insoluble-bound	5.34 ± 0.15f	0.12 ± 0.02g	80.11 ± 3.08c	0.78 ± 0.08e

[a]All data represent the mean of triplicates. Values followed by the same letter are not significantly different ($p > 0.05$) by Tukey's HSD test.

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Table 2. α-Glucosidase and Lipase Inhibitory Activities of Free, Esterified, and Insoluble-Bound Phenolic Fractions from Pomegranate Outer Skin, Mesocarp, and Divider Membrane[a]

sample		α-glucosidase		lipase	
		inhibition (%/mg extract)	IC_{50} (mg/mL extract)	inhibition (%/mg extract)	IC_{50} (mg/mL extract)
outer skin	free	23.66 ± 3.49b	2.14 ± 0.30f	2.40 ± 0.07c	20.87 ± 0.60cde
	esterified	50.68 ± 2.78a	0.99 ± 0.06f	4.64 ± 0.07b	10.77 ± 0.17ef
	insoluble-bound	2.50 ± 0.51de	20.66 ± 4.56c	0.81 ± 0.20d	64.38 ± 16.65a
mesocarp	free	2.09 ± 0.02de	23.95 ± 0.26bc	1.83 ± 0.08cd	27.34 ± 1.21cd
	esterified	16.38 ± 1.96c	3.08 ± 0.37ef	1.06 ± 0.01d	47.13 ± 0.64b
	insoluble-bound	5.08 ± 0.22de	9.86 ± 0.42d	4.34 ± 0.32b	11.57 ± 0.87def
divider	free	1.81 ± 0.02de	27.67 ± 0.36b	1.69 ± 0.03cd	29.52 ± 0.52cd
	esterified	0.57 ± 0.03e	87.26 ± 4.66a	1.90 ± 0.10cd	49.48 ± 2.72ab
	insoluble-bound	5.95 ± 0.52d	8.45 ± 0.75de	15.16 ± 1.19a	3.31 ± 0.25f

[a]All data represent the mean of triplicates. Values followed by the same letter are not significantly different ($p > 0.05$) by Tukey's HSD test.

amounts of total phenolics compared to pomegranate extracts used in this work may account for these differences.

The DPPH radical is another artificial organic nitrogen radical that is more stable compared to the highly reactive and transient peroxyl and hydroxyl radicals that are involved in lipid peroxidation and tissue injury in biological systems.[30] Antioxidative compounds are capable of quenching DPPH radicals by providing either hydrogen atoms or electrons and converting them to a colorless or bleached product (i.e., 2,2-diphenyl-1-hydrazine, or a substituted hydrazine).[40] Electron paramagnetic resonance (EPR) spectroscopy was used in this study to detect the DPPH radical scavenging activity of PS (F, E, B), PM (F, E, B), and PD (F, E, B). Scavenging of DPPH radicals would bring about a decrease in peak intensity of the EPR spectrum. DPPH radical scavenging levels varied between 0.12 ± 0.02 and 6.71 ± 0.06 mmol Trolox equiv/g sample (Table 1), which is nearly 14−60% of DPPH radical scavenging activity. Pomegranate peel extracts with higher total phenolic content exhibited higher DPPH scavenging activity and vice versa. This finding is similar to that reported by Wang et al.,[20] who found a linear relationship (R^2 = 0.9779) between the DPPH scavenging activity and total phenolic content of pomegranate peel. However, no linear relationship with total anthocyanin content was observed. Pomegranate homogenates prepared from 29 different pomegranate accessions showed that there was no correlation between antioxidant activity and anthocyanin content.[41] Thus, total phenolic content plays a vital role in determining the antioxidant activity of a compound; hence, effective phenolic extraction is required for accurate determination. Moreover, the concentration required for achieving 50% DPPH radical inhibition (IC_{50}) ranged from 0.17 to 0.74 mg/mL of pomegranate peel extracts. According to Viuda-Martos et al.,[37] the IC_{50} value for DPPH radical scavenging activity of pomegranate bagasse ranged from 11.6 to 37.8 mg/mL. In comparison with the literature, our study showed a lower concentration of sample for the 50% inhibition of DPPH radical. Another study on pomegranate peel showed an IC_{50} value of 3.88−11.48 μg/mL for inhibition of the DPPH radical.[38]

The hydroxyl radical is highly reactive and could easily react with biomolecules (amino acids, proteins, enzymes, RNA, and DNA), hence leading to cell or tissue injury associated with degenerative diseases.[22,42] Hydroxyl radicals generated in an Fe^{2+}/H_2O_2 system by Fenton reaction will form a spin adduct of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), which could be reflected in a typical EPR signal (1:2:2:1). The height of the third peak of the EPR spectrum represents the relative amount of DMPO−OH adducts that could be decreased in the presence of antioxidants.[43] Hydroxyl radical scavenging activity of pomegranate peel components was expressed as gallic acid equivalents (GAE), and the value ranged between 29.31 ± 5.16 and 171.97 ± 6.97 μmol/g sample (Table 1). PD-E and PM-E exhibited the highest hydroxyl radical scavenging activity, whereas PS-B showed the lowest activity. This assay also showed a similar trend as other antioxidant assays explained above. A few studies have shown hydroxyl radical scavenging activity of pomegranate peel. Singh et al.[16] observed approximately 58% hydroxyl radical scavenging activity from 100 ppm of pomegranate peel extract. Another study showed that the pomegranate peel extract possessed about 25 times higher hydroxyl scavenging activity than the pulp extract.[18] In this method, it is impossible to distinguish whether the pomegranate extract is chelating Fe^{2+} ion or scavenging hydroxyl radical. This is because when the sample is mixed with Fe^{2+}, it may affect the formation of hydroxyl radical by chelating ferrous ion.[44] Hence, the preventive capacity of pomegranate extracts could be related to the metal chelating ability of the samples. Interestingly, in this study, PS-E, which did not exhibit any metal chelation, showed hydroxyl radical scavenging activity (47.14 ± 2.99 μmol GAE/g; Table 1). Thus, this assay measures both the radical scavenging and chelating capacity of antioxidants.

A phenolic group becomes a chelator or metal binder once it is deprotonated, carries a high charge density oxygen center, and next to another oxygen center such as hydroxyl group in the ortho position.[45] Therefore, in this assay, a decrease in the intensity of ferrozine−ferrous color complex (pink color) by pomegranate phenolic extracts was measured at 562 nm using a spectrophotometer and reported as EDTA equivalents (Table 1). Metal chelation values of PS (F, E, B), PM (F, E, B), and PD (F, E, B) varied between 0 and 14.3 μmol EDTA equiv/g sample. PM-E (14.3 ± 0.14 μmol EDTA equiv/g) had the highest ($p < 0.05$) metal chelating ability followed by PD-E (11.97 ± 1.41 μmol EDTA equiv/g), PM-B (7.93 ± 0.18 μmol EDTA equiv/g), and PD-F (4.01 ± 0.23 μmol EDTA equiv/g). PS-E and PM-E also had the highest total phenolic content (Figure 1A) among others. However, PS-E extract did not show any chelating ability. This could be due to the amount and type of phenolic compounds present in the esterified fraction. For example, quercetin has three metal chelating sites, while

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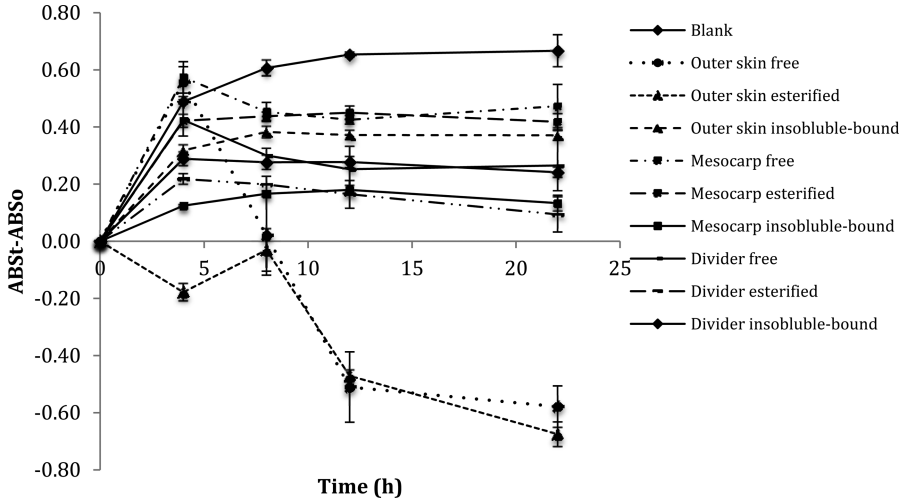

Figure 2. Absorbance (ABS) values for the formation of conjugated dienes in free, esterified, and insoluble-bound phenolic fractions from pomegranate outer skin, mesocarp, and divider membrane during cupric ion induced human LDL oxidation. ABS_t, absorbance at time t after incubation; ABS_0, absorbance at zero time.

kaempferol has only two possible binding sites. In addition, when a phenol group forms glycosidic bond with carbohydrates, it loses the ability to chelate metal ions. For example, rutin possesses only two binding sites due to its one glycosidic bond. All other pomegranate peel fractions showed little metal chelating ability. Mention should be made that metal chelation of pomegranate peel is being reported here for the first time.

Biological Activities of Pomegranate Outer Skin, Mesocarp, and Divider Membrane. *α-Glucosidase and Lipase Inhibitory Activity.* α-Glucosidase hydrolyzes the terminal, nonreducing 1,4-linked α-D-glucose residues of disaccharides (e.g., maltose and sucrose) with release of α-D-glucose, hence enabling gastrointestinal absorption. Thus, α-glucosidase inhibitory effects of PS (F, E, B), PM (F, E, B), and PD (F, E, B) were studied using an in vitro assay. The α-glucosidase inhibitory activity of pomegranate peel components varied from 0.57 ± 0.03 to 50.68 ± 2.78%/mg of extract (Table 2). Among all tested samples, PS-E exhibited the highest ($p < 0.05$) inhibition followed by PS-F and PM-E. In contrast, all other tested extracts showed <6% inhibition. These values were not correlated with total phenolic or anthocyanin contents. The concentration of extracts required for 50% α-glucosidase inhibition (IC_{50}) ranged from 0.99 ± 0.06 to 87.26 ± 4.66 mg/mL of extract (Table 2). This indicates that 50% inhibition of α-glucosidase activity requires about 1 mg/mL of PS-E. As supportive evidence for this value, Kam et al.[46] showed that the methanolic extract of pomegranate peel has selectivity toward α-glucosidase inhibition compared to α-amylase inhibition with the IC_{50} value of 0.835 mg/mL. They also reported that gallic acid and ellagic acid were the major compounds involved in this inhibitory action. Phenolic compounds may form complexes with proteins through hydrogen bonds or addition of nucleophiles to oxidized quinones, and this may explain the ability of phenolics in inhibiting different enzymes.[47] Meanwhile, Zhen-jian[48] reported that 60 mg/mL pomegranate rind peel extracts inhibited only 39.55% of α-glucosidase activity. Recently, Çam et al.[49] incorporated microencapsulated pomegranate peel phenolics (1.0%) in regular ice cream and found α-glucosidase inhibitory activity of IC_{50} = 22.9 mg/mL. Earlier, some investigations were carried out using pomegranate flower and peel extracts and effects on antidiabetic potential as the available literature evidenced their application in traditional medicine for controlling diabetes mellitus. Parmar and Kar[50] showed that administration of 200 mg/kg pomegranate peel to male mice lowered their blood glucose level and showed potential antidiabetic effect.

Pancreatic lipase breaks down triacylglycerols into monoacylglycerols and free fatty acids, which could subsequently be absorbed into enterocytes. Thus, inhibition of lipase activity could reduce the deposition of body fat in the adipose tissues. Lipase inhibitory activity of PS (F, E, B), PM (F, E, B), and PD (F, E, B) ranged between 0.81 ± 0.20 and 15.16 ± 1.19%/mg of extract (Table 2). Lipase inhibition activity of pomegranate peel extracts followed the order PD-B > PS-E ∼ PM-B > PS-F > PD-E ∼ PM-F ∼ PD-F > PM-E ∼ PS-B. It is noteworthy that all other pomegranate extracts exhibited very little lipase inhibitory activity (<5%) as reflected in their higher IC_{50} values. The concentration of pomegranate peel extracts required for inhibiting 50% of enzyme activity ranged from 3.31 ± 0.25 to 64.38 ± 16.65 mg/mL of extract. Insoluble-bound fraction of divider membrane had the best inhibitory activity (15.16 ± 1.19%/mg of extract) against lipase, which had the lowest IC_{50} (3.31 ± 0.25 mg/mL) among all extracts. Pomegranates are good sources of hydrolyzable tannins. Several studies have shown that high tannin content plants exhibit lipase inhibitory activity.[51−53] Although the inhibitory mechanism of phenolic compounds toward lipase is not clear, it has generally been attributed to the ability of tannins to bind, complex, and precipitate proteins via noncompetitive or mixed inhibition mechanism.[54] However, Mathew et al.[55] reported that the consumption of a single drink containing ellagitannin-rich pomegranate extract did not decrease postprandial plasma triacylglycerol concentrations, but suppressed the postprandial increase in systolic blood pressure following a high-fat meal intake.

It is noteworthy that α-glucosidase or lipase inhibition did not show any correlation with total phenolic content. In addition, the pomegranate peel extracts showing highest α-glucosidase inhibition did not necessarily exhibit the highest lipase inhibitory activity. Moreover, both enzyme inhibition mechanisms remain unclear. This reflects that regardless of total phenolic content, the type and amount of phenolics

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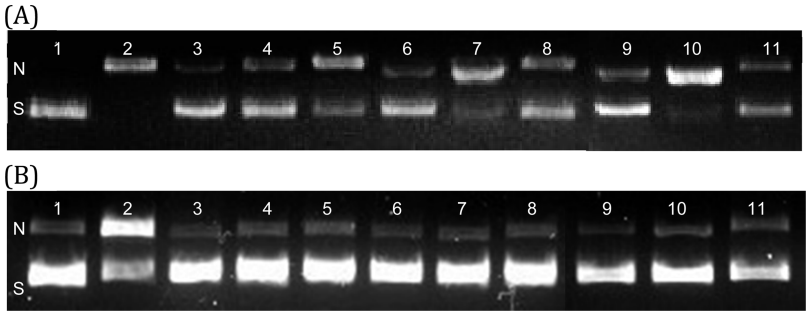

Figure 3. Agarose gel electrophoresis of inhibition of peroxyl (A) and hydroxyl radical (B) induced supercoiled DNA strand scission by pomegranate extracts. Lanes: blank (1); control (2); outer skin free (3), esterified (4), and insoluble-bound (5); mesocarp free (6), esterified (7), and insoluble-bound (8); divider free (9), esterified (10), and insoluble-bound (11). N, nicked DNA; S, supercoiled DNA.

present in each fraction could contribute to the enzyme inhibitory activity of pomegranate peel extracts.

Inhibition of Cupric Ion-Induced Human Low-Density Lipoprotein. Oxidation of LDL plays a vital role in atherogenesis and coronary heart disease in humans. The development of atheromatous plaques occurs due to the uptake of oxidized LDL, through scavenger receptors followed by cholesterol accumulation and foam cell formation.[56] In this assay the oxidation of LDL was measured by the formation of conjugated dienes, which is measured spectrophotometrically at 234 nm. Absorbance (ABS) values for the formation of conjugated dienes measured up to 22 h for PS (F, E, B), PM (F, E, B), and PD (F, E, B) during cupric ion-induced human LDL oxidation is shown in Figure 2. Among all of the tested samples, PS-F and PS-E did not show any inhibitory effect against LDL oxidation and showed a prooxidant effect. This could be due to the poor chelating ability (Table 1) of cupric ions, hence promoting oxidation. The LDL oxidation inhibitory activity of other pomegranate extracts (100 ppm) followed the order PM-B ~ PD-E > PD-B ~ PD-F > PS-B > PM-F ~ PM-E. Interestingly, the insoluble-bound fraction of PS, PM, and PD showed promising inhibitory activity against cupric ion-induced LDL oxidation. This implies the importance of including the insoluble-bound fraction from pomegranate peel components in the analysis. In addition, it is evident (Figure 2) that the formation of conjugated dienes was stabilized after 4 h of incubation during 22 h of assay period. However, the control without any antioxidant showed an increasing trend after 4 h of incubation. Supporting the findings of the present study, Singh et al.[16] showed that the methanol extract of pomegranate peel exhibited 31, 93, and 96% protection at 25, 50, and 100 ppm at the end of 2 h of incubation and suggested a dose-dependent antioxidant effect against LDL oxidation with respect to the concentration of phenolics. Another study reported that the higher amount of phenolics in pomegranate peel extract facilitates the higher LDL oxidation inhibition compared to the pomegranate pulp extract.[18] In addition, Hu and Kitts[57] suggested that the greater the lipophilic property of the antioxidant, the more effective it will be in extending the LDL oxidation lag phase once induced by Cu^{2+}. Moreover, Kaplan et al.[58] found that supplementation of pomegranate juice to mice with advanced atherosclerosis reduces their macrophage oxidative stress and macrophage cholesterol flux and even attenuates the development of atherosclerosis. The authors also found that the tannin fraction isolated from pomegranate juice had a significant antiatherosclerotic activity. Therefore, the LDL oxidation inhibition effect of pomegranate skin parts could be attributed to their tannin content. However, in this study inhibition activity against LDL oxidation was not correlated with its phenolic content. In another study, Aviram et al.[6] showed that pomegranate phenolics (punicalagin, punicalin, gallic acid, and ellagic acid), as well as pomegranate unique complexed sugars, could mimic the antiatherogenic effects of pomegranate extracts. They also found that the uptake rates of oxidized-LDL by atherosclerotic E_0 mice were reduced by ~15% after consumption of pomegranate juice or peel extracts (200 μg GAE/mouse/day). Because the mechanism of LDL oxidation inhibition by phenolic compounds is still unclear, it is difficult to ascertain a confirmed reason for the inhibition mechanism involved. This study also revealed that not all extracts from pomegranate peel could exert inhibitory action against LDL oxidation as some extracts, such as outer skin free and outer skin ester, could promote oxidation.

Inhibition of Peroxyl and Hydroxyl Radical-Induced Supercoiled DNA Strand Scission. DNA strand breakage and DNA damage by free radicals could lead to mutation and carcinogenesis in humans. In our study, pomegranate extracts were evaluated for their inhibition of hydroxyl- and peroxyl-induced supercoiled DNA strand scission. Panels A and B of Figure 3 show the agarose gel electrophoresis of inhibition of peroxyl and hydroxyl radical-induced supercoiled DNA strand scission by PS, PM, and PD, respectively. In this assay we could observe three different forms of DNA, including forms I, II, and III, representing supercoiled DNA, nicked open circular DNA, and linear DNA, respectively (Figure 3). When peroxyl or hydroxyl radical oxidizes DNA, the supercoiled form may be converted to a nicked open circular form followed by a linear form. Supercoiled DNA moves more rapidly through an agarose gel network in comparison with the linear form of DNA. Thus, we could observe two rows of DNA, namely, nicked (N) and supercoiled (S) (Figure 3). Areas of these bands were used to calculate the inhibition percentage by pomegranate extracts. The inhibitory activity of peroxyl- and hydroxyl-induced DNA strand scission of PS (F, E, B), PM (F, E, B), and PD (F, E, B) ranged from 4.94 ± 0.04 to 98.42 ± 0.70 and from 0.29 ± 0.13 to 79.09 ± 7.45, respectively. The insoluble-bound fraction of PD showed the highest inhibition against peroxyl and hydroxyl radical-induced DNA strand scission with IC_{50} values of 0.05 and 0.06 mg/mL extract, respectively ($p < 0.05$). In all parts (PS, PM, and PD) insoluble-bound phenolic extract exhibited significantly ($p < 0.05$) higher inhibitory activity against both radicals compared to their free and esterified fractions. A similar observation was noted in the inhibition of cupric ion-induced LDL oxidation. Although insoluble-bound fractions were not as effective as their esterified counterparts in terms of total phenolics (Figure 1),

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Table 3. Inhibition of Peroxyl and Hydroxyl Radical-Induced Supercoiled DNA Strand Scission by Pomegranate Outer Skin, Mesocarp, and Divider Membrane[a]

sample		DNA scission inhibition (%)			
		peroxyl radical		hydroxyl radical	
		% inhibition/0.1 mg extract	IC_{50} (mg/mL extract)	% inhibition/0.1 mg extract	IC_{50} (mg/mL extract)
outer skin	free	7.88 ± 0.02de	0.63 ± 0.00c	4.54 ± 0.25de	1.11 ± 0.06b
	esterified	21.14 ± 0.06c	0.24 ± 0.00g	15.37 ± 0.88bc	0.33 ± 0.02b
	insoluble-bound	48.80 ± 0.39b	0.10 ± 0.00h	16.11 ± 3.07b	0.32 ± 0.06b
mesocarp	free	10.10 ± 0.04d	0.50 ± 0.00e	7.87 ± 0.53bcde	0.64 ± 0.04b
	esterified	5.17 ± 0.02e	0.97 ± 0.00b	1.43 ± 0.16e	3.54 ± 0.41b
	insoluble-bound	18.44 ± 0.31c	0.27 ± 0.00f	11.17 ± 0.84bcd	0.45 ± 0.03b
divider	free	8.90 ± 0.04de	0.56 ± 0.00d	6.52 ± 0.10cde	0.77 ± 0.01b
	esterified	4.94 ± 0.04e	1.01 ± 0.01a	0.29 ± 0.13e	20.69 ± 7.18a
	insoluble-bound	98.42 ± 0.70a	0.05 ± 0.00i	79.09 ± 7.45a	0.06 ± 0.01b

[a]All data represent the mean of triplicates. Values followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

metal chelation, and radical scavenging activities (Table 2), they showed significantly ($p < 0.05$) higher antioxidant activity in biological systems. This indicates that the type of phenolic compound plays a vital role in their antioxidant activity in biological systems. There were no similar studies reported in the literature for pomegranate peel extract in terms of peroxyl and hydroxyl radical-induced DNA strand scission. However, a study on pomegranate peel extracts on DNA damage using a $CuSO_4$–phenanthroline–Vc–H_2O_2–DNA system by chemiluminescence method showed that 9.64–11.67 μg/mL extract was required for 50% (IC_{50}) inhibition and preventive effect.[59] Recently, Aqil et al.[60] reported that punicalagin isolated from pomegranate husk significantly inhibited oxidative DNA product formation, by about 70% at 40 μM ($p = 0.0017$), resulting from Cu^{2+}-catalyzed redox cycling of 4-hydroxy-17β-estradiol as analyzed by ^{32}P-postlabeling. In addition, pomegranate extract made from fruit skins, which consisted of 95% glycone ellagitannins (mono- and oligomeric) standardized to punicalagins (37–40%) and free ellagic acid (3.4%), down-regulated genes associated with mitosis, chromosome organization, RNA processing, DNA replication, and DNA repair and up-regulated genes involved in the regulation of apoptosis and cell proliferation.[61] Our HPLC analysis showed that the insoluble-bound fraction of divider membrane had the highest content of ellagic acid (121 μg/100 g sample) compared to all other extracts, which had only trace amounts. Thus, ellagic acid may be contributing to the antioxidant activity of the insoluble-bound fraction of divider membrane.

Identification of Phenolic Compounds. *Phenolic Acids.* In this study, 15 phenolic acids were identified from PS, PM, and PD (Table 4). In addition, citric acid was also identified. Hydroxycinnamic acids, namely, *p*-coumaric acid and derivatives of caffeic acid and ferulic acid, as well as hydroxybenzoic acids, namely, protocatecheuic acid, vanillic acid, gallic acid, hydroxycaffeic acid, brevifolin carboxylic acid, coutaric acid, *p*-hydroxybenzoic acid, and derivatives of gallic and vanillic acids, were identified. A representative HPLC chromatogram of the insoluble-bound phenolic fraction of pomegranate outer skin is provided in the Supporting Information (Figure S1).

Compounds **1P**–**5P** were identified by comparing their retention times and UV spectral data with corresponding reference compounds. Compounds **2P** and **3P** exhibited a molecular ion $[M - H]^-$ at m/z 163 with ion fragmentation in MS^2 of m/z 119, which is characteristic of *p*-coumaric acid. Compound **2P** was eluted between 30.8 and 31.2 min, whereas compound **3P** was eluted at 45.4–45.8 min, which indicates the occurrence of *cis* and *trans* isomeric forms of *p*-coumaric acid, respectively. Similarly, Fischer et al.[35] identified isomeric coumaric acid-hexosides in pomegranate. Protocatechuic acid (**1P**), *p*-coumaric acid (**2P** and **3P**), gallic acid (**5P**), and brevifolin carboxylic acid (**8P**) gave deprotonated ions in their respective MS spectra at m/z 153, 163, 169, and 291, respectively. These compounds showed MS^2 ion fragments of m/z 109, 119, 125, and 247, respectively, which are $[M - H - 44]^-$ that is the characteristic loss of CO_2 as reported in the literature.[62] Vanillic acid (**4P**) showed MS^2 fragmentation ions at m/z 123, 125, and 152, among which 123 and 152 corresponded to $[M - H - CO_2]^-$ and $[M - H - CH_3]^-$, respectively.[63] Compounds **6P** and **7P** had deprotonated ions at m/z 187 and 197, which were tentatively identified as hydroxygallic acid and hydroxycaffeic acid, respectively. MS^2 ion fragmentation of m/z 187 was at m/z 169, due to the loss of water $[M - H - 18]^-$, and at m/z 125, due to the loss of H_2O and CO_2 $[M - H - 18–44]^-$. Similarly, compound **7P** showed MS^2 fragments of m/z 179 and 135. However presence of compound **6P** needs to be confirmed, as it has not been previously reported in any foodstuff. de Camargo et al.[34] reported the presence of hydroxycaffeic acid in winemaking byproducts. Compound **9P** had a molecular ion at $[M - H]^-$ obtained by MS at m/z 295 with MS^2 fragments of m/z 119, and m/z 163 was tentatively identified as coutaric acid according to the literature.[62] However, to the best of our knowledge, coutaric acid has not yet been reported in pomegranate. Compound **10P** (m/z 299) was identified as a *p*-hydroxybenzoic-hexoside, on the basis of its prominent fragment at m/z 137 corresponding to the loss of a hexose moiety (162 Da), and represents *p*-hydroxybenzoic hexoside and m/z 93 corresponding to loss of CO_2 from *p*-hydroxybenzoic acid $[M - H - 44]^-$. This identification was supported by the UV spectra and MS data in the literature reported for pomegranate juice.[64] Compound **12P**, producing deprotonated ion at m/z 329 and 167 in the MS^2 experiment, which is characteristic of vanillic acid, was identified as vanillic acid-hexoside due to the loss of a hexose moiety (162 Da).[35] Similarly, compounds **13P** and **14P** were identified as caffeic acid-hexoside and ferulic acid-hexoside by their $[M - H]^-$ at

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Table 4. Phenolic Compounds Identified from Free (F), Esterified (E), and Insoluble-Bound (B) Fractions of Pomegranate Outer Skin, Mesocarp, and Divider Membrane Using HPLC-DAD-ESI-MS^n[a]

					outer skin			mesocarp			divider		
ID	phenolic acids	$[M-H]^-$ (m/z)	RT (min)	MS^2 ion fragments	F	E	B	F	E	B	F	E	B
1P	protocatechuic acid	153	18.8	109		*	*						
2P	*cis*-*p*-coumaric acid	163	30.8	119	*								
3P	*trans*-*p*-coumaric acid	163	45.4	119		*	*		*	*		*	*
4P	vanillic acid	167	27.3	123, 125, 152		*	*			*			
5P	gallic acid	169	14.1	125		*	*		*	*		*	*
6P	hydroxygallic acid	187	5.2	125, 169									*
7P	hydroxycaffeic acid	197	14.1	135, 179			*						
8P	brevifolin carboxylic acid	291	27.9	247			*	*	*	*	*	*	*
9P	coutaric acid	295	19.2	119, 163						*			
10P	*p*-hydroxybenzoic hexoside	299	16.9	93, 137				*					
11P	gallic acid pentoside	301	50.5	125, 169								*	
12P	vanillic acid hexoside	329	36.3	167, 168, 191, 221, 239	*	*				*			
13P	caffeic acid hexoside	341	19	135, 161, 179			*		*				
14P	ferulic acid hexoside	355	30.6	135, 175, 193, 217, 236	*					*			
15P	5-*O*-caffeoylquinic acid	353	6.2	111, 173, 191	*			*					
	other organic acids												
16P	citric acid	191	6.8	111, 173		*	*					*	

					outer skin			mesocarp			divider		
ID	flavonoids	$[M-H]^-$ (m/z)	RT (min)	MS^2 ion fragments	F	E	B	F	E	B	F	E	B
17F	(+)-catechin	289	26	179, 205, 245	*	*							
18F	(−)-epicatechin	289	35.7	179, 205, 245	*	*							
19F	gallocatechin	305	13.7	179	*	*							
20F	dihydroxygallocatechin	341	17.1	305	*	*							
21F	phlorizin	435	51.8	167, 273, 297		*							
22F	epicatechin gallate	441	48.5	125, 169, 289		*							
23F	quercetin 3-*O*-rhamnoside	447	47.8	301		*		*			*		
24F	quercetin hexoside	447	47.7	151, 179, 301	*	*	*				*	*	
25F	kaempferol 3-*O*-glucoside	447	50.3	227, 255, 285	*	*	*	*	*				*
26F	*cis*-dihydrokaempferol hexoside	449	31.3	259, 269, 287, 288		*			*				
27F	*trans*-dihydrokaempferol hexoside	449	50.4	259, 269, 287, 288				*		*	*	*	
28F	syringetin hexoside	507	48.7	295, 312, 315, 327, 343, 345, 441, 471		*					*		

					outer skin			mesocarp			divider		
ID	tannins	$[M-H]^-$ m/z	RT (min)	MS^2 ion fragments	F	E	B	F	E	B	F	E	B
hydrolyzable tannins (ellagitannins and gallotannins)													
29T	ellagic acid	301	48.3	185, 229, 257, 283		*	*	*		*		*	*
30T	monogalloyl hexoside	331	15.5	125, 169, 271	*	*	*		*	*		*	*
31T	ellagic derivative	392	21.9	301, 316, 324	*			*			*		
32T	ellagic derivative I	425	19.5	301					*				
33T	ellagic derivative II	425	38.1	301	*							*	
34T	ellagic derivative III	425	50.7	301				*			*		
35T	ellagic acid pentoside	433	47.2	300, 301	*	*		*		*	*	*	
36T	ellagic acid derivative	441	30.4	300, 397, 398					*				
37T	ellagic acid deoxyhexoside	447	47.3	300, 301, 302	*		*	*	*		*	*	
38T	ellagic acid hexoside	463	8.3	300, 301	*	*		*			*	*	
39T	valoneic acid bilactone I	469	19.3	425	*			*		*	*	*	
40T	valoneic acid bilactone II	469	38.6	425					*				
41T	HHDP hexoside I	481	6.4	301, 302	*	*			*			*	
42T	HHDP hexoside II	481	12.7	301, 302				*					
43T	digalloyl hexoside	483	24.6	169, 271, 331, 439	*			*			*		
44T	monogalloyl-diglucose	493	34.4	157, 169, 331, 379, 457		*					*		
45T	punicalagin isomers	541	20.3	275, 301, 532, 601, 781					*				
46T	HHDP-diglucoside	625	14.6	239, 301, 623		*							
47T	galloyl-HHDP-hexoside (corilagin)	633	34.2	249, 301, 302, 463, 615	*			*					
48T	trigalloylglucopyranose I	635	37.8	301, 465, 483				*			*		
49T	trigalloylglucopyranose II	635	53.9	301, 465, 483		*							

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Table 4. continued

ID	tannins	$[M-H]^-$ m/z	RT (min)	MS^2 ion fragments	outer skin			mesocarp			divider		
					F	E	B	F	E	B	F	E	B
50T	galloyl-HHDP-glucoside (lagerstannin C)	649	15.7	301, 497, 575				*					
51T	bis-HHDP-hexoside (pedunculagin I)	783	20.3	229, 275, 301, 481, 483, 633, 765	*			*			*		
52T	digalloyl-HHDP-gluc (pedunculagin II)	785	31.4	301, 483, 613, 633, 765	*			*					
53T	tetragalloylglucopyranose	787	31.5	301, 465, 617, 635, 766	*			*			*		
54T	ellagic acid derivative	799	27.3	301, 479, 781	*								
55T	digalloyl-HHDP-glucoside (punigluconin)	801	27.4	301, 347, 348, 649	*			*					
56T	galloyl-bis-HHDP-hexoside (casuarinin)	935	29.4	481, 571, 615, 633, 639, 659	*			*					
57T	pentagalloylglucopyranose I	939	38.3	301, 483, 617, 635, 769, 785, 787				*					
58T	pentagalloylglucopyranose II	939	48.5	301, 483, 617, 635, 769, 785, 787							*		
59T	galloyl-HHDP-DHHDP-hex (granatin B)	951	46.6	300, 301, 445, 613, 933	*			*					
60T	HHDP-gallagyl-hexoside I (punicalagin)	1083	7.2	302, 575, 601, 603, 781				*					
61T	HHDP-gallagyl-hexoside II (punicalagin)	1083	14.1	302, 575, 601, 603, 781				*					
62T	HHDP-gallagyl-hexoside III (punicalagin)	1083	20	302, 575, 601, 603, 781				*	*			*	
63T	HHDP-gallagyl-hexoside IV (punicalagin)	1083	24.3	302, 575, 601, 603, 781	*			*	*			*	
proanthocyanidin													
64T	gallocatechin hexoside	487	32.2	305		*							
65T	procyanidin dimer	561	27.4	273, 289, 407, 425, 435, 543		*							
66T	procyanidin dimer B1	562	21.0	285, 287, 289, 407, 423, 435, 447, 449, 539	*								
67T	procyanidin dimer A	575	45.3	285, 287, 289, 407, 423, 435, 447, 449, 539		*							
68T	procyanidin dimer B2	575	21.6	289, 407, 425, 451		*							
69T	procyanidin dimer B3	577	23.4	289, 425, 451		*							
70T	prodelphinidin dimer A	591	27.2	285, 303, 421, 451, 465, 573		*							
71T	procyanidin trimer A	859	57.2	433, 569, 691, 707, 733							*		

ID	anthocyanins	$[M]^+$ (m/z)	RT (min)	MS^2 ion fragments	outer skin			mesocarp			divider		
					F	E	B	F	E	B	F	E	B
72A	cyanidin-3-*O*-pentoside	419	52.4	287	*			*			*		
73A	pelargonidin-3-*O*-glucoside	433	49.2	271	*			*			*		
74A	cyanidin-3-*O*-glucoside	449	49.8	287	*			*			*		
75A	delphinidin-3-*O*-glucoside	465	7	303	*			*			*		
76A	cyanidin-pentoside-hexoside	581	17.5	287, 419, 449							*		
77A	cyanidin-3-*O*-rutinoside	595	19.5	287, 449, 450	*						*		
78A	cyanidin-3,5-*O*-diglucoside	611	7.4	287, 449	*						*		
79A	delphinidin-3,5-*O*-diglucoside	627	51.3	303, 304, 465							*		

[a]RT, retention time; HHDP, hexahydroxydiphenoyl.

m/z 341 and 355 with typical fragment of MS^2 at m/z 179 and 193, respectively, which indicated the loss of the caffeic acid and ferulic acid moiety, respectively, from the $[M-H]^-$. Furthermore, compound **15P** had a molecular ion at m/z 353 with an MS^2 value of m/z 191 detected as 5-*O*-caffeoylquinic acid or chlorogenic acid on the basis of literature data.[35] In addition to phenolic acids, citric acid (compound **16P**) was also detected. Supporting the findings of the present study, Mena et al.[64] identified citric acid along with polyphenols in pomegranate juice, which produced $[M-H]^-$ at m/z 191 with MS^2 fragments m/z 111 and 173.

Quantification of phenolic acids using HPLC-DAD-ESI MS^n (Table 5) reveals that a larger amount of phenolic acids from pomegranate peel exists in the insoluble-bound form (~2743 μg/100 g dry weight) compared to esterified (~171 μg/100 g dry weight) and free (~7 μg/100 g dry weight) forms. Gallic acid was the major phenolic acid present in the insoluble-bound fraction of PS (1131 ± 40 μg/100 g), PM (264 ± 4.4 μg/100 g), and PD (404 ± 5.6 μg/100 g). A small amount of gallic acid was found in the esterified fraction of pomegranate peel parts (~107 μg/100 g). Moreover, PS contained a significantly higher proportion of phenolic acids in comparison with PM and PD. According to Fischer et al.,[35] pomegranate peel extract contained ~27000 μg/100 g DM of total phenolic acids, whereas no phenolic acids were detected in the mesocarp.

Flavonoids. In this study, 12 flavonoid compounds were detected from PS, PM, and PD (Table 4). Compounds **17F** and **18F** were identified as (+)-catechin and (−)-epicatechin,

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Table 5. Quantification of Phenolic Compoundsa from Pomegranate Peel Extracts Using HPLC-DAD-ESI-MS^{n}[a]

Free Phenolics					
ID	compound	MW	outer skin	mesocarp	divider
phenolic acids					
2P	*cis*-*p*-coumaric acid	164	tr		
8P	brevifolin carboxylic acid	292		1.58 ± 0.3	3.63 ± 0.1
10P	*p*-hydroxybenzoic hexoside	300		1.13 ± 0.1	
12P	vanillic acid-hexoside	330	1.47 ± 0.0		
16P	5-*O*-caffeoylquinic acid	354	tr	tr	
14P	ferulic acid hexoside	356	tr		
	total phenolic acids		**1.5**	**2.7**	**3.6**
flavonoids					
17F	catechin	290	12.8 ± 0.74		
17F	epicatechin	290	4.72 ± 0.5		
19F	gallocatechin	306	tr		
20F	dihydroxygallocatechin	342	6.20 ± 0.3		
23F	quercetin 3-*O*-rhamnoside	448		tr	tr
24F	quercetin hexoside	448	52.2 ± 2.4		18.6 ± 0.1
25F	kaempferol 3-*O*-glucoside	448	tr	tr	
27F	*trans*-dihydrokaempferol-hexoside	450		0.62 ± 0.1	4.99 ± 0.4
28F	syringetin-hexoside	508			tr
	total flavonoids		**75.9**	**0.6**	**23.6**
tannins					
hydrolyzable tannins					
29T	ellagic acid	302		tr	
30T	monogalloyl-hexoside	332	1.44 ± 0.0		
33T	ellagic acid derivative II	426	1.69 ± 0.1		
34T	ellagic acid derivative III	426		0.11 ± 0.0	0.21 ± 0.2
35T	ellagic acid pentoside	434	10.3 ± 0.4	4.07 ± 0.0	0.27 ± 0.3
37T	ellagic acid deoxyhexoside	448	tr	tr	tr
38T	ellagic acid hexoside	464	1.08 ± 0.1	0.68 ± 0.3	0.54 ± 0.5
39T	valoneic acid bilactone I	470	4.88 ± 0.3	2.00 ± 0.1	2.48 ± 0.4
41T	HHDP hexoside I	482	23.5 ± 0.9	0.12 ± 0.0	
42T	HHDP hexoside II	482		0.12 ± 0.0	
43T	digalloyl hexoside	484	tr	0.53 ± 0.0	6.30 ± 0.1
44T	monogalloyl-diglucose	494			tr
47T	galloyl-HHDP-hexoside (corilagin)	634	1.67 ± 0.0	0.24 ± 0.0	
48T	trigalloylglucopyranose I	636		0.61 ± 0.1	0.40 ± 0.0
50T	galloyl-HHDP-glucoside (lagerstannin C)	650		1.45 ± 0.2	
51T	bis-HHDP-hexoside (pedunculagin I)	784	0.27 ± 0.0	0.69 ± 0.0	0.86 ± 0.1
52T	digalloyl-HHDP-glucoside (pedunculagin II)	786	4.91 ± 0.3	1.10 ± 0.0	
53T	tetragalloylglucopyranose	788	tr	tr	4.50 ± 0.1
55T	digalloyl-HHDP-glucoside (punigluconin)	802	tr	0.23 ± 0.2	
56T	galloyl-bis-HHDP-hexoside (casuarinin)	936	2.62 ± 0.2	0.63 ± 0.3	
57T	pentagalloylglucopyranose I	940		3.98 ± 0.1	
58T	pentagalloylglucopyranose II	940			35.7 ± 0.1
59T	galloyl-HHDP-DHHDP-hexoside (granatin B)	952	12.3 ± 1.2	1.72 ± 0.0	2.01 ± 0.0
60T	HHDP-gallagyl-hexoside I (punicalagin)	1084		8.78 ± 1.0	
61T	HHDP-gallagyl-hexoside II (punicalagin)	1084		0.09 ± 0.0	
62T	HHDP-gallagyl-hexoside III (punicalagin)	1084		1.89 ± 0.2	
63T	HHDP-gallagyl-hexoside IV (punicalagin)	1084	4.82 ± 0.57	3.72 ± 0.0	-
	total hydrolyzable tannins		**69.5**	**32.8**	**53.3**
proanthocyanidins					
66T	procyanidin dimer B1	562	9.09 ± 1.3		
71T	procyanidin trimer A	860			tr
	total proanthocyanidins		**9.1**		
	total tannins		**78.6**	**32.8**	**53.3**
total phenolic content			**156**	**36.1**	**80.5**
Esterified Phenolics					
ID	compound	MW	outer skin	mesocarp	divider
phenolic acids					
1P	protocatechuic acid	154	tr		

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Table 5. continued

Esterified Phenolics					
ID	compound	MW	outer skin	mesocarp	divider
3P	*trans-p*-coumaric acid	164	35.2 ± 2.1	0.97 ± 0.0	1.24 ± 0.1
5P	gallic acid	170	16.0 ± 0.7	53.8 ± 5.8	38.6 ± 1.3
8P	brevifolin carboxylic acid	292		1.81 ± 0.0	1.76 ± 0.1
11P	gallic acid-pentose	301			tr
12P	vanillic acid-hexoside	330	21.3 ± 0.7		
13P	*trans*-caffeic acid hexoside	342		tr	
	total phenolic acids		**72.5**	**56.6**	**41.6**
flavonoids					
17F	catechin	290	96.2 ± 3.5		
17F	epicatechin	290	44.6 ± 1.6		
19F	gallocatechin	306	tr		
20F	dihydroxygallocatechin	342	55.8 ± 5.8		
21F	phlorizin	436	21.4 ± 0.1		
22F	epicatechin gallate	442	43.5 ± 1.0		
23F	quercetin 3-*O*-rhamnoside	448	tr		
24F	quercetin hexoside	448	2.47 ± 0.1		0.14 ± 0.0
25F	kaempferol 3-*O*-glucoside	448	24.9 ± 0.0	tr	
26F	*cis*-dihydrokaempferol-hexoside	450	31.7 ± 1.9	tr	
	total flavonoids		**320.7**		**0.14**
tannins					
hydrolyzable tannins					
29T	ellagic acid	302	tr		5.31 ± 0.3
30T	monogalloyl-hexoside	332	tr	tr	tr
32T	ellagic acid derivative I	426		0.02 ± 0.01	
34T	ellagic acid derivative III	426			0.01 ± 0.00
35T	ellagic acid pentoside	434	1.76 ± 0.1		tr
36T	ellagic acid derivative	442		tr	
37T	ellagic acid deoxyhexoside	448		tr	tr
38T	ellagic acid hexoside	464	2.70 ± 0.1		tr
39T	valoneic acid bilactone I	470			1.05 ± 0.1
40T	valoneic acid bilactone II	470		3.10 ± 0.3	
41T	HHDP hexoside I	482	52.0 ± 3.2	tr	11.6 ± 0.5
43T	digalloyl-hexoside	484	tr		
44T	monogalloyl diglucose	494			
46T	HHDP-diglucoside	626	4.86 ± 0.1		
49T	trigalloylglucopyranose II	636	0.44 ± 0.1		
62T	HHDP-gallagyl-hexoside III (punicalagin)	1084		1.50 ± 0.1	1.27 ± 0.1
63T	HHDP-gallagyl-hexoside IV (punicalagin)	1084		4.10 ± 0.4	2.78 ± 0.2
	total hydrolyzable tannins		**61.8**	**8.7**	**22**
proanthocyanidins					
64T	gallocatechin hexoside	487	tr		
65T	procyandin dimer	562	42.1 ± 1.5		
68T	procyanidin dimer B2	576	27.8 ± 0.8		
69T	procyanidin dimer B3	578	37.9 ± 1.8		
	total proanthocyanidins		**107.8**		
	total tannins		**168.8**	**8.7**	**22**
total phenolic content			**562**	**65.3**	**63.7**
Insoluble-Bound Phenolics					
ID	compound	MW	outer skin	mesocarp	divider
phenolic acids					
1P	protocatechuic acid	154	106 ± 5.7		
3P	*trans-p*-coumaric acid	164	117 ± 2.5	12.1 ± 0.6	36.1 ± 1.7
4P	vanillic acid	168	107 ± 8.5	tr	
5P	gallic acid	170	1131 ± 40	264 ± 4.4	404 ± 5.6
7P	hydroxycaffeic acid	198	54.9 ± 4.3		
8P	brevifolin carboxylic acid	292	334 ± 30	31.7 ± 0.1	145 ± 1.0
9P	coutaric acid	295		tr	
14P	ferulic acid hexoside	356		tr	
	total phenolic acids		**1850**	**307.7**	**585.1**

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Table 5. continued

			Insoluble-Bound Phenolics		
ID	compound	MW	outer skin	mesocarp	divider
flavonoids					
24F	quercetin hexoside	448	50.8 ± 2.3		
25F	kaempferol 3-*O*-glucoside	448	967 ± 107		tr
27F	*trans*-dihydrokaempferol-hexoside	450		tr	
	total flavonoids		**1017.8**		
tannins					
hydrolyzable tannins					
29T	ellagic acid	302	tr	tr	121.0 ± 0.6
30T	monogalloyl-hexoside	332	tr	tr	97.7 ± 0.8
35T	ellagic acid pentoside	434		38.3 ± 1.0	
37T	ellagic acid deoxyhexoside	448	tr		
39T	valoneic acid bilactone I	470		2.32 ± 0.15	
	total hydrolyzable tannins			**40.6**	**218.7**
	total tannins			**40.6**	**218.7**
total phenolic content			**2867.8**	**348.3**	**803.8**

[a]All data represent the mean of triplicates. tr, trace; HHDP, hexahydroxydiphenoyl.

respectively, on the basis of their UV spectra, MS data, and retention times of corresponding standards. Characteristic fragmentation of $[M - H]^-$ at m/z 289 was m/z 179, 205, and 245, of which the m/z 245 fragment corresponded to the decarboxylation of catechin or epicatechin. Compound **19F** produced a deprotonated ion at m/z 305 and was identified as gallocatechin. It was tentatively identified by the MS^2 fragment ion at m/z 179 according to the literature.[62] In the literature, all three of these flavan-3-ols were identified in pomegranate juice[64] but not in peel extracts. Compound **20F** was tentatively identified as dihydroxygallocatechin by its molecular ion $[M - H]^-$ at m/z 341 with an MS^2 ion fraction at m/z 305, which reflects the loss of two water molecules $[M - H - 36]^-$. Phlorizin (compound **21F**) was tentatively identified according to the $[M - H]^-$ at m/z 435 and MS^2 data at m/z 167, 273, and 297 as reported by Mena et al.[64] Molecular ion m/z 273 is the characteristic molecular ion for phloretin,[65] and MS^2 at m/z 273 indicates the loss of a hexose moiety from phlorizin $[M - H - 162]^-$. Thus, compound **21F** could be named phloretin-hexoside. Compound **22F** was tentatively identified as epicatechin gallate due to its molecular ion $[M - H]^-$ at m/z 441, and MS^2 fragments m/z at 289 and 245 correspond to decarboxylation of epicatechin $[M - H - 44]^-$ as well as those at m/z 169 and 125 that reflect decarboxylation of gallic acid $[M - H - 44]^-$.[34] Compound **23F** with the deprotonated molecular ion at m/z 447 produced a fragment ion at m/z 301 that corresponds to neutral loss of a rhamnosyl group $[M - H - 146]^-$ and corresponds to characteristic quercetin molecular ion $[M - H]^-$. The fragmentation pattern of quercetin was confirmed with an authentic standard; thus, compound **23F** was tentatively identified as quercetin-3-*O*-rhamnoside. Compound **24F** had an m/z 447 $[M - H]^-$ ion, dissociating to yield a fragment ion at m/z 301 $[M - H - 162]^-$ that indicates the loss of a hexose moiety (162 Da), as well as m/z 179 and 151, which are characteristic fragments of quercetin based on the retention time, UV spectra, and MS data from standard. This compound was tentatively identified as quercetin-hexoside according to mass spectral data. Similarly, compound **25F** with a deprotonated ion at m/z 447 gave product ions at m/z 285 (kaempferol), showing the loss of a glucose moiety (162 Da), and other characteristic fragmentation ions of kaempferol at m/z 227 and 255 in MS^2, thus being tentatively identified as kaempferol-3-*O*-glucoside. He et al.[66] reported the presence of quercetin-3-*O*-rhamnoside and kaempferol-3-*O*-glucoside in seeds of pomegranate. Compounds **26F** and **27F** produced a deprotonated molecular ion at m/z 449 with MS^2 fragmentations of m/z 287, which is a characteristic loss of a hexose moiety $[M - H - 162]^-$, and m/z 269 that corresponds to dehydration $[M - H - 162 - 18]^-$. Thus, compounds **26F** and **27F** are identified as isomers of dihydrokaempferol-hexoside, which were eluted at two different retention times of 31.3 and 50.4 min, respectively, and consistent with previous results.[35] However, this compound has so far been detected only in pomegranate juice;[35] thus, these two dihydroflavonols were identified in pomegranate peel for the first time. Compound **28F** was tentatively identified as syringetin-hexoside, which produced a molecular ion $[M - H]^-$ at m/z 507 and fragment m/z 345 indicating the loss of a hexose moiety $[M - H - 162]^-$. Fischer et al.[35] reported the presence of syringetin-hexoside in pomegranate juice.

Kaempferol-3-*O*-glucoside was the major flavonoid identified in pomegranate peel extracts, which followed the order PS-B (967 ± 107 μg/100 g) > PS-E (24.9 ± 0.0 μg/100 g) > PS-F ~ PM-F (trace) ~ PM-E (trace) ~ PD-B (trace). Similar to phenolic acids, HPLC-DAD-ESI-MS^n quantification data showed that a higher proportion of flavonoids was present as insoluble-bound form (~1017 μg/100 g) compared to esterified (~321 μg/100 g) and free (~84 μg/100 g) forms (Table 5). However, the majority of flavonoids were detected in PS (~1414 μg/100 g) compared to PM (<1 μg/100 g) and PD (~24 μg/100 g). Although most of the detected flavonoids were reported for pomegranate juice and seed, none of them were reported for pomegranate peel in the literature.

Tannins. In this study 35 hydrolyzable tannins and 8 proanthocyanidins or condensed tannins were identified in PS, PM, and PD (Table 4).

Hydrolyzable Tannins. Hydrolyzable tannins could be either ellagitannins or gallotannins. Ellagitannins are esters of the hexahydroxydiphenoyl (HHDP) group consisting of a polyol core (glucose or quinic acid), whereas gallotannins contain a glucose molecule in which hydroxyl groups are partly or completely substituted with galloyl groups.[67] In addition, galloyl residues could also be attached to the glucose core via *m*-depside bonds in gallotannins.[67] In general, ellagitannins are

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considered to be more complex than gallotannins due to their various linkages of HHDP residues with glucose moiety as well as formation of C−C and C−O−C linkages.[68]

Compound **29T** was identified as ellagic acid due to its $[M - H]^-$ m/z 301 and MS^2 fragments m/z 185, 229, 257, and 283 based on the retention time, UV spectra, and MS data of authentic standard. The presence of ellagic acid has previously been reported for pomegranate peel and juice.[35,69,70] Compound **30T** produced a deprotonated molecular ion at m/z 331 and its MS^2 fragmentation ions at m/z 125 that is the typical fragment ion for gallic acid due to decarboxylation $[M - H - 44]^-$ and m/z 169 indicating the loss of a hexose moiety $[M - H - 162]^-$. Thus, this compound was named monogalloyl-hexoside according to the literature.[35,69] Compounds **31T** (m/z 392), **32T** (m/z 425), **33T** (m/z 425), **34T** (m/z 425), **36T** (m/z 441), and **55T** (m/z 799) were identified as ellagic acid derivatives due to the characteristic ellagic acid molecular ion fragment m/z 301. Supporting the current findings, Fischer et al.[35] suggested the need for a more detailed characterization of these six ellagic acid derivatives. However, for compound **55T** (m/z 799) the fragment ion at m/z 781 reflects the loss of water $[M - H - 18]^-$ and m/z 479 due to the loss of ellagic acid. Thus, Fischer et al.[35] suggested that this compound could be attributed to either granatin A (HHDP-DHHDP-hexoside) or lagerstannin A (bis-HHDP-gluconic acid), whereas they reported that a more detailed characterization of this ellagitannin is impossible without further information. Recently, García-Villalba et al.[14] also reported the presence of the above ellagitannin (m/z 799) in pomegranate peel. Compound **35T** was tentatively identified as ellagic acid-pentoside due to its molecular ion $[M - H]^-$ obtained by MS at m/z 433. The respective signal in MS^2 was m/z 301, which represents the typical deprotonated ion for ellagic acid by MS and showed the loss of a pentose moiety $[M - H - 132]^-$. Similarly compound **38T** (m/z 463) was tentatively identified as ellagic acid-hexoside due to the loss of a hexose moiety $[M - H - 162]^-$. Compound **37T** produced deprotonated ion at m/z 447 with fragment ions by MS^2 at m/z 300, 301, and 302. According to Fischer et al.[35] compound **37T** was identified as ellagic acid-deoxyhexoside. Two compounds (**39T** and **40T**) were detected with the same molecular ion $[M - H]^-$ at m/z 469, which were obtained at two different retention times of 19.3 and 38.6 min, respectively. According to the literature, these compounds were identified as isomers of valoneic acid bilactone with a fragment ion of m/z 425 that represents the decarboxylation $[M - H - 44]^-$ of valoneic acid bilactone.[14,35] García-Villalba et al.[14] suggested that these isomers could be valoneic acid bilactone and sanguisorbic acid bilactone, and the only structural difference between those two isomers was that the hydroxyl that links the HHDP to the galloyl group belongs either to the HHDP group or to the galloyl group. Compounds **41T** and **42T** had a molecular ion of m/z 481, which eluted at 6.4 and 12.7 min, respectively. MS^2 fragmentation of these compounds produced an ion at m/z 301 (characteristic deprotonated ellagic acid) by losing one hexose moiety $[M - H - 162]^-$. On the basis of the MS^2 fragmentation pattern as well as data from the literature,[35,67] these compounds were tentatively identified as isomers of HHDP-hexoside. Compound **43T** was tentatively identified as digalloyl-hexoside due to its molecular ion at m/z 483 and MS^2 fragment ions m/z 331 due to the loss of galloyl group $[M - H - 152]^-$ and m/z 169 due to the loss of a hexose moiety $[M - H - 162]^-$. A new compound, monogalloyl diglucose (compound **44T**), was tentatively identified in PS-E and PD-F. This identification was according to the literature report for Muscadine grapes.[67] Compound **44T** produced a deprotonated ion at m/z 493 with its MS^2 fragment ions at m/z 331 and 169 after the sequential removal of two glucose moieties, $[M - H - 162]^-$ and $[M - H - 162 - 162]^-$, respectively. Compounds **45T**, **60T**, **61T**, **62T**, and **63T** were tentatively detected as punicalagin (2,3-HHDP-4,6-gallagylglucoside) isomers, which is a well-characterized major phenolic compound in pomegranate.[35,64,71]

Moreover, Gil et al.[69] reported that a quasimolecular ion at 1083 m/z [MH] in accordance with punicalagin (glucose + gallagyl + hexahydroxydiphenoyl), which is a characteristic complex ellagitannin of pomegranate peel, containing glucose, ellagic acid, and gallagic acid. Compound **45T** produced a molecular ion at m/z 541, whereas compounds **60T**, **61T**, **62T**, and **63T** had a deprotonated ion at m/z 1083, which corresponds to a doubly charged ion $[M - 2H]^{2-}$ (equivalent to molecular weight of 1084 Da) and singly charged ion $[M - H]^-$, respectively.[35,60,64] According to the literature, punicalagin isomers give MS^2 fragments at m/z 301 (loss of HHDP), m/z 781 (loss of ellagic acid), and m/z 601 (loss of a gallagic acid moiety), which is consistent with our data. In addition, punicalagin has been shown to occur in two isomeric forms naturally, namely, α and β anomers, on the basis of its behavior in solution.[69,72] However, in our study for the first time four different punicalagin isomers (compounds **60T**, **61T**, **62T**, and **63T**) were identified in pomegranate peel extracts on the basis of their different retention times (7.2, 14.1, 20.0, and 24.3 min), However, additional information is required for full characterization of these isomers. The free phenolic fraction of mesocarp contained all four isomers, whereas others contained only HHDP-gallagyl-hex III and IV. Compound **46T** had a molecular ion $[M - H]^-$ at m/z 625 that dissociated to give an intense MS^2 at m/z 301 due to the loss of two glucose moieties $[M - H - 162 - 162]^-$. On the basis of fragmentation data and literature, compound **46T** was identified as HHDP-diglucoside.[67] Compound **47T** was identified as galloyl-HHDP-hexoside (corilagin) due to its deprotonated ion $[M - H]^-$ at m/z 633 with MS^2 fragment ions m/z 615 (loss of water molecule $[M - H - 18]^-$), m/z 463 (ellagic acid-hexoside), and m/z 301 (loss of galloyl glucose $[M - H - 331]^-$). These data indicate that the galloyl unit was directly linked to the glucose core.[67] Tanaka et al.[73] suggested that galloyl-HHDP-hexoside could exist in three isomeric forms, namely, corilagin, strictinin, and punicacortein A or B, depending on the linkage type, 3,5-position of the glycosyl unit, 4,5-linkage, and 2,3-position of the glycosyl unit and an additional C-glycosidic bond, respectively. However, in this study only one compound was detected. Compounds **48T** and **49T** produced molecular ion $[M - H]^-$ at m/z 635 with two different elution times, 37.8 and 53.9 min, respectively. These compounds were identified as isomers of trigalloylglucopyranose on the basis of their MS^2 fragment ions m/z 483 $[M - H - 152]^-$, m/z 465 $[M - H - 170]$, and m/z 301, indicating the loss of a galloyl group, loss of a gallic acid, and typical ellagic acid molecular ion, respectively. Cai et al.[74] identified the isomers of trigalloylglucopyranose as sanguiin H4 or sanguiin H5, depending on the location of the galloyl group in *Rosa chinensis* flowers. Although trigalloylglucopyranose was identified in pomegranate seed,[66] its isomers were reported for the first time in pomegranate peel. Compound **50T** with its deprotonated ion $[M - H]^-$ at m/z 649 was tentatively

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identified as galloyl-HHDP-gluconic acid (lagerstannin C) due to its MS^2 fragmentation pattern at m/z 497 (releasing HHDP-gluconic acid) and m/z 301 (ellagic acid). Similarly, compounds **52T** (m/z 785) and **55T** (m/z 801) were identified as digalloyl-HHDP-gluconic acid (pedunculagin II) and digalloyl-HHDP-gluconic acid (punigluconin), respectively. Pedunculagin II (compound **52T**) produced MS^2 fragment ions at m/z 633 (loss of HHDP-gluconic acid $[M - H - 152]^-$ and typical molecular ion for galloyl-HHDP-hexoside), m/z 483 (represents digalloyl-hexoside), and m/z 301 (ellagic acid), whereas punigluconin (compound **55T**) had MS^2 fragment ions at m/z 649 (represents galloyl-HHDP-gluconic acid $[M - H - 152]^-$), m/z 348 (loss of ellagic acid $[M - H - 152 - 301]^-$), and m/z 301 (ellagic acid). Compounds **50T**, **52T**, and **55T** have already been identified in pomegranate peel.[35] Compound **51T** produced a deprotonated ion at m/z 783 and MS^2 fragments at m/z 765 and 481, which indicates the loss of water $[M - H - 18]^-$ and ellagic acid $[M - H - 301]^-$. On the basis of this MS^2 fragmentation and the literature data,[35] compound **51T** was identified as bis-HHDP-hexoside (pedunculagin I). Compound **53T** dissociated to give $[M - H]^-$ ions at m/z 787, and the fragmentation in MS^2 yielded ions at m/z 635 $[M - H - 152]^-$, m/z 617 $[M - H - 152 - 18]^-$, m/z 465 $[M - H - 152 - 152 - 18]^-$, and m/z 301, indicating the loss of a galloyl group, loss of a galloyl group and a water molecule, consecutive loss of two galloyl groups and a water molecule, and typical deprotonated ellagic acid, respectively. Thus, compound **53T** was tentatively identified as tetragalloyl-glucopyranose according to the literature.[66,67] Compound **56T** showed an $[M - H]^-$ ion at m/z 935 and was tentatively identified as galloyl-bis-HHDP-hexoside (casuaricitin), which had typical MS^2 fragments at m/z 633, 615, and 481 that represent galloyl-HHDP-hexoside, the dehydrated derivative of galloyl-HHDP-hexoside, and HHDP-hexoside, respectively.[35,73] Compounds **57T** and **58T** were identified as isomers of pentagalloylglucopyranose, which were eluted at 38.3 and 48.5 min, respectively. These compounds gave identical m/z 939 $[M - H]^-$ ions with MS^2 fragment ions at m/z 787 $[M - H - 152]^-$, m/z 769 $[M - H - 152 - 18]^-$, m/z 635 $[M - H - 152 - 18 - 134]^-$, m/z 617 $[M - H - 152 - 152 - 18]^-$, and m/z 483 $[M - H - 152 - 152 - 18 - 152]^-$, indicating the loss of galloyl moieties as well as water molecules. These findings were similar to the data reported by Sandhu and Gu[67] for Muscadine grapes. Compounds tetragalloylglucopyranose and pentagalloylglucopyranose were identified in pomegranate peel for the first time, but were already reported in pomegranate seed by He et al.[66] Khanbabaee and van Ree[68] reported that the gallotannins 2,3,4,6-tetra-*O*-galloyl-D-glucopyranose and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose could be found in many plant families and are considered as key intermediates in the biosynthesis of nearly all hydrolyzable plant polyphenols. Compound **59T** exhibited a deprotonated molecular ion at m/z 951 and MS^2 fragment ions at m/z 933 $[M - H - 18]^-$, m/z 613 $[M - H - 301]^-$, and m/z 301 representing the loss of a water molecule, additional loss of ellagic acid, and typical ellagic acid, respectively. Therefore, on the basis of the above MS^2 fragmentation and the literature data,[35] this compound (**59T**) was confirmed as galloyl-HHDP-DHHDP-hexoside (granatin B).

Total hydrolyzable tannins of pomegranate extracts as determined by HPLC-DAD-ESI-MS^n followed the order (Table 5) insoluble-bound (~259.3 μg/100 g) > free (155.5 μg/100 g) > esterified (92.5 μg/100 g). This indicates that hydrolyzable tannins exist mainly as insoluble-bound in pomegranate peel. Among all samples tested, PD-B had the highest hydrolyzable tannins (218.7 μg/100 g), whereas PD-F contained only trace amounts of hydrolyzable tannins (Table 5). Major hydrolyzable tannins were ellagic acid in PD-B (121.0 $\pm$ 0.6 μg/100 g) followed by monogalloyl-hexoside in PD-B (97.7 $\pm$ 0.8 μg/100 g), HHDP-hexoside I in PS-E (52.0 $\pm$ 3.2 μg/100 g), ellagic acid-pentoside in PS-B (38.3 $\pm$ 1.0 μg/100 g), and pentagalloylglucopyranose II (35.7 $\pm$ 0.1 μg/100 g). Although the PD-B had the highest amount of hydrolyzable tannins, most of the hydrolyzable tannins were detected in the PM-F in trace amounts. According to Fischer et al.[35] no significant difference existed between the amount of hydrolyzable tannins in pomegranate outer skin and mesocarp, and the values ranged between 40625.1 $\pm$ 4434.7 and 43991.2 $\pm$ 395.5 mg/kg DM. Recently, García-Villalba et al.[14] reported that total extractable ellagitannins was 549.1 mg/g dw of pomegranate extract obtained from husk (pericarp), peels (membranes/mesocarp), and arils (containing the seeds), using HPLC-DAD.

Proanthocyanidins. Proanthocyanidins or condensed tannins are oligomeric and polymeric compounds consisting of coupled flavan-3-ol (catechin) units.[68] In this study, eight proanthocyanidins were detected, and most of them were present in the free and esterified fractions of pomegranate outer skin. Procyanidins are the proanthocyanidins that consist exclusively of (epi)catechin, whereas propelargonidin and prodelphinidin are the proanthocyanidins containing (epi)-afzelechin or (epi)gallocatechin as subunits, respectively.[75] Procyanidin dimers could be divided into type A and type B depending on the type of linkages. B-type proanthocyanidins are linked via C4→C8 or C4→C6 linkages, whereas A-type proanthocyanidins have a second linkage through an ether bond at C2→O7.[76]

Compound **64T** was identified as gallocatechin-hexoside according to the literature data;[67,77] it produced a molecular ion $[M - H]^-$ at m/z 487 with its MS^2 fragment ion at m/z 305, which is a typical deprotonated ion of gallocatechin. Compound **65T** was identified as A-type of procyanidin dimer due to its molecular ion $[M - H]^-$ at m/z at 561 and its MS^2 fragment ions m/z 289 and 273, which correspond to catechin and afzelechin units, respectively. Gu et al.[75] identified the same compound (m/z 561) and related the MS^2 fragments at m/z 407 $[M - H - 136 - 18]^-$, m/z 425 $[M - H - 136]^-$, m/z 435 $[M - H - 126]^-$, and m/z 543 $[M - H - 18]^-$ due to elimination of ring B from the flavan-3-ol through retro-Diels–Alder reaction of ring C, loss of a water molecule from m/z 425 ion, heterocyclic ring fission, and loss of water molecules, respectively. He et al.[66] identified procyanidin dimer (m/z 561) in pomegranate seeds and reported that for the precise structure of this dimer further confirmation is required. However, Huang et al.[78] identified a similar compound (m/z 561) in the stem of *Spatholobus suberectus* and named it propelargonidin B-type dimer with major components as one (epi)catechin and one (epi)afzelechin units. Compounds **66T**, **68T**, and **69T** were identified as procyanidin dimers B1, B2, and B3, respectively (Supporting Information Figure S2). Compound **66T** produced a molecular ion $[M - H]^-$ at m/z 562 with its MS^2 fragment ions at m/z 285, 287, 289, 407, 423, 435, 447, 449, and 539, which agrees with the literature data.[78] Compound **68T** was identified as procyanidin dimer B2 that had a deprotonated ion at m/z 575 and fragmentation in MS^2 produced ions at m/z 289 (typical deprotonated (epi)catechin)

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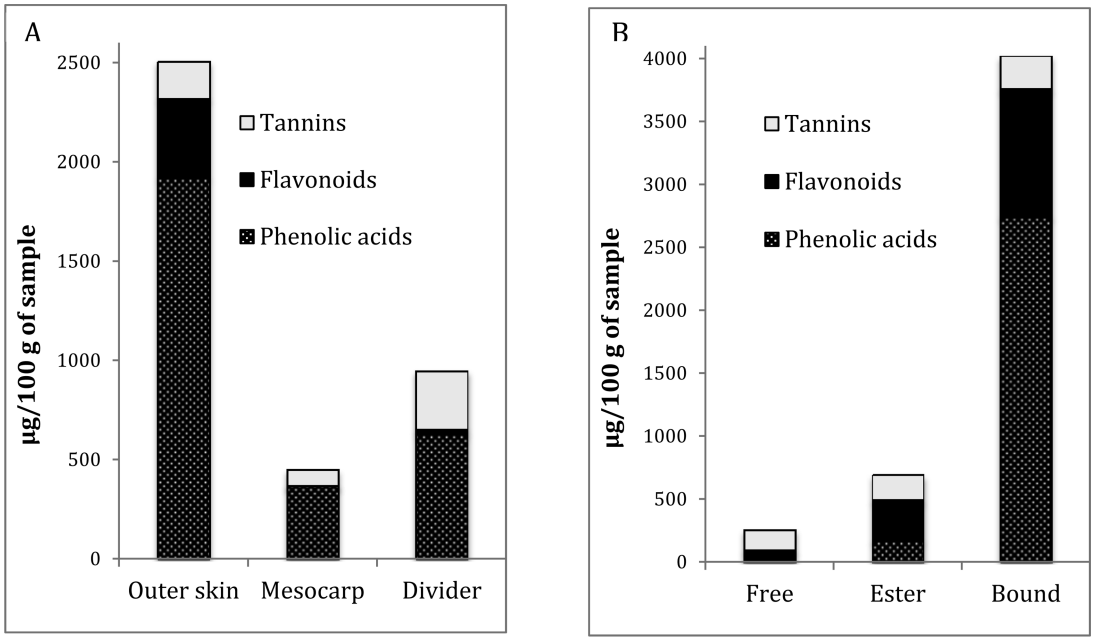

Figure 4. Distribution pattern of phenolic acids, flavonoids, and tannins (μg/100 g) in (A) pomegranate outer skin, mesocarp, and divider membrane and (B) as free, esterified, and insoluble-bound forms determined by HPLC-DAD-ESI-MS^n.

and m/z 407, 425, and 451, which is consistent with previously published literature data.[62,75] Procyanidin dimer B2 consists of epicatechin-(4β-8)-epicatechin.[68] Similarly, compound **69T** was identified as procyanidn dimer B3 on the basis of its molecular ion $[M - H]^-$ at 577 and MS^2 fragments at m/z 289, 425, and 451. In addition, procyanidin dimer A (compound **67T**) was detected in pomegranate peel. Compound **67T** contained a deprotonated ion at m/z 575 and MS^2 ions at m/z 285, 287, 289, 407, 423, 435, 447, 449, and 539, which is consistent with the data in the literature.[62,78] Compound **70T** was identified as prodelphinidin dimer A, which had a molecular ion $[M - H]^-$ at m/z 591 and a fragment ion in MS^2 at m/z 465 that represents the characteristic heterocyclic ring fusion (C ring). A similar observation was reported for the skins of roasted hazelnut by Esatbeyoglu et al.[76], who found two new A-type dimeric prodelphinidins, EGC-(2β→O7, 4β→8)-C and EGC-(2β→O5, 4β→6)-C. Furthermore, a procyanidin trimer A (compound **71T**) was detected in the free fraction of divider membrane. Compound **71T** had a molecular ion $[M - H]^-$ at m/z 859 and MS^2 fragments at m/z 433, 569, 691, 707, and 733 $[M - H - 126]^-$, which are consistent with the data reported by de Camargo et al.[62]

Procyanidin dimers m/z 561 and 577 (procyanidin dimer B3) were identified in pomegranate seed by He et al.[66] However, to the best of our knowledge this is the first time five different procyanidin dimers (m/z 561, B1−B3, A), procyanidin trimer A, and prodelphinidin dimer A were identified in pomegranate peel extracts. Except procyanidin trimer A, all other proanthocyanidins were detected in the esterified and free phenolic fractions of pomegranate outer skin. Proanthocyanidins were not found in PM, whereas PD contained only a trace amount of procyanidin trimer A in its free phenolic fraction. Proanthocyanidins existed mainly in the esterified form (~108 μg/100 g), followed by the free (~9 μg/100 g) form, but did not exist in the insoluble-bound form in pomegranate (Table 5). Procyanidin dimer m/z 561 contributed most (42.1 ± 1.5 μg/100 g) followed by procyanidin dimer B3 (37.9 ± 1.8 μg/100 g), procyanidin dimer B2 (27.8 ± 0.8 μg/100 g), and procyanidin dimer B1 (9.09 ± 1.3 μg/100 g). However, total tannin contributed significantly ($p < 0.05$) to the insoluble-bound fraction (~259 μg/100 g) compared to the esterified (~200 μg/100 g) and the free (165 μg/100 g) phenolic fractions of the pomegranate byproduct extracts.

Figure 4 depicts the distribution pattern of phenolic acids, flavonoids, and tannins in PS, PM, and PD according to the HPLC-DAD-ESI-MS^n analysis. Total phenolic acid and flavonoid contents followed the same order PS > PD > PM, whereas total tannin content followed the order PD > PS > PM. It is interesting to see that the highest amount of phenolic compounds of pomegranate existed as insoluble-bound in pomegranate peel (Figure 4B). This implies the importance of extracting the insoluble-bound fraction during total phenolic determination. In addition, phenolic acids are the major phenolic compounds present in pomegranate peel followed by tannins and flavonoids. Moreover, according to the Folin−Ciocalteu's reagent method, total phenolic content was significantly ($p < 0.05$) higher in PD followed by PM and PS of pomegranate. Although PD had the highest content of tannins in HPLC analysis, it did not show the highest total phenolic content. Any purification method was used in the present study. It is well-known that Folin−Ciocalteu's reagent may also react with nonphenolic reducing substances such as certain sugars, amino acids, vitamin C, and other organic acids. Therefore, this may explain the discrepancies found in the present study. Furthermore, this also supports the need for identification and quantification of individual phenolics using reliable methods, including HPLC-DAD-ESI-MS^n.

Anthocyanins. Anthocyanins of PS, PM, and PD were tentatively identified using HPLC-DAD-ESI-MS^n in a positive mode with UV spectra at 520 nm. Because of limited availability of reference samples, HPLC-DAD-ESI-MS^n was used for identification purposes. Typical mass spectrometric behavior of anthocyanins in ESI positive mode is that they show M$^+$ ions in the MS^1 experiments and then release the aglycones in MS ^{2}

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and MS ^{3} due to the sequential loss of their sugar moieties.[35] For confirmation of anthocyanins, MS^2 ion fragments were compared with the literature data. Positive mode analysis of HPLC-MS/MS resulted in the identification of eight anthocyanin glycosides (Table 5).

Compound **72A** was tentatively identified as cyanidin-3-*O*-pentoside due to its molecular ion $[M - H]^+$ obtained by MS at m/z 419. Its respective signal in MS^2 was m/z 287, corresponding to the cyanidin aglycone due to the loss of a pentose moiety (132 Da). According to Yoshimura et al.,[79] the identified compound at m/z 419 ion was an $[M]^+$ ion of cyanidin-3-*O*-pentoside. Compound **73A** had a molecular ion at m/z 433 with a MS^2 value of m/z 271 (characteristic pelargonidin aglycone molecular ion) due to the loss of 162 Da, which could be attributed to the hexose moiety (glucose or galactose). Thus, compound **73A** was identified as pelargonidin-3-*O*-glucoside.[35] The aforementioned tentative identification was used to identify compounds **74A** and **75A** as cyanidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside due to its characteristic loss of 162 Da (hexose moiety) as well as their moleculars ion $[M - H]^+$ obtained by MS at m/z 449 and 465, respectively.[35] A molecular ion $[M - H]^+$ obtained by MS at m/z 581 for compound **76A** was identified as cyanidin-pentoside-hexoside as reported by Fischer et al.[35] The MS^2 of compound **76A** showed two major fragments m/z 419 and 449, which show the characteristic loss of hexose and pentose moieties, respectively, as well as another fragment ion corresponding to cyanidin aglycone molecule (m/z 287). Compound **77A** with the molecular ion at m/z 595 produced a fragment ion at m/z 449 that corresponds to neutral loss of rhamnose and at m/z 287, which is cyanidin aglycone cation. Therefore, compound **77A** was identified as cyanidin-3-*O*-rutinoside.[80] Compound **78A** showed the molecular ion $[M - H]^+$, obtained by MS at m/z 611 with two fragment ions in MS^2 including m/z 449 and 287, which correspond to the loss of one (162 Da) and two glucose moieties (324 Da), respectively. According to Fischer et al.[35] compound **78A** was cyanidin-3,5-*O*-diglucoside. Similarly, compound **79A** showed a molecular ion at m/z 627 with fractions m/z 303, 304, and 465, identified as delphinidin-3,5-*O*-diglucoside. Noda et al.[15] reported that delphinidin-3,5-*O*-diglucoside is the major anthocyanin present in pomegranate juice.

All of the aforementioned anthocyanins were detected only in the free phenolic fraction of PS, PM, and PD, which was also evidenced from our total monomeric anthocyanin assay (Figure 1B). All eight anthocyanins were present in PD, which closely attaches to the pulp and aril part of the fruit. However, only four monoacylated anthocyanins were detected in PM (**72A**–**75A**). Cyanidin-pentoside-hexoside (**76A**) and delphinidin-3,5-*O*-diglucoside (**79A**) were not detected in the PS extract. Fischer et al.[35] identified nine anthocyanins from pomegranate peel and juice, whereas no anthocyanins were detected in the mesocarp. They found pelargonidin-3,5-*O*-diglucoside as the ninth compound that was not detected in this study. Few other studies have identified anthocyanins from pomegranate peel.[81] To the best of our knowledge, this is the first time the presence of anthocyanins in PM and PD is reported.

This study revealed that pomegranate peel contained 79 phenolic compounds, including 16 phenolic acids, 12 flavonoids, 35 hydrolyzable tannins, 8 proanthocyanidins, and 8 anthocyanins. Phenolic acids were the major phenolic compounds in pomegranate peel followed by hydrolyzable tannins, proanthocyanidins, and flavonoids, mostly present in the insoluble-bound form. In this study, 5 phenolic acids (coutaric acid, *p*-hydroxybenzoic-hexoside, gallic acid-pentoside, and possibly hydroxygallic acid, hydroxycaffeic acid), 12 flavonoids (catechin, epicatechin, gallocatechin, dihydroxygallocatechin, phlorizin, epicatechin gallate, quercetin 3-*O*-rhamnoside, quercetin-hexoside, kaempferol 3-*O*-glucoside, *cis*-dihydrokaempferol-hexoside, *trans*-dihydrokaempferol-hexoside, syringetin-hexoside), 6 hydrolyzable tannins (monogalloyl-diglucose, 2 more punicalagin isomers, trigalloylglucopyranose tetragalloylglucopyranose pentagalloylglucopyranose), and 7 proanthocyanidins (procyanidin dimer m/z 561, procyanidin dimer B1, procyanidin dimer B2, procyanidin dimer B3, procyanidin dimer A, procyanidin trimer A, and prodelphinidin dimer A) were identified for the first time in pomegranate peel extracts. In addition, pomegranate peel parts, namely, PS, PM, and PD, demonstrated their potential as promising antioxidants with biological activities; hence, they could be a potential source of functional food ingredients and nutraceuticals.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b02950.

Figures S1 and S2 (PDF)

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Notes

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■ ABBREVIATIONS USED

PS, pomegranate outer leathery skin; PM, pomegranate mesocarp; PD, pomegranate divider membrane; F, free; E, esterified; B, insoluble-bound

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