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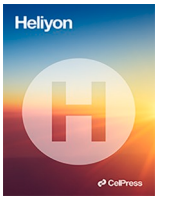

Research article

Ellagitannin content and anti-enterohemorrhagic *Escherichia coli* activity of aqueous extracts derived from commercial pomegranate products

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ABSTRACT

This study compared the efficacy of aqueous extracts of commercially available pomegranate peel products and a juice powder in inhibiting the growth of two enterohemorrhagic *Escherichia coli* strains. Cell suspension of each *E. coli* strain (5 Log CFU/ml) was added into tryptic soy broth amended with 9 or 23% of each extract prepared with two different methods. After treatment for 5, 10, and 24 h at 25 °C, surviving *E. coli* cells were enumerated on tryptic soy agar to determine cell population reduction compared to the controls. The concentrations of six different ellagitannins and titratable activity in each treatment system were determined and correlated to *E. coli* cell population reduction. The extracts from three powdered pomegranate peels caused a significantly greater ($p \leq 0.05$) reduction in *E. coli* population than the extract from the whole peel and juice powder. The higher dose of extracts resulted in a greater cell population reduction than the lower dose. The level of *E. coli* population reduction correlated positively with the total ellagitannins content (R^2 0.67–0.98) and the titratable acidity (R^2 0.69–0.98) in the treatment systems. The study suggests that pomegranate peels are promising natural additives or preservatives to control pathogens like EHEC.

1. Introduction

Indigenous to Central Asia and the Middle East, pomegranate (*Punica granatum* L.) is a popular fruit cultivated in several regions worldwide. As one of the first domesticated fruits in history, it has been used as a traditional medicine to treat diarrhea and dysentery due to its therapeutic value that is attributed to the broad range of phytochemicals [1,2]. Roughly, five hundred pomegranate cultivars are spreading all over the world, and fruit size, husk and aril color, as well as juice flavor, vary from region to region [3]. In the modern age, the fast-growing population of health-conscious consumers has led to sharply increased production and consumption of pomegranate, which inevitably generates vast amounts of byproduct waste: inedible pomegranate peels, which comprise around 30–40 % of the pomegranate fruit [4].

Studies have revealed that pomegranate peels contain a significantly higher content of hydrolyzable tannins, plant metabolites with associated health benefits to humans, than the edible arils. Hydrolyzable tannins are esters of gallic acids and polyols, mainly D-glucose [5]. Ellagitannins, the main hydrolyzable tannins of pomegranate, consist of a central molecule, such as glucose, linked by galloyl units

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via 3, 4, 5, 3′, 4′, 5′- hexahydroxydiphenoyl moiety [6]. The number of free galloyl groups, the degree of hydroxylation, and the configuration of digalloyl or trigalloyl groups on ellagitannins are core factors to exert antibacterial and antivirulence activities [7]. Free galloyl groups chelate ferric ions that are crucial to the growth of bacteria [8,9]. Hydrogen bonds formed between ellagitannins and bacterial cells change the permeability of their membranes, inactivate the enzymes that participate in the cell wall and fatty acid biosynthesis, and damage cell wall integrities [10].

Ellagitannins including punicalagin, punicalin, ellagic acid, and gallic acid have been found in pomegranate peels in previous studies [11,12]. Punicalagin was reportedly to inhibit the growth of pathogenic clostridia and *Staphylococcus aureus* [7,13]. Thus, these disease-preventing compounds in pomegranate peels can be potential candidates for developing natural antimicrobials or food preservatives.

Foodborne bacteria typically cause gastrointestinal infections in humans. As one of the major foodborne pathogens, enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 produces Shiga toxins that cause severe human diseases, including hemorrhagic diarrhea and hemolytic-uremic syndrome that can be life-threatening to immune-compromised population [14]. In the United States, multiple foodborne outbreaks caused by EHEC O157:H7 were linked to the consumption of raw, lightly cooked, or ready-to-eat foods such as sprouts and salads [15]. Therefore, chemical preservatives are crucial to prevent contamination by *E. coli* O157:H7 and other foodborne pathogens. However, the excessive use of chemical preservatives in foods may contribute to developing antimicrobial resistance in bacteria. Cross-resistance between chemical preservatives and antibiotics has been reported, a growing public health threat that results in substantial economic loss and an increase in mortality rate in hospital settings [16,17]. Therefore, alternative natural antimicrobials are preferred by modern and health-conscious consumers.

Although studies have reported the antimicrobial activities of pomegranate peel extracts against various foodborne pathogens [18–21], the relationship between the reduction in *E. coli* O157:H7 cell population and the concentration of ellagitannins quantified by High-Performance Liquid Chromatography (HPLC) has not been revealed. The present study aimed to evaluate the antibacterial effect of aqueous extracts derived from a few pomegranate products by testing their efficacies against selected strains of EHEC and to determine whether there is a correlation between EHEC population reduction and ellagitannins concentration or titratable acidity (TA) in the treatment systems.

2. Materials and methods

2.1. Pomegranate products and E. coli strains

Five commercially available pomegranate products, distributed by eSutras superfood, Bixa, Naturevibe, Ragarden, and Navitas Organic, respectively were purchased from Amazon.com. Three of the products were powdered peels (product I, II, III), one was dried whole peel (product IV), and the remaining one was pomegranate juice powder (product V). The health claims made by the manufacturers/distributors included beneficial effects on human skin, hair, and gastrointestinal health. The products were kept at refrigeration temperature upon arrival at the laboratory located in Griffin, GA USA.

E. coli O157:H7 F4546 and K4492, previously involved in sprout and spinach-related outbreaks, respectively were selected for the study. The bacterial cultures were obtained from Dr. Mark Harrison's laboratory at the University of Georgia.

2.2. Preparation of pomegranate extracts

2.2.1. Method one - unconcentrated extracts

Twenty milliliters of distilled water was heated on a hotplate stirrer (Fisher Scientific, Fair Lawn, NJ USA) until boiling. Boiled water was mixed with 20 g of each pomegranate product described above at room temperature (20 °C) for an hour, while stir rods were used occasionally to avoid product settling. The mixtures were subsequently centrifuged at 10,000 *g* for 10 min at 5 °C. Obtained supernatants were filtered through Whatman No. 1 filter paper (GE Healthcare Systems, Princeton, NJ USA) to remove the remaining product debris. Collected filtrates were sterilized using Corning disposable cellulose acetate filtration systems (0.45 μm, Fisher Scientific). All extracts were kept at 4 °C before use.

2.2.2. Method two - concentrated extracts

Twenty grams of each pomegranate product was added to 200 ml of boiling water and heated on a hotplate stirrer for 30 min. Crude extracts were precipitated and filtered using the conditions described above. Each resulting filtrate was concentrated at 75 °C for 15 h before being sterilized with the Corning disposable cellulose acetate filtration system described above. The extracts were kept at 4 °C before being used in the following experiments.

2.3. Antimicrobial activity assay

Individual colonies of *E. coli* O157:H7 F4546 and K4492 were inoculated in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD USA) and incubated at 37 °C for 16–18 h. The bacterial cultures were serially diluted in TSB to *ca.* 5 Log CFU/ml. Each of the ten extracts described above was added to a test tube with the diluted bacterial suspension, at a concentration of 9% or 23% (v/v). The control groups did not contain any extract, and sterilized distilled water was used to make up the missing volumes. The susceptibility of *E. coli* O157:H7 F4546 and K4492 to different concentrations of the extracts was determined using the plate count assay. After 5, 10, and 24 h of incubation at room temperature on an orbital platform shaker (Model: 3520, Lab-line, IL USA), an aliquot of each sample

was serially diluted and spread plated onto tryptic soy agar (BD Diagnostic Systems) plates in duplicate. The inoculated plates were then incubated at 37 °C for 16–18 h. The inhibitory effect of the extracts was expressed as *E. coli* cell population reduction in the experimental groups compared to the population in the control groups. All experiments were reproduced twice.

2.4. Ellagitannin analysis using HPLC

2.4.1. Preparation of ellagitannin standards and extract samples

Standards for quantification of gallic acid, ellagic acid, punicalagin A&B, and punicalin A&B were purchased from the INDOFINE Chemical Company Inc. (Hillsborough, NJ USA) or Sigma-Aldrich (St. Louis, MO USA). Stock solutions at a concentration of 5 mg/ml were prepared by dissolving 5 mg of each standard in 1 ml of HPLC-grade methanol. The standard solutions were vortexed, followed by dilution with HPLC-grade methanol to create a sequence of concentrations ranging from 5 to 500 μg/ml. The prepared solutions were filtered into a Whatman syringeless filter vial (Cytiva, Marlborough, MA USA) with a 0.45 μm pore size to collect purified samples.

Aqueous extracts were diluted in ultra-filtered water to reach appropriate concentrations that fall within the range of the calibration curve. Each extracted sample was filtered using the method described above.

2.4.2. HPLC system and chromatographic conditions

Agilent 1100 series HPLC system (Agilent Technologies, Germany) equipped with a binary high-pressure pump, Diode-Array Detector, and an autosampler, operated by Agilent OpenLAB ChemStation C.01.09 software was used to perform the chromatographic analyses. Extract and standard sample vials were loaded into the autosampler. A ZORBAX Eclipse XDB-Phenyl column (3 × 150 mm, 5 μm particle size, Agilent Technologies, Germany) with a guard column (4.6 × 12.5 mm, 5 μm particle size, Agilent Technologies, Germany) was used as the stationary phase, the temperature was maintained at 40 °C. Two solvents, A (0.2% formic acid in water) and B (2% acetonitrile), were selected as mobile phases for gradient elution. All solvents used were HPLC grade. The gradient program adopted for the analyses included 2% solution B for 0–5 min; 2–26% solution B for 5–15 min; and 26–40% solution B for 15–20 min. The flow rate was 0.6 ml/min, and the detection wavelength was set at 275 nm for the quantitation of gallic acid and 375 nm for ellagic acid, α & β-punicalin, α & β-punicalagin.

2.4.3. Identification and quantification of ellagitannin compounds in pomegranate extracts

The retention times and calibration curves were established using standard analysis. The ellagitannins in pomegranate extracts were identified, and their concentrations were quantified by comparing the retention times of identified ellagitannins to those of the standards and by fitting integration areas of the peaks into calibration curves of the standards.

2.4.4. Quantification of TA in treatment systems

The TA in the treatment systems was determined using a method previously described by Wu et al. [21].

2.5. Statistical analysis

The results obtained were analyzed using the ANOVA linear model of the statistical analysis software (Statistical Analysis Systems Institute, ver. 9.4, Cary NC). The strain of bacteria, the type of pomegranate peel products, the method of extraction, the concentration of the extracts, as well as treatment time were independent variables, and the dependent variable was the difference in *E. coli* cell

Table 1
F-test of each independent variable and their interactions, with the reduction in enterohemorrhagic *Escherichia coli* cell population as a dependent variable.

Dependent variable			Reduction in *Escherichia coli* Cell Population (Log CFU/ml)		
Source	DF	Type III SS	Mean Square	F value	Pr > F
Culture	1	2.98	2.98	19.81	<0.0001
Time	2	43.52	21.76	144.62	<0.0001
Extract	4	72.35	18.09	120.21	<0.0001
Method	1	84.68	84.68	562.77	<0.0001
Concentration	2	371.72	185.85	1235.20	<0.0001
Culture*time	2	1.00	0.50	3.32	0.0386
Culture*extract	4	0.56	0.14	0.93	0.4500
Culture*method	1	1.51	1.51	10.00	0.0018
Culture*concentration	2	2.28	1.14	7.57	0.0007
Time*extract	8	43.13	5.39	35.83	<0.0010
Time*method	2	15.22	7.61	50.56	<0.0001
Time*concentration	4	37.09	9.27	61.63	<0.0001
Extract*method	4	1.38	0.35	2.30	0.0608
Extract*concentration	8	39.91	4.99	33.15	<0.0001
Method*concentration	2	53.84	26.92	178.91	<0.0001

DF: Degrees of freedom, Type III SS: Type III sum of squares, F value: Test statistic for F-test under the null hypothesis, and Pr: *p*-value, the probability of obtaining test statistic given the null hypothesis is true.

populations generated under conditions of various variables compared to the cell population in the control groups. Results are presented as mean values of the reduction. The statistical significances ($p \leq 0.05$) were determined using Fisher's least significant difference test based on a 95% interval. The same statistical protocol was used to analyze the concentrations of ellagitannins in the treatment systems. The correlation coefficients between the concentrations of ellagitannins or the contents of organic acid and the reduction in bacterial populations were determined using the Pearson correlation analysis.

3. Results

3.1. The overall mean reduction in E. coli cell population

Results in Table 1 revealed that all independent variables (bacterial strain, sampling time, type of extract, extraction method, and extract concentration) were significant factors ($p \leq 0.05$) influencing the reduction in *E. coli* cell population. Significant interactions were observed between these independent variables, except for the interactions between bacterial strain and pomegranate extract, and between the type of extract and extraction method used.

Extract of product I exhibited the highest inhibitory effect against *E. coli* cells with a mean reduction of 1.98 Log CFU/ml which is significantly higher ($p \leq 0.05$) than the reduction caused by the other extracts evaluated in the study (Table 2). The reductions caused by extracts of product II and III (1.46 and 1.38 Log CFU/ml) were similar ($p > 0.05$) and so were the reductions caused by extracts of product IV and V (0.92 and 0.73 Log CFU/ml). However, the inhibitory activities of products II and III were significantly higher than those of products IV and V. In general, the unconcentrated extracts had significantly lower antimicrobial activity than the concentrated extracts. Systems with a higher concentration (23%) of the extracts were more effective and resulted in a significantly higher level of reduction in cell population compared to the system with a lower concentration (9%) of the extracts. The reduction in *E. coli* population peaked at the 10 h sampling point which was significantly higher than the levels of reduction observed in samples collected at the 5 h and 24 h sampling points. Overall, no significant cell population difference between the two *E. coli* strains was observed.

3.2. E. coli cell population reduction affected by individual extracts of pomegranate products

A trend that is similar to what was observed in the overall statistical analysis was demonstrated in TSB supplemented with individual extracts of the pomegranate products (Table 3). For instance, for all five types of extracts, significantly ($p \leq 0.05$) higher antimicrobial activity was observed in TSB supplemented with the concentrated extracts and in the system containing a higher concentration of the extracts (23 %). Extracts showed the highest inhibitory effect against *E. coli* cells in TSB after 10 h of treatment, except for the extracts of products IV and V that had insignificantly ($p > 0.05$) different cell population reductions at the 24 h and the 10 h sampling points. However, extracts of product I, II, and III had a similar inhibitory effect in reducing the cell population at a shorter period of treatment of 5 h, and the activities of the three extracts were significantly more effective than the extracts of products IV and V. At the 10 sampling points, extract of product I was significantly more effective than the extracts of product II and III which

Table 2
Overall reduction in enterohemorrhagic *Escherichia coli* cell population as affected by the supplementation of tryptic soy broth with aqueous extracts of five pomegranate products.

	Reduction in *Escherichia coli* Cell Population (Log CFU/ml)	
Product for phenolic extraction		
(n = 72)	I	1.98A
	II	1.46B
	III	1.38B
	IV	0.92C
	V	0.73C
Extraction method		
(n = 180)	I	0.81B
	II	1.78A
Extract concentration in TSB		
(n = 120)	0%	0.00C
	9%	1.41B
	23%	2.48A
Treatment interval (h)		
(n = 120)	5	1.15B
	10	1.78A
	24	0.96B
E. coli strain		
(n = 180)	F4546	1.39A
	K4492	1.20A

I, II, and III: Powdered pomegranate peels, IV: Whole pomegranate peel, and V: Pomegranate juice powder. TSB: Tryptic soy broth.
Means within the same variable in the same column not followed by the same letter are significantly different ($p \leq 0.05$).

Table 3
Reduction in enterohemorrhagic *Escherichia coli* cell population as affected by the supplementation of tryptic soy broth with aqueous extracts of individual pomegranate peels.

	Reduction in *Escherichia coli* Cell Population (Log CFU/ml)				
	I	II	III	IV	V
Time (n = 120)					
5 h	1.45Ba	1.69Ba	1.60Ba	0.60Bb	0.41Bb
10 h	2.87Aa	2.08Ab	1.98Ab	1.15Ac	0.80Ac
24 h	1.65Ba	0.64Cc	0.59Cc	1.03Ab	0.90Abc
Extraction method (n = 180)					
I	1.63Ba	0.94Bb	0.89Bb	0.42BCE	0.18BCE
II	2.35Aa	2.00Ab	1.89Ab	1.44Ac	1.23Ac
Extract conc. (n = 120)					
0%	0.00Ca	0.00Ca	0.00Ca	0.00Ca	0.00Ca
9%	2.27Ba	1.80Bb	1.67Bb	0.89BCE	0.40Bd
23%	3.70Aa	2.61Ab	2.50Ab	1.90Ac	1.71Ac

Means in the same row not followed by the same lowercase letters are significantly different ($p \leq 0.05$) regarding pomegranate peel extracts. Means of the same variables in the same column not followed by the same uppercase letters are significantly different ($p \leq 0.05$) regarding treatment time, extraction method, and concentration of pomegranate peel extracts in tryptic soy broth.

were, in turn, more effective than those of product IV and V. The extract of product I was more effective than the extracts of product IV which more were effective than product II and III at the 24 h sampling points. But EHEC population reduction caused by the extract of product V was only significantly different from that caused by the extract of product I at this sampling point.

3.3. The profiles and concentrations of six types of ellagitannins in individual extracts

The R^2 values for the standard curves of the six phenolics ranged from 0.99961 to 0.99996. Fig. 1 shows the concentrations of six different ellagitannins and their total contents in milligrams per milliliter (mg/ml) of each aqueous extract prepared by two different methods. The identified ellagitannins included *α* & *β*-punicalin, *α* & *β*-punicalagin, ellagic acid, and gallic acid. The total content of ellagitannins, labeled as ellagitannins, was calculated by summing up the concentrations of all six detected ellagitannins. Table 4 shows the average concentrations of aforementioned ellagitannins in extracts prepared by the two methods. The result revealed the average quantities of ellagitannins in the five extracts ranged from 0.70 to 27.24 mg/ml, whereas the extracts of the three powdered peel products had significantly ($P \leq 0.05$) higher ellagitannins contents (25.15–27.24 mg/ml) than those of the whole peel and juice powder (0.70–3.70 mg/ml). The extracts prepared by method two contained a significantly higher average concentration of ellagitannins than those prepared by method one, which was in the amounts of 24.70 and 8.52 mg/ml, respectively (Fig. 1). Among all the observed ellagitannins, *α* & *β*-punicalagins were the most abundant in all extracts (0.42–22.34 mg/ml), followed by *α* & *β*- punicalins (0.26–10.37 mg/ml), ellagic acid (0.01–0.65 mg/ml), and gallic acid (0.00–0.49 mg/ml).

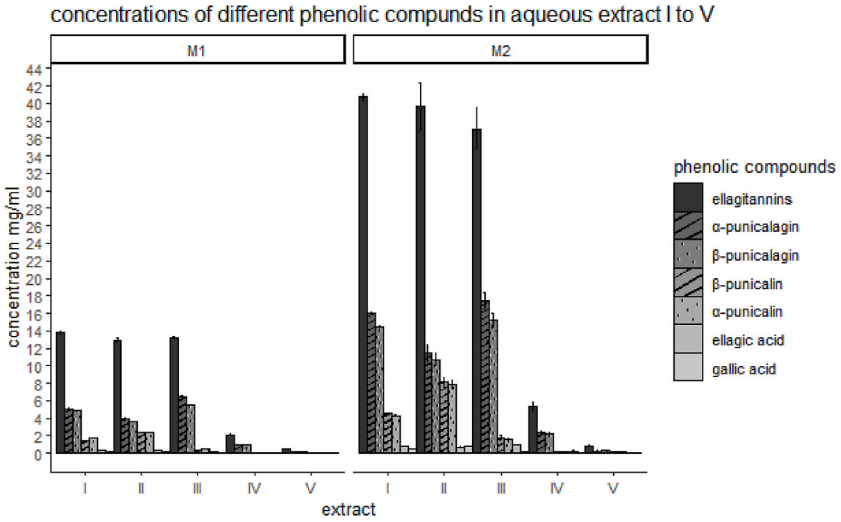

Fig. 1. The concentrations of different ellagitannins in the extracts of pomegranate products prepared with method I or method II and analyzed using high-performance liquid chromatography. I, II, and III: Powdered pomegranate peels, IV: Whole pomegranate peel, and V: Pomegranate juice powder. M1: Unconcentrated method, M2: Concentrated method.
Fig. 1.

Table 4
The average concentrations of different ellagitannins in the extracts of pomegranate products prepared by method I and method II and analyzed using high-performance liquid chromatography.

The Concentrations of ellagitannins (mg/ml)							
Ellagitannis	α-punicalagin	β-punicalagin	α-punicalin	β-punicalin	Ellagic Acid	Gallic Acid	Total Ellagitannins
Product for extraction							
I	10.54B	9.65A	3.06B	3.02B	0.65A	0.32B	27.24A
II	7.71C	7.21B	5.07A	5.30A	0.50B	0.49A	26.28A
III	11.97A	10.37A	1.01C	1.08C	0.59A	0.12C	25.15A
IV	1.68D	1.57C	0.13D	0.13D	0.16C	0.02D	3.70B
V	0.20E	0.22D	0.12D	0.14D	0.01D	0.00D	0.70C

Means in the same column not followed by the same letters are significantly different ($p \leq 0.05$).

3.4. Relationship between total contents of ellagitannins in the treatment systems and reductions in E. coli population

Table 5 shows the Pearson correlation coefficients between the total contents of ellagitannins and the reductions in the populations of *E. coli* O157:H7 F4546 and K4492 during various lengths of treatments. Positive correlations were noticed with R^2 ranging from 0.68 to 0.98 and from 0.67 to 0.97, respectively in the system with strain F4546 or K4492. In general, a slightly lower correlation was observed with treatment in TSB with different extracts for 5 h.

3.5. Relationship between TA in the treatment systems and reductions in E. coli populations

The correlation coefficients between the TA of all treatment systems and the reductions in the *E. coli* population ranged from 0.69 to 0.98 (Table 6). The bivariate correlation between the TA of the treatment systems amended with extracts of products I, IV, and V and the bacterial population reductions was relatively lower after 5 h of treatment.

4. Discussion

All evaluated pomegranate aqueous extracts in this study had inhibitory activity against the two EHEC strains, even though powdered peel products demonstrated significantly ($p \leq 0.05$) higher antibacterial activities than the whole peel and juice powder (Table 2). Similar observations were made by several previous studies, and the extracts from peel powders had significantly higher inhibitory activity against *P. stutzeri*, *Streptococcus mutans, Streptococcus mitis*, *Salmonella* Enteritidis, and *Lactobacillus acidophilus* than those from the whole peels [21–23]. In comparison, the extract of pomegranate juice powder had the lowest inhibitory activity against the EHEC strains tested in the study (Table 2). This observation could be attributed to the difference in chemical compositions between the extracts of pomegranate peel and juice products. It was reported that the principal constituents of pomegranate peels are phenolic compounds with a substantial quantity of hydrolyzable tannins, more specifically ellagitannins, whereas the major compounds of pomegranate juice are sugars, flavonoids, and organic acids [24,25].

In HPLC analysis of the present study, α & β-punicalagin, α & β-punicalin, ellagic acid, and gallic acid were identified as the major ellagitannins (Fig. 1). The extracts of all powdered peel products had significantly higher ($p \leq 0.05$) concentrations of individual and total ellagitannins than did the juice powder (Table 4). John et al. [26] found that the quantities of punicalagins, punicalins, and ellagic acid were significantly lower in the arils than in the peel and the inner membrane of tested pomegranate fruits. This finding may explain the difference in antibacterial effect between the extracts of pomegranate peel vs. juice powder against the two EHEC strains observed in the current study. Fig. 1 and Table 4 showed that α & β-punicalagin were the predominant ellagitannins in all extracts,

Table 5
Correlation between total ellagitannins of individual pomegranate extracts and the population reductions of *E. coli* strains in tryptic soy broth.

	Pearson Correlation Coefficients, n = 12 Prob > |r| under H0: Rho = 0				
Extract	I	II	III	IV	V
F4546					
Treatment time (h)					
5	0.68*	0.70*	0.69*	0.86***	0.81**
10	0.71**	0.88***	0.96***	0.95***	0.86***
24	0.96***	0.95***	0.94***	0.98***	0.90***
K4492					
Treatment time (h)					
5	0.72**	0.68*	0.72**	0.79**	0.67*
10	0.69*	0.95***	0.92***	0.96***	0.90***
24	0.97***	0.91***	0.87***	0.93***	0.89***

I, II, and III: Powdered pomegranate peels, IV: Whole pomegranate peel, and V: Pomegranate juice powder.
The magnitude of the *P*-value of the correlations is represented by the stars: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 6
Correlation between the titratable acidity of tryptic soy broth amended with phenolic extracts and the reduction in *E. coli* cell population at different sampling points.

	Pearson Correlation Coefficients, n = 12 Prob > |r| under H0: Rho = 0				
Extract	I	II	III	IV	V
Treatment time (h)					
5	0.82**	0.81**	0.97***	0.79**	0.69*
10	0.83***	0.91***	0.96***	0.97***	0.90***
24	0.96***	0.85***	0.88***	0.97***	0.98***

I, II, and III: Powdered pomegranate peels, IV: Whole pomegranate peel, and V: Pomegranate juice powder.
The magnitude of the *p*-value of the correlations is represented by the stars: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

which constituted 72%–91% of the total ellagitannins in the extracts of pomegranate peel products. This result is consistent with the finding of Talekar et al. [27] who reported that 75–78% of total phenolics were punicalagin in pomegranate peels. In the current study, all concentrated extracts contained higher concentrations of ellagitannins (Fig. 1). This result coincided with the higher EHEC cell population reduction contributed by the extracts produced with method two (Table 1).

The average growth inhibition contributed by oligomeric ellagitannins was reportedly higher than the ellagitannin monomers [8]. Among the six identified ellagitannins in the current study, α & β-punicalagin and α & β-punicalin were the oligomeric ellagitannins. Although the extract of product III contained higher concentrations of α & β-punicalagin (22.34 mg/ml in total) than the extract of product II (14.92 mg/ml in total), the latter extract had a higher concentration of α & β-punicalin (10.37 mg/ml in total) than the former one (2.09 mg/ml in total) (Table 4). The sum concentrations of the four ellagitannins were 26.27, 25.29, and 24.43 mg/ml in the extracts of products I, II, and III, respectively. This result was consistent with the order of strength of the inhibitory activity of the three extracts (Table 2). The level of *E. coli* population reduction caused by extracts I to III were 1.98, 1.46, and 1.38 Log CFU/ml, respectively (Table 2). However, it is worth noting that although oligomer ellagitannins presented a high antibacterial effect, the inhibitory activity against multiple gram-negative and gram-positive bacteria by gallic acid, a monomer ellagitannin, was also reported [28].

The highest mean reduction of 3.70 Log CFU/ml in the mean population of *E. coli* was observed in the test system supplemented with 23% of the extract of peel product I (Table 3). A significantly ($p \leq 0.05$) lower level of 2.27 Log CFU/ml reduction was noticed when 9% of the same extract was used in the treatment system. These observations in dose-response are in accordance with the results of previous studies where either the disc diffusion assay or minimum inhibitory concentration was used [20,29,30].

Using hot water extracts prepared by two different methods with a 1:10 solid-water ratio, Wu et al. [21] reported that the extracts from powdered peels reduced the mean *Salmonella* populations by 1.67–2.03 Log CFU/ml. An approximate 2.7 Log CFU/ml reduction was observed when *E. coli* was growing in Mueller Hinton broth amended with 20.3% (w/v) extracts prepared by soaking pomegranate peels in hot water for 18 h with 1:2 of solid-water ratio [29]. In contrast, McCarrell et al. [31] found that the aqueous extracts of pomegranate peels prepared using a 1:3 solid-water ratio for 10 min before filtration and sterilization, were inactive against an avian pathogenic *E. coli* strain. Other than hot water extracts, pressurized water, and microwave heating were also used in a previous study to improve the yield of phenolic compounds from pomegranate peel [32].

Ethanol and methanol have been used as common solvents to extract phenolic compounds from pomegranate peels. Wang et al. [33] revealed that the use of methanol extracted the highest quantity of polyphenolics from pomegranate peels, followed by water and ethanol. Yassin et al. [34] also reported that a methanolic extract of pomegranate peel had a higher phenolic content than its aqueous counterpart. However, given the toxic nature of methanol, it is not practical when it comes to food application unless the residue can be reduced to a safe level in the downstream process. In contrast, the use of ethanol to extract phenolics from pomegranate peels for food application is safe, and thus, the antimicrobial activity of ethanolic extracts of pomegranate peels could be further explored.

Other than phenolic compounds, the organic acid may also contribute to the antimicrobial effect of pomegranate peel/juice extracts. In the present study, a positive correlation was observed between the percentage of TA and the inhibitory effect of aqueous extracts of pomegranate peel and juice powder (Table 6). A wide range of citric acid (0.2–37.5 %) was found in different cultivars of pomegranate juice, while a 3 - to 5-fold lower concentration of citric acid was detected in peels than in the juice [35].

The inhibitory mechanisms of polyphenols vary according to their chemical structures. Lipophilic polyphenols can interact with, and change the integrity and permeability of, bacterial cell membranes. Furthermore, hydrogen bonding between polyphenols and bacterial enzymes that are involved in many important metabolic pathways could lead to the interruption of many metabolic activities in bacterial cells [36]. Different from ellagitannins, the antimicrobial properties of citric acid are due to its acidulation and chelating capacity with a metal ion [37]. However, *E. coli* O157:H7 was documented to be more resistant to organic acids than other common foodborne pathogens such as *Salmonella* and *Listeria monocytogenes* [38,39]. The acid tolerance may have contributed to the relatively lower susceptibility of EHEC strains to the extract of pomegranate juice powder (Table 2).

5. Conclusion

Pomegranate peels, often considered food waste, have the potential to be used as food preservatives, given their nature of being rich in phenolic compounds with antimicrobial activities. The fact that *E. coli* O157:H7 had significant population reduction in the test

system amended with an aqueous extract of pomegranate peel powder proved itself a promising natural preservative to control food spoilage and pathogen contamination. The higher amounts of ellagitannins extracted from powdered pomegranate peels than from whole peels or juice powder correlated to their activities in inhibiting the growth of *E. coli.* The results of ellagitannin quantification further revealed the major phenolic compounds that contributed to the observed antibacterial activity. Reprocessing inedible pomegranate peels into natural food preservatives can largely decrease the waste of by-products and reduce the use of synthetic chemical preservatives in food. Further research is needed to explore more effective methods to extract functional compounds from pomegranate peels. Meanwhile, the regulation regarding the use of plant polyphenols as natural preservatives in food should be established to warrant their safe application.

Data availability statement

Data will be available upon request.

CRediT authorship contribution statement

Weifan Wu: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Kevin Mis Solval:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Jinru Chen:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

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