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Deep Eutectic Solvent-Based Ultrasound-Assisted Extraction of Pomegranate Peel: Phenolic Composition and Biological Activities of the Extracts

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Abstract

Large amounts of pomegranate peel, which contains abundant bioactive polyphenols, are discarded during industrial processing. Here, pomegranate peel extracts were prepared by ultrasound-assisted extraction (UAE) with either a deep eutectic solvent (DES) or water under identical extraction conditions, and their phenolic composition and in vitro biological activities were compared. The DES extracts were additionally purified on a macroporous resin to remove the solvent. Expressed per gram of purified (DES) or crude (water) dry extract, total phenolic content was higher in the DES extracts, with a maximum of 88.15 mg GAE/g, and the major phenolic compounds, especially punicalagin (130.65 mg/g), were also present at higher levels. The DES extract prepared under the punicalagin-optimized UAE conditions was the most potent inhibitor of α-glucosidase (IC_{50} = 6.49 μg/mL) and pancreatic lipase (IC_{50} = 14.58 μg/mL), and it also showed stronger antioxidant activity and suppressed nitric oxide production more effectively. In exploratory multivariate analyses, differences in biological activity among the extracts were associated with phenolic composition, although individual compounds did not behave uniformly. These differences reflect the combined extraction and purification procedure rather than the solvent alone. The results support further investigation of DES-based UAE for the valorization of pomegranate peel; however, residual solvent, safety, and bioavailability need to be evaluated before the extracts can be considered as functional food ingredients.

Keywords: *Punica granatum*; green extraction; phenolic compounds; functional food ingredient; multivariate analysis

Academic Editor: Adane Tilahun Getachew

Received: 14 October 2026
Revised: 17 November 2026
Accepted: 18 November 2026
Published: 23 November 2026

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1. Introduction

Pomegranate (*Punica granatum* L.) is a member of the Lythraceae and has long been prized both as a food and as a medicinal plant. Its fruit supplies organic acids, minerals, vitamins, and phenolic compounds, and interest in its possible health benefits has grown steadily [1]. The fruit is made up of edible arils and seeds enclosed by inedible inner and outer peels. The arils are eaten fresh or made into juice, oil, wine, and jam, and this processing leaves behind a large volume of peel [2]. Although the peel has potential value, most of it is thrown away, and only a small portion ends up as animal feed [3].

Pomegranate peel is richer in polyphenolic compounds than the edible parts of the fruit and contains tannins, flavonoids, and anthocyanins [4,5]. Its main phenolics, namely ellagitannins such as punicalagin and punicalin, together with ellagic acid and proanthocyanidins, show antioxidant, anti-inflammatory, antidiabetic, antimicrobial, anticancer, and anti-obesity activities [6]. Du et al. [7], for instance, found that polyphenols from pomegranate peel attenuated inflammation in macrophages, and Šavikin et al. [8] observed antioxidant and antidiabetic effects in phenolic fractions obtained from pomegranate. Even so, most of the peel is still discarded, which creates an environmental burden and wastes a potentially useful source of bioactive compounds. Using the peel as a raw material for functional food ingredients could therefore add value to this agro-industrial by-product [9].

Many solvents and extraction techniques have been tested for recovering bioactive compounds from plants. Deep eutectic solvents (DESs) are among the newer green solvents and are of interest because they have low toxicity, are easy to prepare, and are inexpensive [10]. A DES is formed by mixing a hydrogen bond acceptor (HBA) with a hydrogen bond donor (HBD). Because its physicochemical properties change with the choice of HBA and HBD and with the amount of water added, a DES can be tailored to extract many different classes of phytochemicals [11]. DESs are now often paired with techniques such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction to raise the yield of functional compounds [12]. In the UAE, cavitation breaks down plant cell walls, which improves extraction efficiency and can shorten extraction time relative to conventional methods [13].

We previously optimized DES-based UAE conditions for recovering punicalagin and ellagic acid from pomegranate peel by response surface methodology [14]. That study defined the extraction conditions on the basis of the yields of these two compounds, but it did not characterize the broader phenolic profile of the resulting extracts, assess their biological activities, or compare them with water extracts obtained under the same conditions. Such combined chemical and biological characterization, which has been applied to other plant extracts [15], is needed to judge whether optimizing extraction for marker compounds also yields extracts with greater bioactivity. In the present work, extracts were prepared under the two optimized conditions, and their phenolic composition and biological activities were compared with those of extracts obtained by UAE with water under the same conditions. Ten phenolic compounds were quantified; antioxidant, α-glucosidase inhibitory, pancreatic lipase inhibitory, and nitric oxide production inhibitory activities were measured in vitro; and exploratory multivariate analyses were used to describe how these activities related to the phenolic profiles. Our aim was to determine whether the DES-based UAE workflow yields pomegranate peel extracts with higher phenolic contents and stronger in vitro activities than water-based UAE.

2. Materials and Methods

2.1. Materials

Pomegranates (*Punica granatum* L.) harvested in Goheung-gun, Jeollanam-do, Republic of Korea, in November 2023 were purchased from a local market. The fruits were washed, and the peels were removed from the arils and seeds. The peels were then freeze-dried (Freeze Dryer, IlshinBioBase, Dongducheon, Republic of Korea), pulverized with a food mixer (FM-681C, Hanil, Incheon, Republic of Korea), and sieved through a 45-mesh screen (Chung Gye Indus MFG Co., Seoul, Republic of Korea). The resulting powder was kept at −30 °C in a deep freezer (MDF, Sanyo, Osaka, Japan) until analysis.

2.2. Preparation of DES

DES composed of choline chloride (Daejung, Siheung, Republic of Korea) and urea was prepared as previously described [14]. The two components were combined at a 1:2 molar ratio and heated at 80 °C in a shaking water bath (BS-11, JeioTech, Seoul, Republic of Korea) at 100 rpm until the mixture turned into a clear, homogeneous liquid. Before use, the DES was left at room temperature for 24 h to stabilize.

2.3. Preparation of Pomegranate Peel Extracts

Extraction was carried out under the optimized conditions established by Kim and Yoon [14]. To obtain punicalagin-optimized DES extracts, 2.26 g of peel powder was combined with 100 mL of DES (25.65% water, *v*/*v*) and sonicated for 20 min at 120 W with an ultrasonic probe system (KFS-600N, Korprotech, Seoul, Republic of Korea). Ellagic acid-optimized DES extracts were obtained by sonicating 1.67 g of peel powder in 100 mL of DES (33.10% water, *v*/*v*; the optimum predicted in [14] was 33.13%, which was rounded to 33.10% for practical preparation) for 20 min at 300 W with the same system. The system operates at a frequency of 20 kHz with a probe tip diameter of 20 mm and a maximum output of 600 W; the applied powers of 120 W and 300 W therefore correspond to 20% and 50% of the maximum output. Sonication was performed in pulsed mode (5 s on, 5 s off), and the extraction time of 20 min refers to the total sonication (on) time. To prevent a rapid rise in temperature, the beaker containing the sample was placed in an ice bath during sonication, and the extract temperature was monitored with a temperature sensor (Korprotech, Seoul, Republic of Korea) and kept at 40 ± 2 °C.

After extraction, DES was separated from the extracts on Diaion HP-20 resin (Sigma-Aldrich, St. Louis, MO, USA). A slurry of 20 g resin in distilled water was poured into a 19 × 250 mm column, giving a bed volume of 36 mL. The column was equilibrated for 20 min, and 5 mL of extract was applied. DES was washed out with 30 mL of distilled water, after which the retained compounds were recovered with 80 mL of 80% ethanol. Loading, washing, and elution were carried out by gravity flow without a pump. The solids content of the applied extract was not measured, so the load relative to resin capacity is not known. The recovery of phenolic compounds from the resin and the amounts of residual choline chloride and urea in the eluates were not determined; these points are considered in Sections 3.2 and 3.8.

For comparison, water extracts were produced by replacing DES with distilled water while keeping the UAE conditions unchanged. Ethanol was removed from the DES extracts with a rotary evaporator (N-1300, Eyela, Tokyo, Japan) under reduced pressure, and the concentrates were freeze-dried. Because the water extracts contained no DES, they were freeze-dried directly without resin treatment. The comparison between DES and water extracts therefore reflects the complete extraction and work-up procedure applied to each solvent, not the extraction solvent alone. Extracts prepared with DES and water under the punicalagin-optimized conditions are referred to as PE-DES and PE-water, and those prepared under the ellagic acid-optimized conditions as EE-DES and EE-water; these abbreviations are used throughout the manuscript. Because only the DES extracts were purified, phenolic contents are expressed per gram of purified dry extract for the DES extracts and per gram of crude dry extract for the water extracts.

2.4. Measurement of Total Phenolic and Flavonoid Contents

Total phenolic content (TPC) was measured by the Folin–Ciocalteu assay [16]. An aliquot of extract (0.2 mL) was combined with 0.2 mL of Folin–Ciocalteu's phenol reagent (Jusei, Tokyo, Japan) and left at room temperature for 3 min. Next, 0.4 mL of 10% Na_2CO_3 solution (OCI, Seoul, Republic of Korea) and 4 mL of distilled water were added. After

the mixture had stood in the dark for 1 h at room temperature, its absorbance was read at 725 nm. TPC was calculated from a gallic acid (Sigma-Aldrich) standard curve.

Total flavonoid content (TFC) was measured following Lim et al. [17]. Extract (0.5 mL) was combined with 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 M potassium acetate, and 4.3 mL of 80% ethanol. The mixture was kept at room temperature for 40 min, and its absorbance was then read at 415 nm. TFC was calculated from a quercetin (Sigma-Aldrich) standard curve.

2.5. HPLC Analysis of Phenolic Compound Contents

Individual phenolic compounds were quantified by high-performance liquid chromatography (HPLC; Waters 2695, Waters Co., Milford, CA, USA) on a system fitted with a UV detector (Waters 2489, Waters Co.) and an Atlantis® dC18 column (4.6 × 150 mm, 5 μm, Waters Co.). Each extract was passed through a 0.45 μm membrane filter (SV25P045NL, Hyundai Micro, Seoul, Republic of Korea) before injection. The column was held at 30 °C, and acetonitrile (A) and 1% phosphoric acid in water (B) served as the mobile phases. The 60 min gradient was programmed as follows: 0–27 min, 60% B; 28–55 min, 56% B; and 56–60 min, 90% B. Samples (10 μL) were injected at a flow rate of 1.0 mL/min, and the eluate was monitored at 280 nm. The following authentic standards were used: gallic acid, catechin, chlorogenic acid, epicatechin, p-coumaric acid, ferulic acid, and protocatechuic acid (Sigma-Aldrich); procyanidin (Cayman Chemical, Ann Arbor, MI, USA); punicalagin α and β (Biofron, Goyang, Republic of Korea); and ellagic acid (Tokyo Chemical Industry, Tokyo, Japan). Each standard was injected individually, and peaks in the extract chromatograms were identified by comparing their retention times with those of the standards. Because identification relied on retention time at a single wavelength, catechin is reported as tentatively identified. Quantification was based on an external calibration curve for each standard, and punicalagin was reported as the sum of the α and β anomers. Linear regression was used for all compounds except catechin and chlorogenic acid, whose detector response deviated from linearity at high concentrations; these two compounds were quantified with quadratic calibration functions. The calibration range of each compound was chosen to bracket the concentrations measured in the extracts wherever the available standards allowed. Procyanidin, whose calibration was less linear ($R^2 = 0.977$) and whose contents in three of the four extracts exceeded the highest standard, is reported as semi-quantitative, and the ellagic acid content of EE-DES, which also exceeded the highest standard, is reported as an approximate value. The calibration ranges, regression parameters, coefficients of determination (R^2), and retention times are listed in Table S1, and representative chromatograms of the extracts, with the identified peaks numbered, are shown in Figure S1. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as $3.3\sigma/S$ and $10\sigma/S$, respectively, where σ is the standard deviation of the y-intercept, and S is the slope of the calibration curve. Vanillic acid was also analyzed with an authentic standard, but because its peak was not clearly resolved and its response was not reproducible, it was not quantified and is not reported. Compounds below the LOD were reported as not detected (ND), and values between the LOD and LOQ are reported as estimates. Because detection was performed at a single wavelength, spectral peak purity could not be assessed, and co-elution of minor components with the quantified peaks cannot be completely excluded. Recovery and precision were not determined in this study.

2.6. Antioxidant Activity Assays

Three assays were used to assess antioxidant activity: DPPH radical scavenging, ABTS radical scavenging, and Fe^{2+}-chelating activity. The DPPH assay followed Adhikari et al. [18] with minor changes. In a 96-well plate, 100 μL of extract at different

concentrations was combined with 200 μL of 0.2 mM DPPH solution (Sigma-Aldrich). The plate was kept in the dark at room temperature for 30 min before absorbance was read at 517 nm. Radical scavenging activity was obtained from Equation (1):

$$\text{Radical scavenging activity (\%)} = \left(1 - \frac{A-B}{C}\right) \times 100 \quad (1)$$

where A is the absorbance of the reaction mixture containing the extract, B is the absorbance of the sample blank in which distilled water replaced the radical or chromogenic reagent, and C is the absorbance of the control in which distilled water replaced the extract.

ABTS radical scavenging activity was measured by a modified version of the method of So et al. [19]. To produce ABTS radical cations, 7 mM ABTS (Sigma-Aldrich) was combined with 2.45 mM potassium persulfate and kept in the dark at room temperature for 16 h. The resulting solution was diluted with 80% ethanol until its absorbance at 734 nm reached 0.700 ± 0.002. In a 96-well plate, 15 μL of extract was added to 300 μL of the diluted ABTS solution, and absorbance at 734 nm was recorded after 6 min. Scavenging activity was then calculated with Equation (1).

Fe^{2+}-chelating activity was measured following Nam et al. [20]. Extract (1 mL) at different concentrations was combined with 25 μL each of 2 mM $FeCl_2$ and 5 mM ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4′-disulfonic acid; Sigma-Aldrich]. Absorbance was read at 562 nm after 10 min at room temperature, and chelating activity was calculated with Equation (1). Results of the three antioxidant assays are given as IC_{50} values, i.e., the extract concentration that produced 50% radical scavenging or metal chelation. The extracts were tested at 50–200 μg/mL (PE-DES) and 100–1000 μg/mL (other extracts) in the DPPH assay, at 10–100 and 25–200 μg/mL in the ABTS assay, and at 50–300 and 50–400 μg/mL in the Fe^{2+}-chelating assay (concentrations of the solutions added to the assay). IC_{50} values were obtained by linear regression of activity (%) against concentration, with the IC_{50} calculated from the regression equation as the concentration giving 50% activity. The same procedure was used for the enzyme inhibition assays. IC_{50} values were calculated for each replicate and are expressed as means ± standard deviations. The concentration–response data and the regression statistics for all assays are provided in Table S2.

2.7. α-Glucosidase Inhibitory Activity Assay

α-Glucosidase inhibition was measured using a slightly modified version of the method of Kim et al. [21]. The enzyme was α-glucosidase from *Saccharomyces cerevisiae* (Type I, G5003; Sigma-Aldrich). Extract (50 μL; 1–20 μg/mL for PE-DES and 10–40 μg/mL for the other extracts) was combined with 50 μL of 200 mM potassium phosphate buffer (pH 6.8) and 50 μL of α-glucosidase solution (0.1 U/mL; Sigma-Aldrich) and preincubated for 15 min at 37 °C. The reaction was started by adding 100 μL of 3 mM *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG; Sigma-Aldrich) as a substrate, allowed to proceed for 10 min at 37 °C, and stopped with 50 μL of 0.1 M NaOH. The *p*-nitrophenol released was quantified from its absorbance at 405 nm. Acarbose (A8980; Sigma-Aldrich) served as the positive control and was tested at 125–1000 μg/mL. All concentrations of the extracts and acarbose, including the IC_{50} values, refer to the solutions added to the assay; the final concentrations in the 250 μL reaction mixture were one-fifth of these values. At each extract concentration, a sample blank without enzyme was included, and its absorbance was subtracted to correct for the color of the extract, and a control containing distilled water instead of the extract defined 0% inhibition. The two water extracts were assayed in a separate run in which the control absorbance was lower (0.19–0.21, compared with 1.38–1.45 for the DES extracts and 1.71–1.79 for acarbose); in each run, inhibition was calculated

relative to the control of that run. IC_{50} values were calculated as described in Section 2.6, and inhibitory activity was calculated with Equation (2):

$$\text{Enzyme inhibitory activity (\%)} = \left(1 - \frac{A-B}{C}\right) \times 100 \quad (2)$$

where A is the absorbance of the reaction mixture containing the extract, B is the absorbance of the sample blank without enzyme, and C is the absorbance of the control in which distilled water replaced the extract.

2.8. Pancreatic Lipase Inhibitory Activity Assay

Pancreatic lipase (PL) inhibition was assessed using a slightly modified version of the method of Ryu et al. [22]. Porcine pancreatic lipase (Type II, L3126; Sigma-Aldrich) was dissolved at 10 mg/mL as crude enzyme powder following Ryu et al. [22] in a buffer containing 10 mM 3-[N-morpholino]propanesulfonic acid (MOPS; Daejung, Siheung, Republic of Korea) and 1 mM ethylenediaminetetraacetic acid (pH 6.8). Extract of orlistat (Tokyo Chemical Industry) at 10–50 μg/mL (100 μL; concentrations, including IC_{50} values, refer to the solutions added, and the final concentrations in the 1 mL reaction mixture were one-tenth of these values) was combined with 850 μL of Tris buffer (100 mM Tris, 5 mM $CaCl_2$, pH 7.0) and 30 μL of the PL solution and kept at 37 °C for 15 min. Then 20 μL of 10 mM **p**-nitrophenyl butyrate (Sigma-Aldrich) was added as a substrate, and incubation continued for another 60 min at 37 °C before absorbance was read at 400 nm. At each concentration, a sample blank without enzyme was included, and its absorbance was subtracted to correct for the color of the extract. Inhibition was calculated with Equation (2) and reported as the IC_{50} value, the concentration at which PL activity was reduced by half, obtained as described in Section 2.6.

2.9. Cell Viability and Nitric Oxide Production Assay

RAW 264.7 macrophages from the Korean Cell Line Bank (Seoul, Republic of Korea) were maintained in DMEM (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin (both from Welgene Inc., Gyeongsan, Republic of Korea) in an incubator at 37 °C and 5% CO_2 (MCO-17AIC, Sanyo). Cell viability was assessed by the MTT assay. Cells were plated in 96-well plates (5×10^4 cells/well), cultured for 24 h, and exposed to the extracts at different concentrations for another 24 h. MTT solution (5 mg/mL; Duchefa Biochemie, Haarlem, The Netherlands) was then added for 3 h. The supernatant was discarded, the formazan crystals were dissolved in dimethyl sulfoxide, and absorbance was read at 540 nm.

Nitric oxide (NO) production was determined with a Griess reagent system (Promega, Madison, WI, USA). Cells plated at 5×10^4 cells/well in 96-well plates were cultured for 24 h, exposed to 1 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich) for 1 h, and then treated with the extracts at different concentrations for 24 h in the continued presence of LPS. The culture supernatant (100 μL) was combined with sulfanilamide solution and N-(1-naphthyl)ethylenediamine solution, and absorbance was read at 540 nm.

2.10. Statistical Analysis

For each extraction condition, the extracts obtained from three separate extractions were pooled, and all chemical and biochemical analyses were performed in triplicate on the pooled extract; the standard deviations therefore reflect analytical rather than extraction variability. Cell-based assays were likewise performed in triplicate. Data are expressed as means ± standard deviations. IBM SPSS Statistics (Ver. 23, IBM Corp., Armonk, NY, USA) was used for one-way analysis of variance (ANOVA). Because each

group comprised only three replicates, formal tests of normality and homogeneity of variance have little power and were not performed. Duncan's multiple range test ($p < 0.05$) was then applied for post hoc comparisons among means. Hierarchical clustering analysis, principal component analysis (PCA), and Pearson correlation analysis were carried out in Python (version 3.13.18) on the mean values of the four extracts (PE-DES, EE-DES, PE-water, and EE-water). Compounds not detected were entered as zero, and each variable was autoscaled by mean-centering and dividing by its standard deviation. Clustering was based on Euclidean distance with Ward's linkage. PCA was used to visualize how the extracts were distributed, and Pearson correlation analysis was used to relate phenolic composition to biological activities. Because only four extract groups were included (n = 4), the multivariate analyses were used only to describe patterns among the extracts. With so few observations, the correlation coefficients are sensitive to individual values, and neither the correlations nor the proportion of variance explained by PCA indicate causal relationships or predictive validity.

3. Results and Discussion

3.1. Total Phenolic and Flavonoid Contents

Table 1 shows the total phenolic and flavonoid contents of the extracts. TPC was highest in PE-DES (88.15 mg GAE/g purified dry extract), followed by EE-DES (84.05 mg GAE/g purified dry extract), PE-water (59.95 mg GAE/g crude dry extract), and EE-water (56.34 mg GAE/g crude dry extract). Under both sets of extraction conditions, the TPC per gram of purified DES extract was significantly higher than that per gram of the corresponding crude water extract. Because only the DES extracts were purified on HP-20 resin, their higher TPC per gram of purified dry extract may result, at least partly, from the removal of non-phenolic material, such as sugars and organic acids, during the water wash rather than a greater release of phenolics from the peel. The masses of the freeze-dried extracts were not recorded, so extraction yields and phenolic contents per gram of dry peel could not be calculated. The present data therefore show differences in the composition of the extracts but do not show whether DES released more phenolics from the peel than water did. Rajha et al. [23] reported that DES recovered polyphenols from pomegranate peel more efficiently than water, but this could not be tested here. The TPC of pomegranate peel is known to vary widely with cultivar, growing region, and extraction conditions [24,25].

Table 1. Total phenolic and flavonoid contents (per gram of purified dry extract for DES extracts and of crude dry extract for water extracts) of pomegranate peel extracts obtained using DES or water under optimized extraction conditions.

Sample	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg QE/g)
PE-DES	88.15 ± 0.75 ^{a}	22.63 ± 0.84 ^{b}
PE-water	59.95 ± 0.12 ^{c}	19.40 ± 0.84 ^{d}
EE-DES	84.05 ± 0.12 ^{b}	26.76 ± 0.19 ^{a}
EE-water	56.34 ± 0.64 ^{d}	20.69 ± 0.56 ^{c}

Values are means ± standard deviations (n = 3). Different superscript letters within the same column indicate significant differences ($p < 0.05$) according to Duncan's multiple range test. GAE, gallic acid equivalent; QE, quercetin equivalent. PE and EE indicate extracts prepared under punicalagin- and ellagic acid-optimized UAE conditions, respectively; DES and water indicate the extraction solvents.

TFC ranged from 19.40 to 26.76 mg QE/g and decreased in the order EE-DES > PE-DES > EE-water > PE-water. As with TPC, flavonoid contents were significantly higher in the DES extracts than in the water extracts. DES has also outperformed conventional solvents in extracting flavonoids from other plant materials, such as *Platycladus orientalis* fruits and rice [26,27]. The TFC values obtained here were considerably higher than those reported for pomegranate peel extracted with ethanol or water [28,29].

Part of this difference may be due to the tunable polarity and strong hydrogen-bonding interactions of DES, which facilitate the solubilization of phenolic compounds from plant matrices [30], but in the present design the contribution of the solvent cannot be separated from that of the resin purification. Since phenolics and flavonoids are closely linked to antioxidant, anti-inflammatory, and other biological activities, their higher levels in the DES extracts may account for differences in bioactivity between the DES and water extracts.

3.2. Phenolic Compound Contents

Table 2 lists the phenolic compound contents of the extracts. Nine compounds were identified by comparison with the retention times of authentic standards (procyanidin, gallic acid, punicalagin, chlorogenic acid, epicatechin, *p*-coumaric acid, ferulic acid, protocatechuic acid, and ellagic acid), and catechin was tentatively identified. Punicalagin was the most abundant compound in every extract and was the principal phenolic constituent identified.

Table 2. Phenolic compound contents (mg/g purified dry extract for DES extracts; mg/g crude dry extract for water extracts) of pomegranate peel extracts obtained using DES or water under optimized extraction conditions.

Sample	Procyanidin †	Gallic Acid	Punicalagin	Catechin ‡	Chlorogenic Acid	Epicatechin	*p*-Coumaric Acid	Ferulic Acid	Protocatechuic Acid	Ellagic Acid
PE-DES	39.37 ± 2.02 ^{a§}	4.85 ± 0.26 ^{c}	130.65 ± 5.92 ^{a}	70.34 ± 6.13 ^{a}	4.79 ± 0.14 ^{a}	6.29 ± 0.63 ^{a}	0.39 ± 0.01 ^{a}	0.46 ± 0.02 ^{a}	1.13 ± 0.16 ^{a}	1.83 ± 0.10 ^{b}
PE-water	22.74 ± 1.16 ^{b}	4.31 ± 0.10 ^{d}	118.04 ± 1.12 ^{b}	52.83 ± 4.19 ^{b}	ND	ND	0.14 ± 0.01 ^{b*}	0.18 ± 0.13 ^{b*}	ND	1.09 ± 0.02 ^{c}
EE-DES	19.69 ± 3.13 ^{b§}	9.39 ± 0.23 ^{a}	63.39 ± 0.48 ^{c}	53.51 ± 0.52 ^{b}	4.46 ± 0.14 ^{b}	3.65 ± 0.00 ^{b}	0.09 ± 0.01 ^{c*}	0.37 ± 0.02 ^{a}	0.57 ± 0.05 ^{b}	2.93 ± 0.16 ^{a§}
EE-water	12.09 ± 0.13 ^{c§}	6.82 ± 0.41 ^{b}	60.44 ± 1.96 ^{c}	38.73 ± 1.40 ^{c}	ND	ND	0.13 ± 0.00 ^{b*}	0.10 ± 0.01 ^{b*}	ND	1.17 ± 0.02 ^{c}

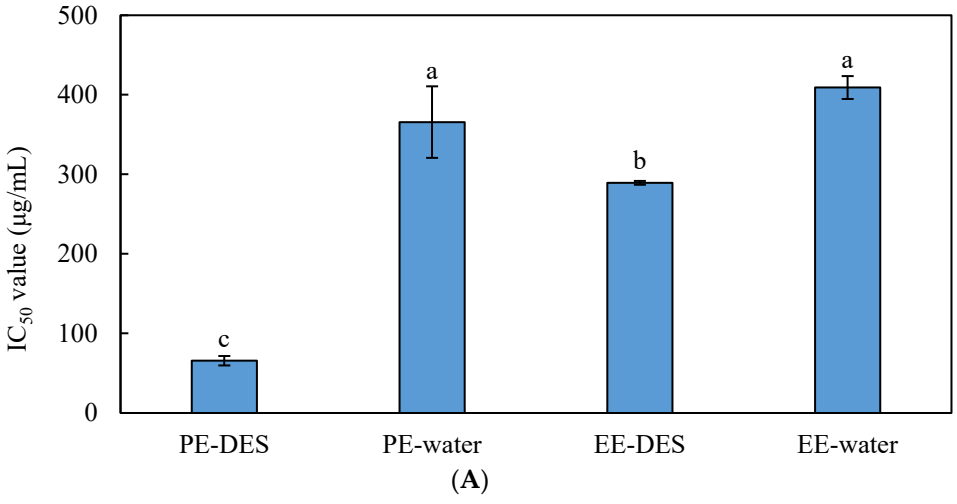
Values are expressed as means ± standard deviations (n = 3). Different superscript letters within the same column indicate significant differences ($p < 0.05$) according to Duncan's multiple range test. ND, not detected (below the LOD). * Between the LOD and LOQ; estimated value. † Semi-quantitative (calibration $R^2 = 0.977$). ‡ Tentatively identified on the basis of retention time only; if peak 4 contains punicalagin α, the punicalagin contents are underestimated. § Above the highest calibration standard; extrapolated, approximate value.

Punicalagin content ranged from 60.44 to 130.65 mg/g, with PE-DES showing the highest concentration, followed by PE-water, EE-DES, and EE-water. The punicalagin content of PE-DES (130.65 mg/g) was obtained in the same series of experiments used to validate the optimized extraction conditions in our previous study, where it was first reported [14]. The tentatively identified catechin and the semi-quantitative procyanidin were also highest in PE-DES, but these values are approximate and are not interpreted further. Because peak 4 (Figure S1) may contain punicalagin α, the punicalagin contents may be underestimated. Previous studies reported punicalagin contents of 64.98 mg/g in ethanol extracts [3] and 47.55 mg/g in methanol extracts [31]. Although direct comparison

should be made with caution because of differences in extraction conditions, the high punicalagin content of PE-DES indicates that the DES workflow yielded an extract enriched in this compound. Ellagic acid was present at 1.09–2.93 mg/g, with the highest level in EE-DES, the extract prepared under ellagic acid-optimized conditions. Chlorogenic acid, epicatechin, and protocatechuic acid were found only in the DES extracts and were below the LOD in the water extracts, indicating a broader phenolic profile in the DES extracts. The HP-20 step, however, may have affected individual compounds differently. As a nonpolar polystyrene adsorbent, HP-20 generally retains ellagitannins and flavan-3-ols well, whereas highly polar constituents can be partly lost during the water wash; at the same time, the enrichment of retained phenolics may have raised minor compounds above the LOD. Because phenolic recovery from the resin was not measured, compound-specific losses or enrichment during purification cannot be excluded. Punicalagin, ellagic acid, proanthocyanidins, and other phenolics are regarded as the main bioactive components of pomegranate peel and exert antioxidant, anti-inflammatory, antidiabetic, and anticancer effects either individually or synergistically [24,32]. The higher contents and broader phenolic profile of the DES extracts thus reflect the combined influence of the extraction solvent and the purification step on the phenolic composition of the peel extracts.

3.3. Antioxidant Activities

Antioxidant activity was assessed by DPPH radical scavenging, ABTS radical scavenging, and Fe^{2+}-chelating assays and expressed as IC_{50} values (Figure 1). PE-DES had the lowest DPPH IC_{50} value (65.69 μg/mL) and thus the strongest scavenging activity, followed by EE-DES (289.34 μg/mL), PE-water (365.62 μg/mL), and EE-water (409.19 μg/mL) (Figure 1A); the two water extracts did not differ significantly. This activity of PE-DES is likely related to its higher phenolic content, especially of punicalagin, a high-molecular-weight ellagitannin with multiple hydroxyl groups that is known to scavenge radicals effectively [33]. Antioxidant activity has also been reported in different pomegranate cultivars [34]. In various fruit peel extracts, total phenolic content has been positively correlated with antioxidant activity, particularly DPPH radical scavenging [35,36]. Our results agree with this relationship, since PE-DES had both the highest phenolic content and the strongest DPPH scavenging activity.



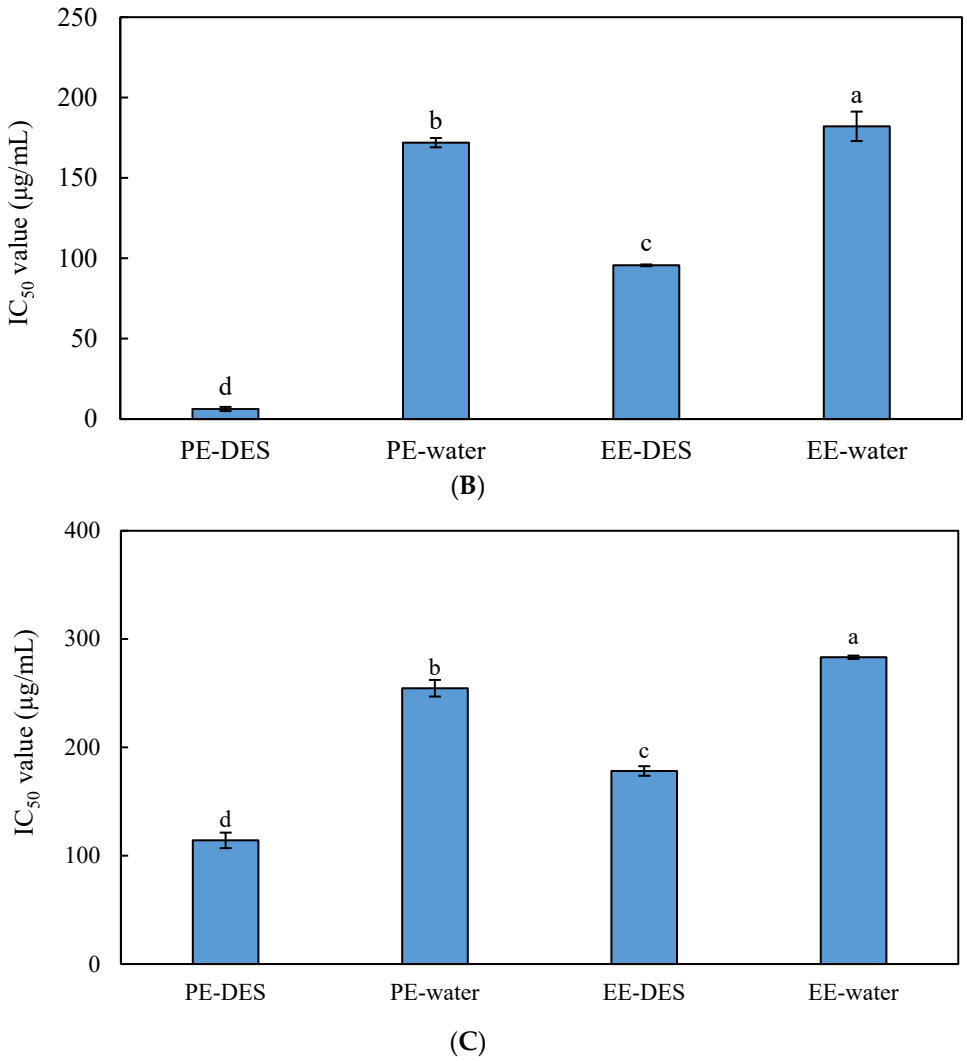


Figure 1. IC_{50} values of DPPH radical scavenging activity (**A**), ABTS radical scavenging activity (**B**), and Fe^{2+}-chelating activity (**C**) of pomegranate peel extracts obtained using DES or water under optimized extraction conditions. Extract concentrations refer to purified dry extract (DES extracts) or crude dry extract (water extracts). Bars represent the means ± standard deviations (n = 3). Different letters indicate significant differences among the samples ($p < 0.05$) according to Duncan's multiple range test.

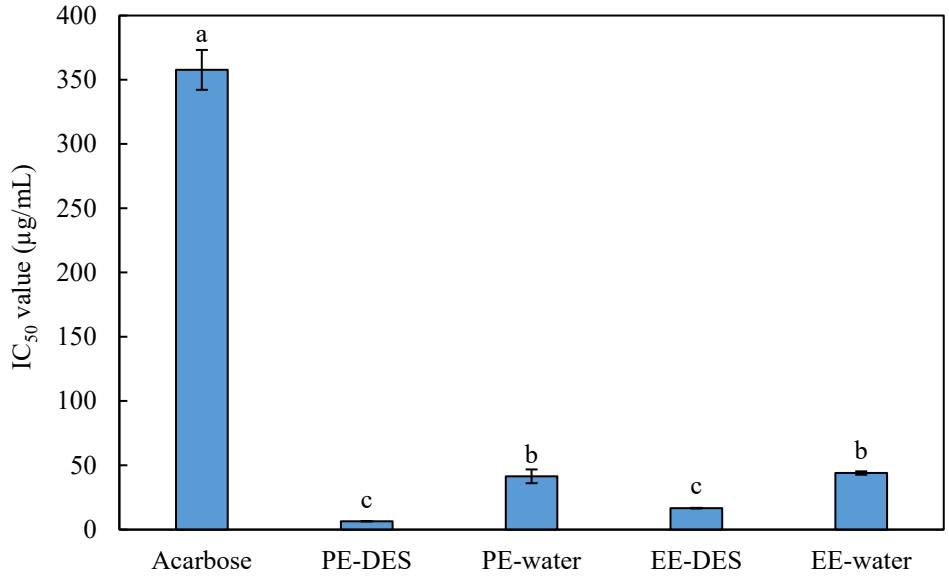
The ABTS assay showed the same pattern (Figure 1B). PE-DES again had the lowest IC_{50} value (6.29 μg/mL), followed by EE-DES (95.63 μg/mL), PE-water (171.91 μg/mL), and EE-water (182.05 μg/mL). Because PE-DES already scavenged about 49% of ABTS radicals at the lowest concentration tested (10 μg/mL), its IC_{50} lies below the tested range; it was obtained by extrapolation and should be regarded as approximate. IC_{50} values were generally lower in the ABTS assay than in the DPPH assay, probably because the two radicals react by different mechanisms and differ in their affinity for phenolic compounds [37]. Ellagitannins, including punicalagin, have been shown to scavenge ABTS radicals more strongly than DPPH radicals [38], in line with our observations.

Fe^{2+}-chelating activity followed a similar trend (Figure 1C). PE-DES chelated metal ions most effectively (IC_{50}, 114.21 μg/mL), followed by EE-DES (178.28 μg/mL), PE-water (254.61 μg/mL), and EE-water (283.25 μg/mL). Phenolic compounds can bind transition metal ions and thereby inhibit metal-catalyzed oxidation [39]. The consistently lower IC_{50} values of the DES extracts suggest that their higher phenolic contents contributed to their stronger antioxidant activity. Because the activities were compared per mass of purified

(DES) or crude (water) dry extract, part of this difference may reflect the purification step rather than the extraction solvent alone. Taken together, the antioxidant results matched the differences in phenolic composition among the extracts.

3.4. α-Glucosidase Inhibitory Activity

Figure 2A shows the IC_{50} values of the extracts for α-glucosidase inhibition. Under the present assay conditions, the positive control acarbose had an IC_{50} value of 357.71 μg/mL. PE-DES was the most potent inhibitor (IC_{50}, 6.49 μg/mL), followed by EE-DES (16.64 μg/mL), PE-water (41.47 μg/mL), and EE-water (44.04 μg/mL). Because 50% inhibition was not reached at the highest concentration tested for the two water extracts (40 μg/mL), their IC_{50} values were obtained by slight extrapolation of the concentration–response curves. All pomegranate peel extracts had substantially lower IC_{50} values than acarbose under these conditions. This comparison applies only to the present in vitro assay and does not indicate greater physiological efficacy than acarbose. Acarbose inhibits yeast α-glucosidase much less effectively than mammalian intestinal α-glucosidases [40], which probably explains its relatively weak inhibition in this assay. The DES-based workflow, which included resin purification, also markedly enhanced α-glucosidase inhibitory activity compared with water extraction, reducing the IC_{50} value by approximately 6.4-fold for the punicalagin-optimized extract (PE-DES vs. PE-water) and 2.6-fold for the ellagic acid-optimized extract (EE-DES vs. EE-water).



(**A**)

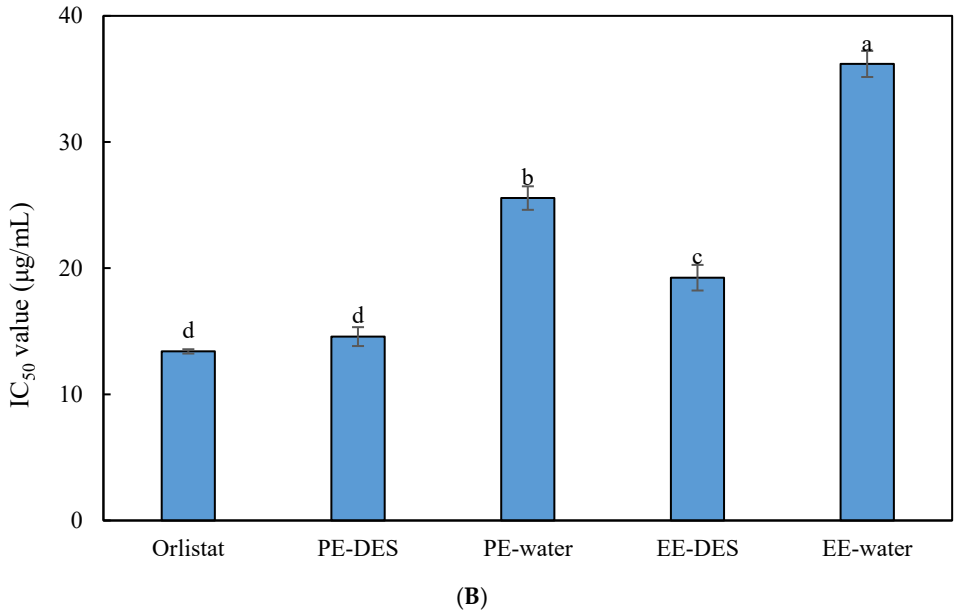


Figure 2. IC_{50} values of α-glucosidase inhibitory activity (**A**) and pancreatic lipase inhibitory activity (**B**) of pomegranate peel extracts obtained using DES or water under optimized extraction conditions. Extract concentrations refer to purified dry extract (DES extracts) or crude dry extract (water extracts). Bars represent the means ± standard deviations (n = 3). Different letters indicate significant differences among the samples ($p < 0.05$) according to Duncan's multiple range test.

Comparable α-glucosidase inhibitory activity has been reported for pomegranate peel extracts. Alsataf et al. [41] reported an IC_{50} value of approximately 30 μg/mL for ethanol extracts of pomegranate peel. Although direct comparison is limited by differences in extraction and assay conditions, the low IC_{50} values obtained here indicate that the DES extracts are potent α-glucosidase inhibitors in vitro. Their stronger inhibition may be related to their higher contents of ellagitannins, particularly punicalagin, and of ellagic acid; these compounds have been reported to bind proteins through multiple hydroxyl groups, which can alter enzyme conformation and limit substrate access to the catalytic site [42]. Other co-extracted phenolic compounds may also have contributed. Such mechanisms are usually confirmed by inhibition kinetics combined with spectroscopic and calorimetric binding analyses; feruloylated oligosaccharides from rice bran, for example, were shown in this way to be mixed-type α-glucosidase inhibitors that altered the conformation of the enzyme [43]. Because these analyses were not performed in this study, the explanations given here remain tentative.

Acarbose, an α-glucosidase inhibitor in clinical use, slows carbohydrate digestion and blunts the rise in blood glucose after meals [44]. The DES extracts, especially PE-DES, inhibited α-glucosidase strongly in vitro, but whether this would translate into effects on postprandial glycemia depends on the stability, bioavailability, and safety of the extracts, which must be evaluated in vivo.

3.5. Pancreatic Lipase Inhibitory Activity

Figure 2B presents the PL inhibitory activities of the extracts as IC_{50} values. Orlistat, the positive control, had an IC_{50} value of 13.41 μg/mL. Of the extracts, PE-DES inhibited PL most strongly (IC_{50}, 14.58 μg/mL), and its IC_{50} did not differ significantly from that of orlistat; it was followed by EE-DES (19.25 μg/mL), PE-water (25.56 μg/mL), and EE-water (36.19 μg/mL). When extraction conditions were held constant, DES significantly increased PL inhibition relative to water; the IC_{50} values of PE-DES and EE-DES were about 1.8 and 1.9 times lower than those of PE-water and EE-water, respectively.

Pancreatic lipase inhibition by pomegranate peel extracts has been documented previously; Mayasankaravalli et al. [45], for instance, obtained an IC_{50} value of 603.50 μg/mL for an ethanolic peel extract. Differences in extraction methods and assay conditions limit direct comparison, but the much lower IC_{50} values found here indicate that the DES extracts are strong PL inhibitors.

The polyphenols and flavonoids abundant in pomegranate peel, especially ellagitannins and proanthocyanidins, have been linked to pancreatic lipase inhibition and related biological activities [1]. Tannins have been reported to inhibit pancreatic lipase through interactions with the enzyme, in which their multiple hydroxyl groups bind to amino acid residues and interfere with catalytic activity [46]. The stronger PL inhibition of PE-DES may therefore reflect its higher phenolic content, in particular its higher level of punicalagin, although this was not tested directly.

3.6. Anti-Inflammatory Activity

In the MTT assay, cell viability remained above 80% for all extracts at 125–1,000 μg/mL, so none of the extracts was significantly cytotoxic under these conditions. Anti-inflammatory activity was then assessed from NO production in LPS-stimulated RAW 264.7 macrophages (Figure 3). Every extract reduced NO production in a dose-dependent manner. PE-DES was the most effective, lowering NO production to 32.95%, 24.51%, 19.93%, and 17.31% of the LPS-treated control at 125, 250, 500, and 1,000 μg/mL, respectively, followed by EE-DES, PE-water, and EE-water. Under the same extraction conditions, each DES extract suppressed NO production significantly more than the corresponding water extract ($p < 0.05$).

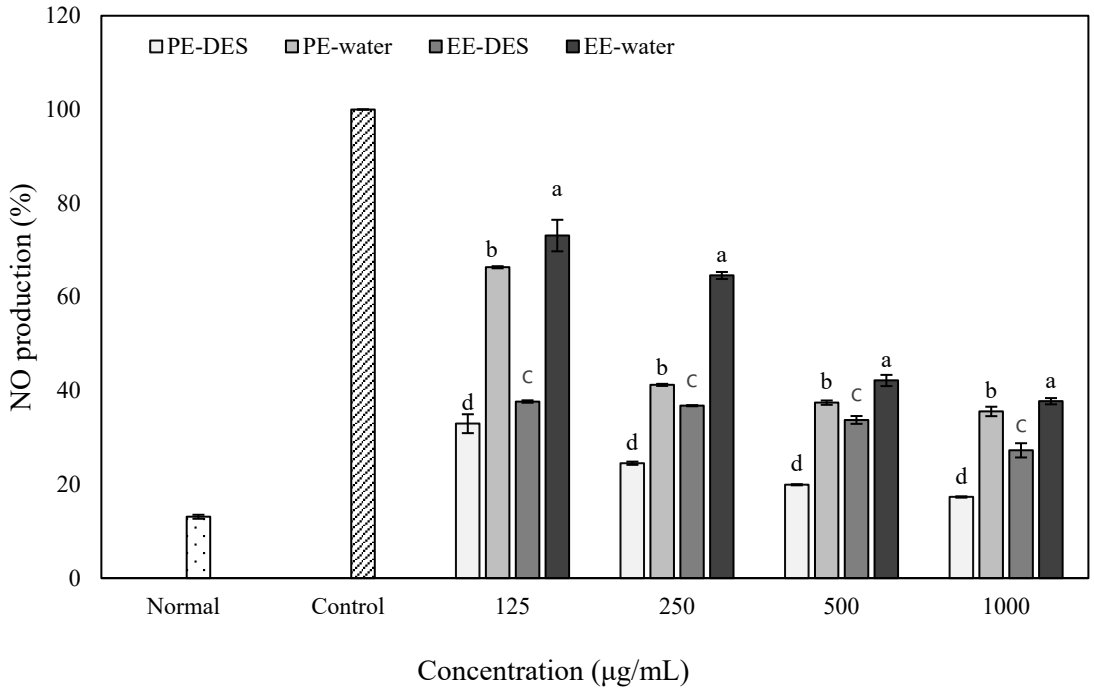


Figure 3. Effects of pomegranate peel extracts obtained using DES or water under optimized extraction conditions on nitric oxide (NO) production. Extract concentrations refer to purified dry extract (DES extracts) or crude dry extract (water extracts). Bars represent the means ± standard deviations (n = 3). NO production was expressed relative to the LPS-treated control (100%). Different letters indicate significant differences among the samples ($p < 0.05$) according to Duncan's multiple range test.

Our observations agree with earlier reports on the anti-inflammatory effects of pomegranate peel and its main phenolics. Lee et al. [47] showed that hydrolyzable tannins from pomegranate peel markedly decreased NO production and the expression of inducible nitric oxide synthase (iNOS) in RAW 264.7 macrophages. Du et al. [7] also found that pomegranate peel extract, punicalagin, and ellagic acid each inhibited NO production and the expression of the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β, and punicalagin was the most potent of the three.

The stronger inhibition of NO production observed in the DES extracts, particularly PE-DES, may be related to their higher phenolic contents and broader phenolic profiles. Punicalagin, the dominant phenolic in PE-DES and a compound with reported anti-inflammatory effects [7], may be one of the contributors, together with other co-extracted phenolic compounds, including ellagic acid. However, only NO accumulation in the culture medium was measured. Because iNOS expression, pro-inflammatory cytokines, and upstream signaling pathways were not examined, the present results show that the extracts reduced NO production at non-cytotoxic concentrations, but they do not establish a molecular anti-inflammatory mechanism. In addition, cell-free optical blanks containing the extracts were not included in the Griess assay. Any absorbance of the extracts at 540 nm would have raised the apparent NO level, so this omission would tend to underestimate rather than overestimate the inhibitory effect.

3.7. Multivariate Analysis of Phenolic Composition and Biological Activities

The phenolic composition and biological activities of the extracts were compared by hierarchical clustering analysis (Figure 4). Catechin, procyanidin, and ellagic acid were excluded from the multivariate analyses because their values were tentative or extrapolated (Section 2.5). The heatmap separated the four extracts into two main clusters, with the DES extracts (PE-DES and EE-DES) grouped together and distinguished from the water extracts (PE-water and EE-water). PE-DES showed relatively high levels of punicalagin, *p*-coumaric acid, protocatechuic acid, and epicatechin, whereas EE-DES was richer in gallic acid and total flavonoids. The DES extracts also exhibited lower IC_{50} values for antioxidant and enzyme inhibitory activities than the corresponding water extracts. Among the four extracts, PE-DES generally showed the strongest biological activities. These differences were reflected in the clustering patterns, indicating distinct phenolic profiles and biological properties among the extracts. Other studies have likewise found that phenolic composition and antioxidant activity differ among fruit peel extracts [28] and that phenolic recovery depends on the properties of the DES-based extraction system [30].

PCA was then applied to visualize how the extracts were distributed according to their phenolic composition and biological activities (Figure 5A). Together, PC1 (77.23%) and PC2 (20.06%) explained 97.29% of the variance. The two DES extracts had negative PC1 scores, and the two water extracts had positive scores. PE-DES fell in the region where both PC1 and PC2 were negative, in the direction of the punicalagin and *p*-coumaric acid loadings. EE-DES had a negative PC1 score and a positive PC2 score and lay closer to gallic acid and total flavonoid content. The water extracts were oriented toward higher IC_{50} values for antioxidant and enzyme inhibitory activities and toward higher NO production. This arrangement agrees with the differences in phenolic composition and bioactivity described above. Earlier work has similarly shown that the phenolic make-up and bioactivity of pomegranate-derived extracts vary with their phytochemical profiles [48,49]. Because these results are based on four group means, the PCA is presented only as an exploratory description, and the high proportion of variance explained by the first two components does not imply predictive validity.

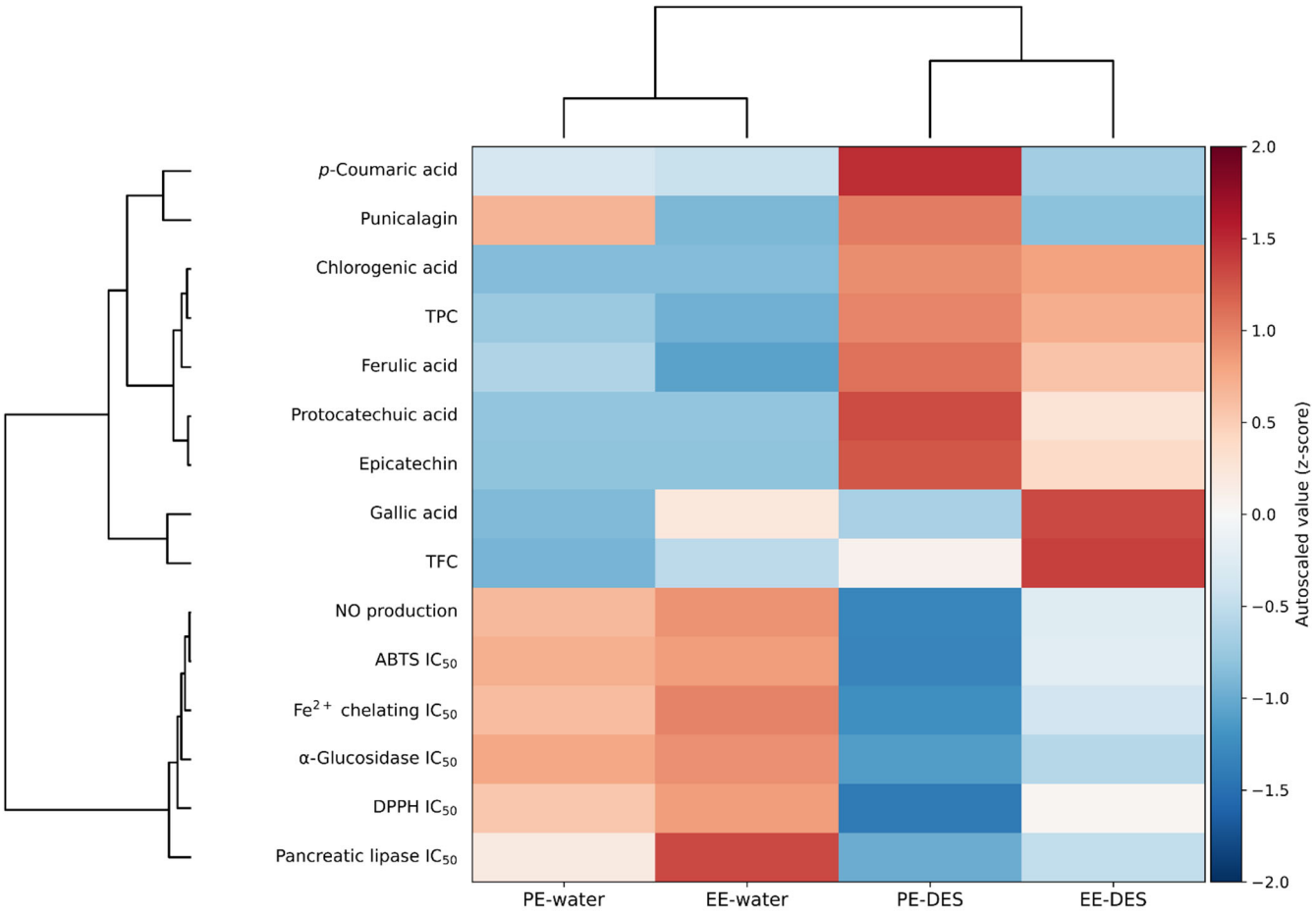


Figure 4. Hierarchical clustering heatmap of phenolic composition and biological activities of pomegranate peel extracts. Colors indicate autoscaled values (z-scores), with red and blue representing relatively higher and lower values, respectively. Clustering was based on Euclidean distance and Ward's linkage, using the means of the four extracts. Catechin, procyanidin, and ellagic acid were excluded because their values were tentative or extrapolated (Section 2.5).

Relationships between phenolic constituents and biological activity parameters were assessed by Pearson correlation analysis (Figure 5B). The IC_{50} values for antioxidant and enzyme inhibitory activities and NO production generally showed positive correlations with one another. In contrast, total phenolic content and several individual phenolic compounds were negatively correlated with these parameters. Total phenolic content was strongly and negatively correlated with the IC_{50} values for α-glucosidase inhibition ($r = -0.992$), pancreatic lipase inhibition ($r = -0.917$), and DPPH radical scavenging ($r = -0.858$). Epicatechin, ferulic acid, and protocatechuic acid also showed strong negative correlations, but because these compounds were not detected, or were present only at estimated levels, in the water extracts, their correlations largely reflect the contrast between the DES and water extracts. The correlation patterns, however, differed among individual phenolic compounds. Punicalagin showed only moderate correlations with the activity parameters ($r = -0.325$ to -0.623), while gallic acid exhibited a different pattern from most other phenolic constituents. Since lower IC_{50} values and reduced NO production indicate stronger biological activities, the negative correlations indicate that higher levels of certain phenolic compounds co-occurred with greater biological activities. Comparable links between phenolic content and antioxidant activity have been observed

in tropical fruit peel extracts [36]. These correlations, however, rest on the mean values of only four extracts; they should be regarded as exploratory and cannot establish the effects of individual compounds.

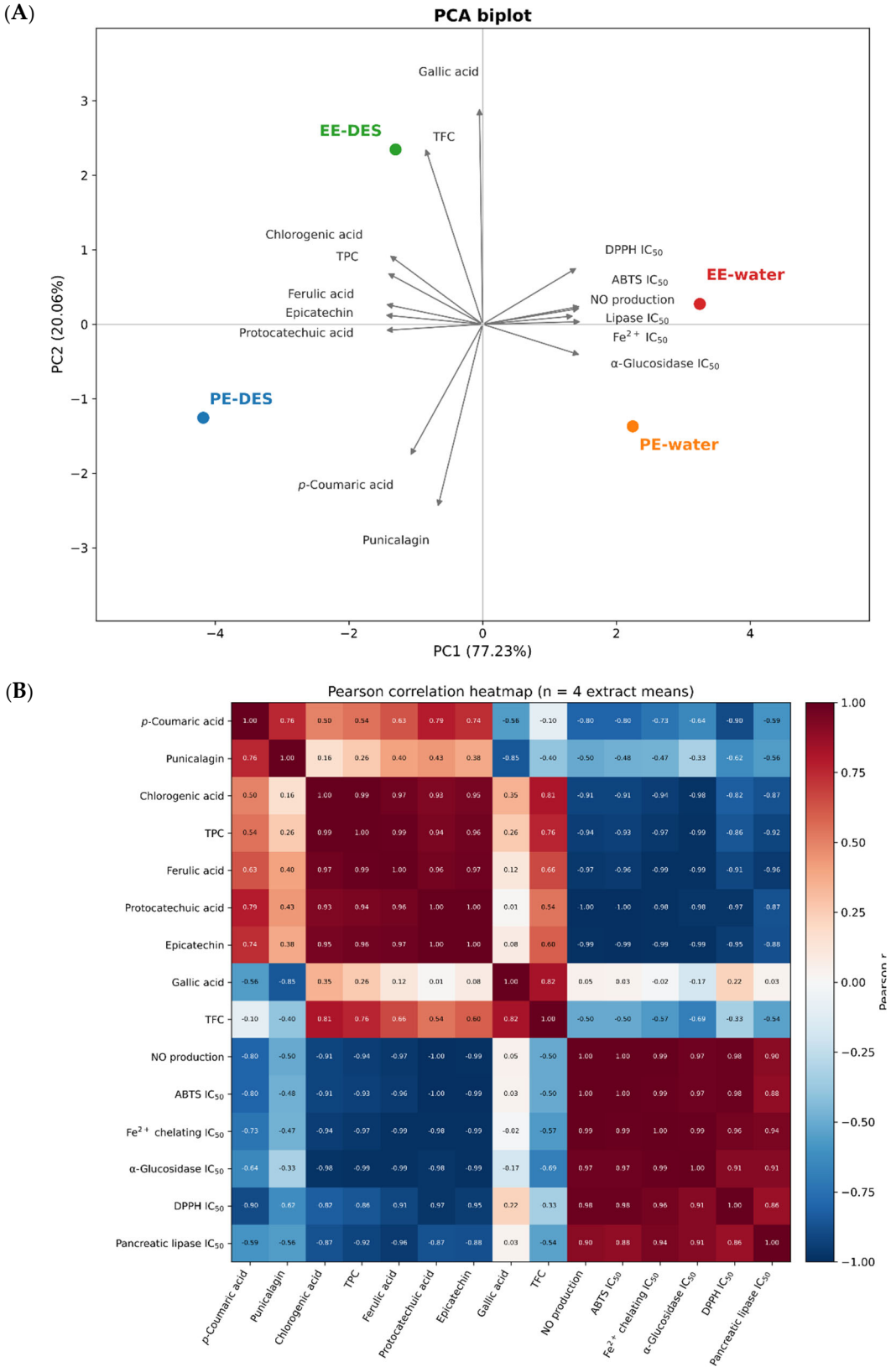


Figure 5. Principal component analysis (PCA) biplot (**A**) and Pearson correlation heatmap (**B**) of phenolic composition and biological activities of pomegranate peel extracts, based on the means of

the four extracts (n = 4). In (**A**), Lipase IC_{50} and Fe^{2+} IC_{50} denote the IC_{50} values for pancreatic lipase inhibition and Fe^{2+} chelation. In (**B**), red and blue indicate positive and negative correlations, respectively, and the correlation coefficients are shown in each cell.

Overall, the multivariate analyses separated the DES extracts from the water extracts on the basis of phenolic profile and bioactivity. PE-DES had the most punicalagin and the strongest activities. Yet punicalagin did not track consistently with the activity parameters, whereas total phenolic content correlated more strongly (negatively) with several IC_{50} values. These patterns are consistent with a contribution of the overall phenolic composition rather than of punicalagin alone, but correlations among four group means cannot attribute the activities to specific compounds. Testing of isolated fractions or individual compounds, alone and in combination, would be needed to determine how much each phenolic compound contributes.

3.8. Limitations and Implications for Application

Although DESs are often described as green solvents, the environmental merit of the present procedure should be judged over the whole workflow rather than from solvent composition alone. The choline chloride–urea DES is inexpensive, has low toxicity, and requires only mixing and heating at 80 °C for its preparation. However, each extraction consumed 100 mL of DES, and DES recovery and reuse were not examined. Removal of the DES also required HP-20 resin, water for washing, and 80% ethanol for elution, followed by rotary evaporation and freeze-drying, whereas the water extracts required freeze-drying only. Resin regeneration, ethanol recovery, energy consumption, and waste generation were not quantified. The term "green extraction" is therefore used here in a limited sense, and a life-cycle or green-metrics assessment would be needed for a quantitative comparison with conventional extraction.

Residual choline chloride and urea in the purified DES extracts were not quantified, so the completeness of DES removal was not verified, and controls containing DES alone were not included in the biological assays. A contribution of residual solvent components or resin-derived impurities to the measured activities therefore cannot be ruled out. In addition, the DES and water extracts were obtained by different work-up procedures, so the differences between them cannot be attributed to the extraction solvent alone. Likewise, the α-glucosidase assay of the water extracts was performed in a separate run with a lower control absorbance than that of the DES extracts; although inhibition was calculated relative to the control of each run, differences in enzyme activity between runs may have affected this comparison. Extraction yields were not determined, so all contents and activities are expressed per gram of purified (DES) or crude (water) dry extract, and the extraction efficiency of DES relative to water on a dry-peel basis remains to be established. Moreover, because the replicate extracts were pooled before analysis, the variability between extractions was not assessed, and the statistical comparisons reflect analytical replicates. The phenolic compounds were identified by retention time with single-wavelength detection, so the identification of catechin is tentative, and procyanidin was quantified only semi-quantitatively; confirmation by LC–MS would strengthen the identification and quantification. All biological activities were measured in vitro, using chemical antioxidant assays, isolated enzymes, and a macrophage cell line, and they do not demonstrate health benefits in humans. Before the extracts can be considered as functional food ingredients, residual solvent levels, safety, stability, bioavailability, and the relevant regulatory requirements need to be addressed, and the bioactivities should be confirmed in vivo.

4. Conclusions

Compared with water under the same UAE conditions, the DES-based workflow, which included resin purification of the DES extracts, produced pomegranate peel extracts with higher phenolic contents per gram of purified dry extract than those of the crude water extracts and stronger in vitro biological activities. Total phenolics, flavonoids, and several individual phenolic compounds were all present at higher levels in the DES extracts. PE-DES contained the most punicalagin and showed the strongest antioxidant, α-glucosidase inhibitory, pancreatic lipase inhibitory, and NO production inhibitory activities. Exploratory multivariate analyses associated differences in phenolic composition with the observed activities. Although punicalagin was the predominant phenolic compound, total phenolic content correlated more strongly with several activity parameters in only four extracts; however, these correlations cannot identify the compounds responsible for the activities. DES-based UAE therefore merits further study for the valorization of pomegranate peel, but the effects of purification, residual solvent, safety, and in vivo efficacy must be clarified before the extracts can be proposed as functional food ingredients.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/foods15234204/s1. Table S1: Retention times, calibration functions, coefficients of determination (R^2), limits of detection (LOD), and limits of quantification (LOQ) of the phenolic compound standards used for HPLC analysis; Table S2: Concentration–response data, linear regression statistics, and IC_{50} values for the antioxidant and enzyme inhibition assays of pomegranate peel extracts and positive controls; Figure S1: HPLC chromatograms (280 nm) of pomegranate peel extracts obtained using DES or water under optimized extraction conditions: (A) PE-DES, (B) PE-water, (C) EE-DES, and (D) EE-water.

Author Contributions: Conceptualization, K.Y.Y.; Methodology, H.J.K. and K.Y.Y.; Formal analysis, H.J.K.; Validation, H.J.K. and K.Y.Y.; Writing—original draft, H.J.K.; Writing—review and editing, K.Y.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) in 2026 (Grant No. RS-2026-25586869, Project Title: Elucidation of Functional Origin and Gut Microbiota-Mediated Mechanisms of Coffee Spent Ground-Derived Melanoidin-Rich Fractions).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article and Supplementary Materials. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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https://doi.org/10.3390/foods15234204