Antioxidant Assay-Guided Purification and LC Determination of Ellagic Acid in Pomegranate Peel

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Abstract

On the basis of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay-guided purification, ellagic acid was isolated from the methanol extract of pomegranate fruit peel by liquid–liquid extraction and chromatographic techniques. A reversed-phase high-performance liquid chromatography was described for determination of ellagic acid in pomegranate fruit peel extract. The method involved the use of a TSK-gel ODS-80Tm column with a mixture of 2% aqueous acetic acid and methanol (gradient elution mode: 0–15 min, 40–60% v/v methanol and 15–20 min, 60% v/v methanol) as the mobile phase and detection at 254 nm. The parameters of linearity, repeatability, reproducibility, accuracy, and specificity of the method were evaluated. The recovery of the method was 98.5% and linearity ($r^2 > 0.9995$) was obtained for ellagic acid. A high degree of specificity as well as repeatability and reproducibility (relative standard deviation values less than 5%) were also achieved. The limits of detection and quantification were 1.00 and 2.50 µg/mL, respectively. The solvent for extraction of ellagic acid from pomegranate fruit peel was examined in order to maximize the ellagic acid content of the extract. A solution of 10% v/v water in methanol was capable of increasing the ellagic acid content in the extract up to 7.66% w/w. The ellagic acid content and antioxidant activity of the ethyl acetate fraction separated from the crude extract using water and ethyl acetate partition was higher than that of the crude extract.

Introduction

Pomegranate (*Punica granatum L.*) fruits are widely consumed fresh and can be found in commercial products as juice, jam, and wine. Pomegranate fruit husk/peel is a rich source of hydrolyzable tannin belonging to ellagitannins. In the commercial pomegranate juice industry, these ellagitannins are extracted from the husk in significant quantities into the juice due to their hydrophilic properties. Pomegranate fruit peel, a by-product of the pomegranate juice industry, is therefore an inexpensive and abundant source of ellagitannins. Although the fruit peel has also been used in folklore medicine as astringent for treatment of diarrhea and dysentery (1), it has been consumed in very small amounts. Pomegranate extracts are also being investigated for their potential use as natural food preservatives and nutraceuticals (2). It has been reported that the methanol extract of the fruit peel contained 192 mg of total phenolics per g and exhibited antioxidant activity with Trolox equivalent antioxidant capacity (TEAC) values of 394.66 and 316.29 mmol/100 g dry weight, as evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay, respectively (3). The major types of phenolic compounds were reported as hydrolyzable tannin (punicalin, punicalagin, gallagic acid, ellagic acid) and gallic acid. The aqueous and ethyl acetate extracts of the fruit peel also showed antioxidant activity when evaluated by DPPH test, 5-lipoxygenase assay, and chemiluminescence assay (4). Because pomegranate fruit peel extracts are reported to show antioxidant activity, we are interested in the isolation of the antioxidant active compound using bioassay-guided isolation in order to use it as the standard marker for standardization of the extract.

In this study, we report a rapid high-performance liquid chromatography (HPLC) method for quantitative analysis of ellagic acid, a well-known natural antioxidant isolated from pomegranate fruit peel. Although HPLC methods for identification and quantification of ellagic acid are available from published literature (5–7), they were established for the determination of free ellagic acid or ellagic acid in other foodstuffs. In addition, the HPLC quantitative determination of ellagic acid in pomegranate has been developed (8,9). However, the method is time consuming; a total run time of about 45 min is required, and validation of the analytical procedure is not yet established. The solvents for extraction and fractionation that can improve ellagic acid content of the extract are also reported herein.

Experimental

Plant material

Pomegranate fruits were collected from Mengzhi pomegranate garden (Yunnan, China). The fruit peels were dried at 50–60°C in a hot air oven for 24 h and were ground to coarse powder using a grinder.

Chemicals and reagents

Standard ellagic acid and DPPH were purchased from Fluka (Buchs, Switzerland). Quercetin, kaempferol, and β-carotene were purchased from Sigma (Buchs, Switzerland). HPLC-grade and analytical-grade methanol was purchased from Labscan Asia (Bangkok, Thailand). Acetic acid was from J.T. Baker (Phillipsburg, NJ). Water was purified in a Millipore Milli-Q system (Bedford, MA).

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Extraction and purification of the antioxidant constituent

The dried powder of pomegranate fruit peels (500 g) was macerated with methanol for 3 days (1 L × 3). The methanol extracts were filtered and evaporated to dryness under reduced pressure. The extract (40 g) was then suspended in water (500 mL) and partitioned with ethyl acetate (500 mL × 3). The pooled ethyl acetate fractions were evaporated to dryness under reduced pressure. The ethyl acetate fraction of pomegranate fruit peels was subjected to silica gel vacuum chromatography. A sintered glass column, 13 cm in diameter, was packed with silica gel No. 9385, approximately 6 cm high. The ethyl acetate fraction (20 g), which pre-adsorbed on silica gel, was loaded on the surface of the silica gel column and eluted with chloroform–methanol (9:1). The fractions (200 mL) were collected. The fractions were then subjected to DPPH radical scavenging assay. The pooled antioxidant active fractions (fraction 11) were further purified by Sephadex LH-20 column (3.5 × 80 cm) chromatography eluted by methanol. The fractions (30 mL) were collected. The fractions were then subjected to DPPH radical scavenging assay. White colored needles (TP1) were obtained from the pooled antioxidant active fractions (fraction 3).

Determination of antioxidant activity

The antioxidant activities of the extracts and fractions were determined according to DPPH radical scavenging assay (10) and β-carotene bleaching test (11).

DPPH radical scavenging assay

The stock solution (1 mg/mL) of the sample was prepared in absolute ethanol. Five concentrations of the sample were produced by twofold dilutions. A portion of the sample solution (0.1 mL) was mixed with the same volume of 6×10^{-5} M DPPH in absolute ethanol. After the mixture had been allowed to stand for 30 min at room temperature, its absorbance was measured at 520 nm using a spectrophotometer (Genesis 5 Model, Miltonroy, Ivyland, PA). The scavenging activity of the sample against DPPH radical was calculated according to the following equation: DPPH radical scavenging activity (%) = (1 – absorbance of sample/absorbance of control) × 100. A mixture of absolute ethanol (500 µL) and 6×10^{-5} M DPPH in absolute ethanol (500 µL) was used as the control. Dose response curve was plotted between % inhibition and concentrations. Linear regression analysis was carried out calculating the effective concentration of the sample required to scavenge DPPH radical by 50% (EC_{50}). All tests were carried out in triplicate.

β-carotene bleaching test

Approximately 10 mg of β-carotene was dissolved in 10 mL of chloroform. The carotene-chloroform solution (0.2 mL) was transferred into a boiling flask containing 20 mg linoleic acid and 200 mg Tween 40. Chloroform was removed using a rotary evaporator at 40°C for 5 min, and to the residue 50 mL of distilled water was slowly added with vigorous agitation to form an emulsion. Five mL of the emulsion was added to a tube containing 0.2 mL of sample solution, and the absorbance was immediately measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The tubes were placed in a water bath at 50°C and monitored spectrophotometrically by measuring absorbance at 470 nm over a 60-min period. Control samples contained 10 µL of water instead of sample solution. The antioxidant activity was expressed as inhibition percentage with reference to the control after a 60 min incubation using the following equation: AA = 100(DRC–DRS)/DRC, where AA = antioxidant activity; DRC = degradation rate of the control = (ln(*a*/*b*)/60); DRS = degradation rate in presence of the sample = (ln(*a*/*b*)/60); *a* = absorbance at time 0; *b* = absorbance at 60 min. All tests were performed in triplicate.

HPLC Conditions

HPLC analysis was carried out using Agilent 1100 series equipped with an Agilent 1100 series photodiode array detector (PDA) and autosampler. Data analysis was performed using Agilent 3D ChemStation software (Agilent, Santa Clara, CA). Separation was achieved at 25°C on a 150 mm × 4.6 mm i.d. TSK-gel ODS-80Tm column. The mobile phase consisted of methanol and 2% aqueous acetic acid, gradient mode (0–15 min, 40–60% v/v methanol and 15–20 min, 60% v/v methanol) with a flow rate of 1 mL/min. The injection volume was 20 µL. The quantitation wavelength was set at 254 nm.

HPLC method validation

Sample preparation

Pomegranate fruit peel powder (100 mg) was extracted with methanol (20 mL) under reflux conditions for 1 h. The extract was then filtered and concentrated under reduced pressure. The sample was reconstituted and adjusted to 10 mL with methanol. Samples were analyzed immediately after extraction in order to avoid possible chemical degradation. The experiments were carried out in triplicate.

Calibration curve

A stock solution of the reference standard, ellagic acid was made in methanol and subsequently diluted to provide a series of the standard ranging from 3–50 µg/mL for use in constructing calibration curve of ellagic acid. Calibration curve was constructed on three consecutive days by analysis of the solution of standard ellagic acid at five concentrations and plotting peak area against the concentration of the reference standard. The linearity of the detector response for the standard was determined by means of linear regression.

Accuracy

Sample portions were fortified with known quantities of the standard ellagic acid in order to assay accuracy data. Prior to standard ellagic acid fortification, the background levels of ellagic acid in pomegranate fruit peel extracts were determined so as to calculate actual recoveries. The amount of ellagic acid was determined in triplicate, and percentage recoveries were then calculated.

Precision

Precision experiments were conducted for intra-day and inter-day. The solution of one sample was used to achieve repeatability testing. The data of repeatability was the content of six injections separately in the same day. The data used to calculate percent relative standard deviation (%RSD) of inter-day precision was the

content of three samples analyzed in three days (three injections in succession each day).

Specificity

Peak identification was carried out using the standards and diode array detector. The UV spectra were taken at various points of the peaks to check peak homogeneity.

Limits of detection and quantification

The limits of detection and quantification were determined by means of serial dilution based on signal-to-noise ratios of 3:1 and 10:1, respectively.

Determination of solvent for extraction and fractionation

The dried powder (100 mg) was extracted with various solvents (20 mL × 2), including ethyl acetate, methanol, 5, 10, 15, and 20% v/v water in methanol under reflux conditions for 1 h. After filtration, the pooled extracts of the same solvent were concentrated under reduced pressure, adjusted to 10 mL with methanol and subjected to HPLC analysis.

Table I. Antioxidant Activity of the Fractions Isolated by Silica Gel Vacuum Chromatography

Fraction	EC_{50} (µg/mL)*	Fraction	EC_{50} (µg/mL)*
Fraction 1	> 100.0	Fraction 8	7.8 ± 0.10
Fraction 2	> 100.0	Fraction 9	5.7 ± 0.04
Fraction 3	> 100.0	Fraction 10	11.0 ± 0.12
Fraction 4	> 100.0	Fraction 11	1.8 ± 0.02
Fraction 5	37.6 ± 2.58	Fraction 12	6.8 ± 0.13
Fraction 6	65.0 ± 1.49	Quercetin	8.3 ± 0.23
Fraction 7	12.7 ± 0.22		

* Evaluated by DPPH radical scavenging assay.

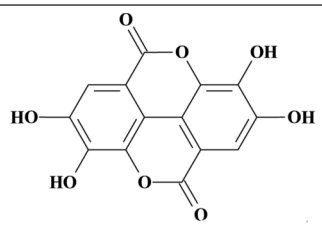

Figure 1. Structure of ellagic acid.

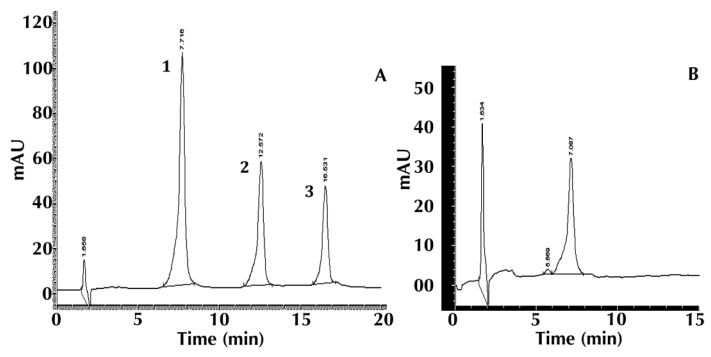

Figure 2. HPLC-chromatograms of (A) authentics: 1, ellagic acid; 2, quercetin; and 3, kaempferol; (B) methanol extract of pomegranate fruit peel.

Fractionation of the extract was performed using liquid–liquid extraction between water and organic solvents including ethyl acetate, *n*-butanol, and a mixture of ethyl acetate and *n*-butanol (1:1). The organic phase was concentrated under reduced pressure and adjusted to 10 mL with methanol and subjected to HPLC analysis. The extracts were also evaluated for antioxidant activity by DPPH radical scavenging assay. All experiments were carried out in triplicate.

Results and Discussion

DPPH radical scavenging assay of the ethyl acetate extract of pomegranate fruit peel showed that it possessed a strong antioxidant activity with EC_{50} value of 5.8 µg/mL. Further purification of the ethyl acetate extract using a silica gel vacuum chromatography gave 12 fractions of the isolate. Evaluation of the antioxidant activity of each fraction showed that the fraction 11 gave an antioxidant activity (EC_{50} 1.8 µg/mL) that was not only stronger than other fractions but also stronger than standard quercetin (EC_{50} 2.5 µg/mL) (Table I). Using Sephadex LH-20 gel filtration chromatography, we further purified the fraction 11 to produce the highest antioxidant fraction with the white colored needles (TP1) obtained. TP1 was identified as ellagic acid (Figure 1) by comparison of the spectroscopic data, including ^{1}H NMR and MS with previous report (12). On the basis of DPPH radical scavenging assay and β-carotene bleaching test, ellagic acid exhibited stronger antioxidant activity than standard quercetin with the EC_{50} values of 2.1 ± 0.02 and 4.3 ± 1.21 µM, respectively, while those of the standard quercetin were 8.3 ± 0.23 and 4.4 ± 0.77 µM, respectively. These data further implicate pomegranate fruit peel extract as a potential antioxidant agent for cosmetic and nutraceutical applications. In addition, ellagic acid should be considered as an indicative marker for standardization of the fruit peel extract.

It has been reported that the antioxidant compounds found in pomegranate fruit peel extract were phenolic compound: ellagic acid and flavonol as well as quercetin and kaempferol (13). We, therefore, examined the optimal conditions for the simultaneous quantitative determination of ellagic acid, quercetin, and kaempferol in the fruit peel extract using gradient reversed-phase HPLC system. As the compounds have good absorption at 254 nm, this wavelength was used for quantitation. Mixture of methanol and 2% aqueous acetic acid was examined as the mobile phase, and the ratios as well as gradient elution system were optimized. The result indicated that a mixture of methanol and 2% aqueous acetic acid, gradient mode (0–15 min, 40–60% v/v methanol and 15–20 min, 60% v/v methanol) could separate all components in less than 20 min with satisfactory peak resolution. Ellagic acid, quercetin, and kaempferol were eluted with the retention time of 7.7, 12.6, and 16.5 min, respectively (Figure 2). This HPLC method is simple and rapid. The previously reported HPLC method takes a longer time. A total run time of about 45 min is required, and validation of the analytical procedure is not yet established (8,9). On the basis of the HPLC analysis, only ellagic acid was found as the major

constituent in the methanol extract of pomegranate fruit peel (Figure 2). Neither quercetin nor kaempferol were found. This implies that quercetin and kaempferol are rarely found in the pomegranate fruit peel extract.

Defining the linearity, accuracy, intra-day and inter-day precision, specificity, and limits of detection and quantitation validated the HPLC method. Linearity was evaluated using standard ellagic acid over five calibration points (3.0–50.0 µg/mL) with six measurements for each calibration point. Ellagic acid exhibited linearity over the evaluated ranges with a linear equation of $y = 139159x + 26.146$ ($r^2 = 0.9995$).

Intra-day precision was estimated by the RSD of six measurements for ellagic acid. Analysis of three independently prepared samples of pomegranate fruit peel extracts determined the inter-day precision. The RSD values for both intra-day (1.36%) and inter-day (4.40%) analysis of ellagic acid were less than 5%.

Method accuracy was determined by analyzing pomegranate fruit peel extracts fortified with known quantities of the standard analyte. Recoveries in the range of 98.4 ± 1.36% were observed for ellagic acid. Utilizing the PDA makes it possible to obtain the UV spectra. Specificity of the method was evaluated using UV-absorption spectra produced by diode array detector. The spectra were taken at three points of the peak of ellagic acid. When it was compared with the standard ellagic acid, homogeneity for spectra of the peak was observed. Finally, it was found that the HPLC method was very sensitive for ellagic acid with limit of detection and limit of quantitation at 1.00 and 2.50 µg/mL, respectively. Although limit of detection of ellagic acid has been reported at 0.1 µg/mL (7), it was the HPLC method developed for quantitative determination of the pure compound.

A few different extraction solvents were examined to maximize the ellagic acid content in pomegranate fruit peel extract. With regards to the polarity of ellagic acid, combinations of water and methanol were examined as the extraction solvent. The results showed that 10% v/v water in methanol gave significantly higher ellagic acid content than those of the other extraction solvents (Table II). The extract was further purified by partitioning between water and a few organic solvents including ethyl acetate, *n*-butanol, and a mixture of *n*-butanol and ethyl acetate (1:1). It was found that the ethyl acetate fraction showed the highest ellagic acid content as well as the antioxidant activity (Table III). Increasing ellagic acid content resulted in an increase in the antioxidant activity of the extract. Partitioning between water and ethyl acetate was, therefore, an appropriate method for the preparation of the high antioxidant potency extract of pomegranate fruit peel.

Table II. Ellagic Acid Content in Pomegranate Peel Extracted Under Reflux with Various Concentration of Water in Methanol

Water concentration (% v/v)	Ellagic acid content Mean (% w/w) ± SD
0	5.84 ± 0.031*
5	7.11 ± 0.174*
10	7.66 ± 0.006
15	6.82 ± 0.379*
20	6.18 ± 0.617*

* Significant difference ($P < 0.05$) when compared with 10% v/v water.

Table III. Ellagic Acid Content and Antioxidant Activity in Pomegranate Fruit Peel Extracts, Which were Partitioned Between Water And Various Organic Solvents

Extract/fraction	Ellagic acid content Mean (%w/w) ± SD	Antioxidant activity* EC_{50} (µg/mL)
Methanol extract	7.06 ± 0.025†	38.21 ± 0.144†
Ethyl acetate fraction	9.48 ± 0.065	15.17 ± 0.398
n-Butanol fraction	8.16 ± 0.014†	22.16 ± 0.512†
Ethyl acetate and *n*-butanol fraction	8.57 ± 0.063†	18.11 ± 0.497†

* Evaluated by DPPH radical scavenging assay.
† Significant difference ($P < 0.05$) when compared with ethyl acetate fraction within the same column.

Conclusion

A simple, specific, precise, accurate, rapid, and reproducible HPLC method has been established to quantify the active principle ellagic acid in pomegranate fruit peel. The solution of 10% v/v water in methanol is the suitable extraction solvent of ellagic acid from pomegranate fruit peel. Fractionation of the extract through partitioning between water and ethyl acetate significantly improves ellagic acid content and antioxidant property of pomegranate fruit peel extract.

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