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RESEARCH ARTICLE

Preparation method and stability of ellagic acid-rich pomegranate fruit peel extract

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Abstract

A simple one-step purification using liquid–liquid extraction for preparing pomegranate peel extract rich in ellagic acid has been demonstrated. The method involved partitioning of the 10% v/v water in methanol extract of pomegranate peel between ethyl acetate and 2% aqueous acetic acid. This method was capable of increasing the ellagic acid content of the extract from 7.06% to 13.63% w/w. Moreover, the antioxidant activity of the extract evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was also increased (ED_{50} from 38.21 to 14.91 µg/mL). Stability evaluations of the ellagic acid-rich pomegranate peel extract in several conditions through a period of four months found that the extracts were stable either kept under light or protected from light. The extracts were also stable under 4° ± 2°C, 30° ± 2°C and accelerated conditions at 45°C with 75% relative humidity. However, study on the effect of pH on stability of the extract in the form of solution revealed that the extract was not stable in all tested pH (5.5, 7 and 8). These results indicated that the ellagic acid-rich pomegranate peel extract was stable when it was kept as dried powder, but it was not stable in any aqueous solution.

Keywords: Punica granatum*; pomegranate; ellagic acid; stability; antioxidant*

Introduction

Pomegranate [*Punica granatum* L. (Punicaceae)] fruit peel, a by-product of the pomegranate juice industry, is a rich source of hydrolysable tannin belonging to ellagitannins. It is therefore an inexpensive and abundant source of ellagitannins. A wide range of clinical applications of pomegranate fruit peel extract for the treatment and prevention of cancer, as well as other diseases where chronic inflammation is believed to play an essential etiologic role, is suggested (Lansky & Newman, 2007). The reports have revealed that pomegranate fruit peel extract is a potential antioxidant agent for nutraceutical and cosmetic applications (Aguilar et al., 2008). Ellagic acid is considered as an indicative marker for standardization of the fruit peel extract (Zhou et al., 2008) and as a biomarker for human bioavailability studies involving consumption of ellagitannins from food sources (Seeram et al., 2004). In addition, ellagic acid also has the benefit of being anti-inflammatory (Ogawa et al., 2002), anti-allergy (Rogerio et al., 2008), anti-cancer (Larrosa et al., 2006; Mertens-Talcott & Percival, 2005), reducing microbial growth (Zambuchini et al., 2008), inhibiting many toxic effects caused by phospholipase A_2 (Da Silva et al., 2008) and a protective effect against testicular toxicity (Turk et al., 2008). Thus, a method for ellagic acid enrichment in pomegranate fruit peel extract is needed. As part of our interest in a simple purification method that can improve the ellagic acid content in pomegranate fruit peel extract, liquid–liquid extraction method was used to prepare ellagic acid-rich pomegranate fruit peel extract. Stability of the extract was also studied in order to get useful information for future studies on development of herbal products from the extract.

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Materials and methods

Plant material

Pomegranate fruits were collected from Mengzhi pomegranate garden, Yunnan, China, in August 2006. The voucher specimen (no. SKP 158 16 07 01) was identified by Pharkphoom Panichayupakaranant and deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The fruit peels were dried at 50°C for 24 h in a hot air oven and were reduced to powder using a grinder and a no. 45 sieve.

Chemicals and reagents

Standard ellagic acid and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Fluka (Buchs, Switzerland). Methanol (High Performance Liquid Chromatography (HPLC) grade and analytical grade) and ethyl acetate (analytical grade) were purchased from Labscan Asia (Bangkok, Thailand). Acetic acid was from J.T. Baker (Phillipsburg, NJ, USA). Water was purified in a Milli-Q system (Millipore, Bedford, MA).

Preparation of ellagic acid-rich pomegranate fruit peel extract

The dried powder of pomegranate fruit peel (0.5 kg) was extracted with methanol containing 10% (v/v) water (2 L) under reflux conditions for 1 h (×2). The pooled extracts were dried *in vacuo*. The extract (4 g) was then suspended in 2% aqueous acetic acid (400 mL) and partitioned with ethyl acetate (400 mL × 4). The pooled ethyl acetate fractions were then evaporated to dryness *in vacuo*.

Quantitative analysis of ellagic acid

HPLC conditions and calibration curves

HPLC analysis was carried out using Agilent 1100 series equipped with Photodiode-array detector (PDA) and autosampler. Data analysis was performed using Agilent 3D ChemStation software (Agilent, Santa Clara, CA, USA). Separation was achieved at 25°C on a 150 mm × 4.6 mm TSK-gel ODS-80Tm column. The mobile phase consisted of methanol and 2% aqueous acetic acid with gradient mode elution (0-15 min, 40-60% v/v methanol and 15-20 min, 60% v/v methanol) at a flow rate of 1 mL/min. The injection volume was 20 µL. The quantitation wavelength was set at 254 nm. The calibration curve was established from the standard ellagic acid at a concentration between 3-50 µg/mL. The linear equation of $Y = 139159X + 26.146$ ($r^2 = 0.9995$) corresponds to ellagic acid.

Sample preparation

The extracts were accurately weighed to 5 mg and diluted to 10 mL volumetric flask with methanol. The solutions were filtered through 0.45 µm membrane filter and subjected to HPLC analysis.

In vitro antioxidant assay

The antioxidant activity of the extracts and fractions was determined according to DPPH radical scavenging assay (Yamasaki et al., 1994). Briefly, the stock solution (1 mg/mL) of the sample was prepared in absolute ethanol. Five concentrations of the sample were produced by two-fold 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 (Milton Roy Spectronic Genesys 5, Champaign IL, USA). The scavenging activity of the sample against DPPH radicals 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. Te 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}). Ellagic acid and quercetin (Sigma, St. Louis, MO, USA) were used as the positive control. All tests were carried out in triplicate.

Stability evaluation

Effect of light on stability of the extract

The ellagic acid-rich pomegranate fruit peel extracts were weighed to 100 mg and kept in well-closed containers. The extracts were then stored at room temperature (30° ± 2°C) either protected from light or exposed to light for 4 months. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 17 weeks and subjected to quantitative analysis of the ellagic acid content using HPLC. The experiments were done in triplicate.

Effect of temperature on stability of the extract

The ellagic acid-rich pomegranate fruit peel extracts were weighed to 100 mg and kept in well-closed containers and protected from light. The extracts were then stored at 4° ± 2°C and at room temperature (30° ± 2°C) for 4 months. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 17 weeks and subjected to quantitative analysis of the ellagic acid content using HPLC. The experiments were done in triplicate.

Effect of accelerated conditions for stability of the extract

The ellagic acid-rich pomegranate fruit peel extracts were divided into aliquots of 100 mg and were kept in well-closed containers and protected from light. The extracts were then stored in a stability chamber at 45°C, 75% humidity for 4 months. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 17 weeks and subjected to quantitative analysis of the ellagic acid content using HPLC. The experiments were done in triplicate.

Effect of pH on stability of the extract

The ellagic acid-rich pomegranate fruit peel extracts were accurately weighed into aliquots of 100 mg and dissolved in phosphate buffer solution to achieve pH value of 5.5, 7, and 8. The sample solutions were kept in well-closed containers and protected from light and stored at room temperature (30° ± 2°C) for 4 months. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 17 weeks and subjected to quantitative analysis of the ellagic acid content using HPLC. The experiments were done in triplicate.

Statistics

Values are expressed as mean ± SD. Data were analyzed by student *t*-test. The level of statistical significance was taken as $P < 0.05$.

Results and discussion

Preparation of ellagic acid-rich pomegranate fruit peel extract

After fractionation of the pomegranate fruit peel extract using liquid–liquid extraction between ethyl acetate and 2% aqueous acetic acid, the ellagic acid-rich extract was obtained from the ethyl acetate fraction with a yield of 16.56 ± 1.132% w/w compared to the weight of dried powder (Table 1). The appearance of the extract was changed from a dark brown semisolid of the crude extract to a brown powder of the ellagic acid-rich extract. On the basis of HPLC analysis, only ellagic acid was found as the major compound in the extract (Figure 1A). This method was capable of increasing the ellagic acid content in the extracts from 7.06 ± 0.025% to 13.63 ± 0.89% w/w as well as the antioxidant activity (Table 1). Preliminary studies on fractionation of the pomegranate fruit peel extract found that liquid–liquid extraction between ethyl acetate and water was an appropriate method for a preparation of the high antioxidant potency extract of pomegranate fruit peel. However, the result from this study indicated that the use of 2% aqueous acetic acid instead of water in the liquid–liquid extraction process produced an extract with a higher ellagic acid content and antioxidant activity (Table 2). This is due to a suppression of ionization of ellagic acid in aqueous acidic conditions that was capable of decreasing the water solubility of ellagic acid. Hence it is more soluble in ethyl acetate. According to the satisfactory antioxidant activity of the ellagic acid-rich pomegranate extract, the extracts used in further studies were standardized to an ellagic acid content not less than 13% w/w.

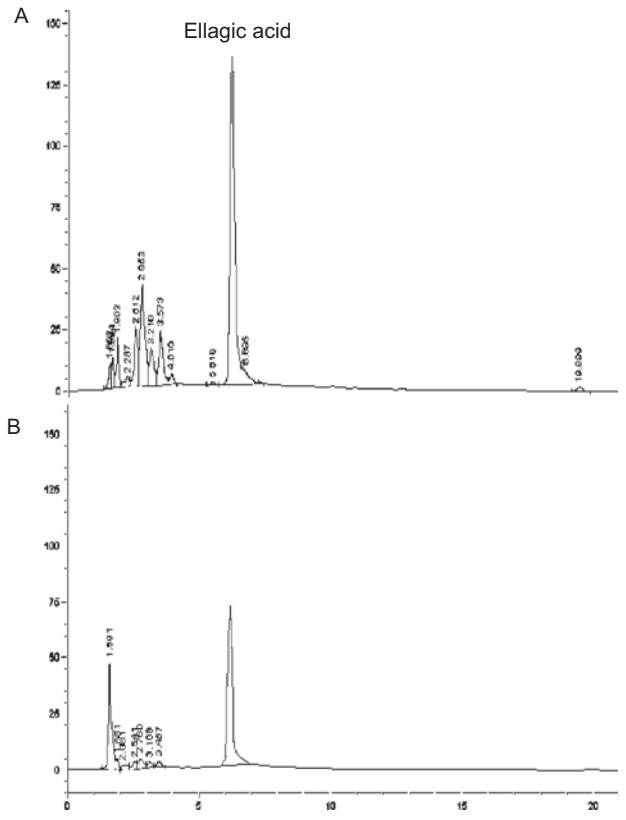

Figure 1. HPLC-chromatograms of the solution of ellagic acid-rich pomegranate extract (pH 8) at the initial time (A) and after keeping for 10 weeks (B).

Table 1. Extraction yield, ellagic acid content and antioxidant activity of the ellagic acid-rich pomegranate fruit peel extract.

Extracts/ compounds	Extraction yield (% w/w*)	Ellagic acid content (%w/w**)	Antioxidant activity† ED_{50} (μg/mL)
Crude extract	32.51 ± 2.14	7.06 ± 0.025	38.21 ± 0.138
Ellagic acid-rich extract	16.56 ± 1.132	13.63 ± 0.891#	14.91 ± 0.239#
Standard quercetin	-	-	3.57 ± 0.637
Standard ellagic acid	-	-	3.12 ± 0.349

*Compared to the weight of dried powder.
**Compared to the weight of the extract.
†Evaluated by DPPH radical scavenging assay.
#Significant difference ($P < 0.05$) when compared with the crude extract.

Stability evaluation

Ellagic acid was used as the marker for monitoring the chemical stability of the ellagic acid-rich pomegranate fruit peel extract.

Study on the effect of light on the stability of the extract demonstrated that the physical appearances (color and solidity) of the extracts were similar, and were unchanged throughout the period of 4 months. In addition, the ellagic acid contents of the extracts kept in both conditions were not significantly decreased through the period of 4 months. Normally, antioxidant compounds can degrade in light conditions, because the antioxidant compounds are oxidized when they are exposed to light. From this result, light has no effect on the ellagic content of the extracts. This may be due to the other polyphenolic compounds or ellagitannins in the extract that may play an important role as antioxidant for ellagic acid.

Study on the effect of temperature on the stability of the extract showed that both tested temperatures did not affect either the physical appearance of the extracts or ellagic acid content through the period of 4 months. It also implies that the ellagic acid-rich pomegranate fruit peel extract is stable at temperatures between 4°C and 30°C at least in the period of 4 months.

Study of the effect of accelerated conditions on the stability of the extracts showed that their physical appearances as well as the ellagic acid contents of the extracts were unchanged even when stored under accelerated conditions in the period of 4 months. This result implies that the ellagic acid-rich pomegranate fruit peel extract should be stable for at least two years when kept in a well-closed container and stored at room temperature.

The acid-base stability evaluation of the ellagic acid-rich pomegranate fruit peel extract in the solution was determined at three different pH including 5.5, 7, and 8. It was found that at pH 5.5 the solution was yellow, at pH 7 the solution was brownish-yellow and at pH 8 the solution was brown. Unfortunately, in all tested pH, the ellagic acid content of the extract was significantly

Table 2. Ellagic acid content and antioxidant activity of the ethyl acetate fractions obtained from liquid-liquid extractions.

Solvents for liquid–liquid extraction	Ellagic acid content (%w/w, mean ± SD)	Antioxidant activity†, ED_{50} (μg/mL, mean ± SD)
Ethyl acetate and water	9.24 ± 0.511	16.31 ± 0.722
Ethyl acetate and 2% aq. acetic acid	13.63 ± 0.891*	14.91 ± 0.239*

†Evaluated by DPPH radical scavenging assay.
*Significant difference ($P < 0.05$).

Table 3. Ellagic acid content of the ellagic acid-rich pomegranate fruit peel extract in the solution at pH 5.5, 7 and 8.

	Ellagic acid content (% w/w, mean ± SD)					
Weeks	pH 5.5	% Remaining	pH 7	% Remaining	pH 8	% Remaining
0	12.81 ± 0.669	100	12.81 ± 0.673	100	12.81 ± 0.671	100
1	12.82 ± 0.408	100.08	12.68 ± 0.533	98.99	12.59 ± 0.809	98.28
2	12.71 ± 0.189	99.22	12.76 ± 0.614	99.61	12.68 ± 0.513	98.98
3	12.59 ± 0.808	98.28	12.62 ± 0.523	98.52	12.76 ± 0.582	99.61
4	11.02 ± 0.321*	86.03	12.02 ± 0.838*	93.83	11.92 ± 0.367*	93.05
6	11.89 ± 0.512*	92.82	11.15 ± 0.789*	87.04	10.03 ± 0.177*	78.3
8	10.41 ± 0.484*	81.26	9.77 ± 0.4111*	76.27	9.31 ± 0.432*	72.68
10	7.01 ± 0.314*	54.72	8.21 ± 0.573*	64.09	7.29 ± 0.514*	56.91
12	4.21 ± 0.332*	32.86	7.66 ± 0.728*	59.8	3.93 ± 0.831*	30.68
14	4.09 ± 0.708*	31.93	7.65 ± 0.424*	59.72	3.17 ± 0.518*	24.75
17	2.69 ± 0.361*	21	3.29 ± 0.253*	25.68	2.57 ± 0.447*	20.06

* Significance at $P < 0.05$ when compared with the content at the initial time.

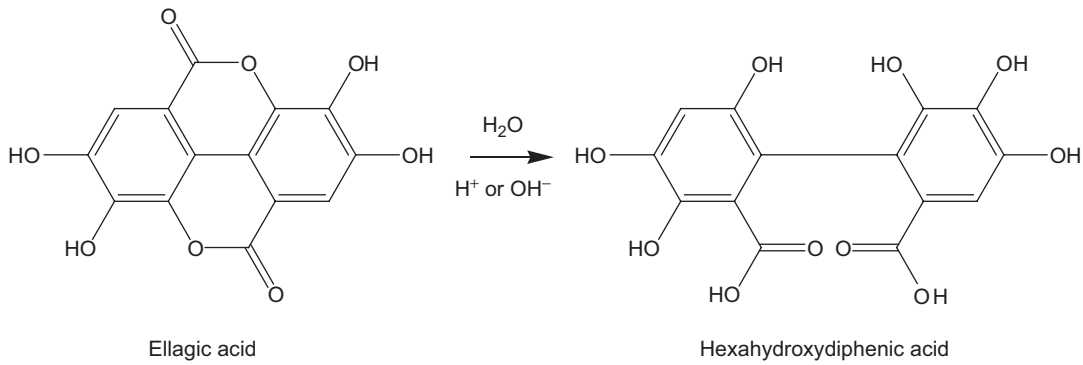

Figure 2. Hydrolysis of ellagic acid to hexahydroxydiphenic acid.

decreased after keeping for four weeks (Table 3). The remaining percentage of ellagic acid in the extract after 4 weeks of storage was significantly lower than the initial time. In these conditions, HPLC chromatograms showed the main peak of the degraded polar compound with the retention time of 1.6 min (Figure 1B). The instability of ellagic acid in the solution should be considered as their hydrolysis of the ellagic acid (Figure 2). The ester group of ellagic acid was hydrolyzed to hexahydroxyphenic acid in aqueous, acid and base solution. Thus, application of the ellagic acid-rich pomegranate fruit peel extract in an aqueous solution should be performed carefully.

The results from these stability tests indicate that the ellagic acid-rich pomegranate fruit peel extract possesses satisfactory stability for further development of herbal medicines. However, an application as aqueous solution should be avoided.

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