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Rapid Separation Method of Ursolic Acid from Pomegranate (*Punica granatum* L.) Flowers using Normal-Phase Flash Chromatography

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Abstract

Ursolic acid (UA) is a major bioactive component in many traditional medicinal plants including pomegranate (*Punica granatum* L.) flower. In vitro and in vivo studies have both revealed that UA has many important biological functions.In this study, a rapid and efficient procedure for preparation of UA from an Iranian pomegranate flower cultivar using ultrasound-assisted extraction followed by normal-phase flash chromatography (NP-FC) was developed. After one flash chromatography run, the purity of UA reached more than 97% with a total yield of 0.1% of dried pomegranate flower powder. The chemical structure of isolated UA was identified by IR, MS and NMR spectra. This is the first report on isolation of UA from pomegranate flowers. The NP-FC method was shown to be a good method for the purification of UA from pomegranate flowers, and it can be an excellent basis for large-scale preparation of UA from pomegranate flowers or other plant extracts.

Keywords: Ursolic acid, Isolation, Pomegranate flowers, Flash chromatography.

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Introduction

Pomegranate (*Punica granatum* L.), a deciduous shrub or small tree, is native to Iran and Afghanistan [1]. Apart from its edible part, its seeds, peels, and flowers all have good health and medicinal values, and they have been widely used for the prevention and treatment of a wide range of diseases in traditional medicine [2]. The pomegranate flowers were reported as a haemostatic, astringent and as a remedy for diabetes in Unani and Ayurvedic medicine [3]. These flowers were also used to treat chronic diarrhea, especially in children, and injuries from falls and graying hair in traditional Chinese medicine [4].They had a medicinal use in traditional Iranian medicine as well as in current studies [3,5-6]. Pomegranate flowers have been reported to improve insulin resistance [7], to inhibit postprandial hyperglycemia [5], to attenuate atherosclerosis [8], and to diminish cardiac fibrosis [9] in animal models. Ursolic acid and oleanolic acid, active components contained in pomegranate flowers have long been recognized to have antihyperlipidemic properties [10]. Wang et al. (2006) reported the chemical composition of pomegranate flowers [11].

Pomegranate flowers contain multiple secondary metabolites, the most abundant of which are polyphenols such as gallic acid, ellagic acid and ethyl brevifolin-carboxylate; followed by triterpenes including ursolic, oleanolic, maslinic and asiatic acids [12]. Ursolic acid (UA, 3β-hydroxy-12-urs-12-ene-28-oic acid) (Figure 1), which is a natural pentacyclic triterpenoid carboxylic acid, was considered biologically inactive for ages [13-14], while it has recently attracted considerable interest for its pharmacological effects with low toxicity [15]. In vitro and in vivo studies have both revealed that UA has many important biological functions such as immunomodulatory [16], hepatoprotective [17-18], anti-inflammatory [19-20], antitumor [21], antidiabetic [22-23], antibacterial [24-25], antiviral [26-27], antiulcer [28] and anticancer [29] activities. UA has also attracted much attention because of its multifunctional anticancer activities [29-30]. Anti-inflammatory and anti-angiogenic, anti-proliferative, anti-metastatic and proapoptotic abilities of UA have been reported in both in vitro and in vivo models of cancer [29,31]. Although the separation and isolation of natural products from plant material is challenging due to the compositional complexity, considering the potential use of UA, it is necessary to develop a simple, rapid and efficient procedure for its preparation separation.

Several methods including silica gel column chromatography (SGCC) [32-33], and high speed counter current chromatography (HSCCC) [34] have been developed for the separation and isolation of UA from plant extracts. Silica gel column chromatography is time-consuming and laborious, and requires large volumes of solvents. Frighettoa and his co-workers have successfully isolated UA from the extracts of apple peels by HSCCC [34]. Nevertheless, its application in large-scale preparation is limited by its high cost, limited flow rate and lower sample load. Flash

chromatography provides a fast, economic and efficient separation technique for the separation of natural products from plant extracts [35-36]. It can support the relatively high flow rate with low pressure, offering good separation in a short time under appropriate chromatographic conditions. In the previous study; we evaluated the variation of UA content in ten cultivars of pomegranate flower grown in Iran. Among the ten pomegranate flower cultivars researched, eight cultivars were characterized by high content of UA and can be used as UA rich sources [37].This study aims to provide a rapid, simple and efficient method for the extraction, separation and isolation of UA, a highly useful natural product for the development of a wide range of novel and bioactive compounds, from pomegranate flowers using normal–phase flash chromatography.

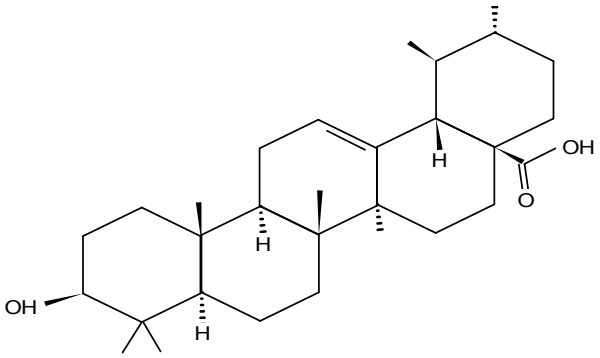

Figure 1. Structureof Ursolic acid (UA, 3β-hydroxy-12-urs-12-ene-28-oic acid).

Experimental

Chemicals

Ursolic acid standard (European Pharmacopoeia Reference Standard) was obtained from Sigma-Aldrich (Milwaukee, WI, USA). Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany) and pure water was used for HPLC analysis. The solvents used for extraction and isolation were analytical grade, and purchased from Merck (Darmstadt, Germany).Silica gel (particle sizes 40-63μm, 63-200μm) was purchased from Merck (Darmstadt, Germany).

Plant materials

Pomegranate flowers of Shirin Siyah Saveh cultivar (one of the cultivars with high levels of UA [37]) were collected from Pomegranate trees, which identified originally by a group of botanists, cultivated in the Pomegranate Genotypes Resources Collection (PGRC) subordinate to AREEO (Agricultural Research, Education, and Extension Organization, Ministry of Agriculture) in Isfahan Province, Iran in May 2019. The perfect flowers were dried at 105°C for 15 min, and then at 65°C for 2 days in a hot-air oven. The powdered pomegranate flowers were kept at 4°C until use [38].

Selection of solvent for extraction of UA

To determine the ideal solvent, the extraction of UA from pomegranate flowers was performed by different solvents including ethanol (90%), ethyl acetate and ethyl acetate followed by 90% ethanol. Pomegranate flower powder (1 g)was dissolved in 20mL solvent followed by 50min ultrasonic extraction at 40°C by MH S3 ultrasonic machine (Soltec Co. Milan, Italy) [13, 38]. Each solvent extraction was repeated three times, and the extracts were passed through a 0.45µm membrane filter For HPLC analysis.

High-performance liquid chromatography system

An SY-8100 system (Tianjin, Beijing, China) equipped with SY-8100 HPLC pump, a 7725i manual sample injector, a variable-wavelength UV detector and SY-8000 HPLC software was used for quantification of UA. The analytical column used was Venusil MP C18 (250mm×4.6 mm, 5 µm), and the isocratic mobile phase was composed of methanol and 0.1 M Phosphate buffer (pH=3, 90:10). The flow rate was 0.9 mL min^{-1}, the eluted material was monitored at 210 nm, and the temperature of the column was kept constant at 21±1°C [39].The peaks related to UA were identified by its retention time and co-injection test with standard compound. The UA concentration for all samples was calculated using the peak area based on the calibration curve.

Extraction of UA from pomegranate flowers

The powder of pomegranate flowers (60g) was ultrasonically extracted with 1200mL 90% ethanol for 50min at 40°C, and then it was filtered. The extract was concentrated under reduced pressure by a rotary evaporator (Rotavapor HS-2005s, Hahnshin, Korea) at 40°C to obtain crude extract (12 g), which was subjected to column chromatography separation on silica gel.

Separation of UA by silica gel column chromatography

Isolation of UA from ethanol extract of pomegranate flowers was done by silica gel column chromatography (SGCC). A glass column of 60×25 mm (I×D) was packed with silica gel (40 g, particle size 63-200µm). After that, the crude extract (12 g) was loaded onto the column and was then eluted with a gradient elution starting from 20% ethyl acetate-hexane to 100% ethyl acetate. Ten fractions with a volume of 75ml were collected and analyzed by HPLC. Fractions containing UA were pooled and concentrated under reduced pressure. Then, the residue was crystallized several times with methanol, and a white amorphous solid was obtained [40].

Separation of UA by normal-phase flash chromatography

Flash column chromatography (FCC) was performed on silica gel (170 g, particle size 40-63µm) in a glass column (300×50 mm (I×D)) designed for FCC. Manually packed flash column was packed using a plug of glass wool and slurry method. After the column was cool to the touch, the crude extract (12 g) was loaded on to the column and the isocratic elution began using dichloromethane: methanol (30:2 V/V) as the mobile phase. The flash chromatographic system was pumped with a continuous flow of nitrogen at a flow rate of 0.5 in. /min. Ten fractions (50 ml) were collected in test tubes and were analysed by HPLC. Fractions containing UA were pooled and concentrated under reduced pressure. The residue was crystallized with methanol several times to obtain pure colourless Ursolic acid.

Characterization of UA

The chemical structure of purified Ursolic acid was confirmed by IR, MS and ^{1}H-NMR spectrometry. Infrared (IR) spectra were recorded on a Jasco 4200 FT-IR spectrometer (Japan) using KBr pellets. The purified Ursolic acid was mixed with KBr in a clean glass pestle and compressed to obtain a pellet. The spectra were recorded from 350 to 4000 cm^{-1}. Background spectra were obtained with KBr pellet. JASCO software was used for data processing. Mass spectra were obtained using GC-MS QP5050 Shimadzu (Kyoto, Japan).The injector and ion source temperatures were set at 250°C. The detector voltage was 70 eV and ion current was 300 µA.Identification of purified UA was based on comparison of its mass spectra and retention indices (RIs) with previously published RIs of UA.^{1}H-NMR spectra were recorded on a Bruker Ultrashield 400 MHz (Germany) spectrometer with 400MHz magnet with QNP probe (5mm)to determine the purified Ursolic acid structure. The purified UA was dissolved in CDCl3 (Deuteriated Chloroform) and tetramethylsilane (TMS) was used as an internal standard. Signal multiplicities are represented by:s (singlet), d (doublet), t (triplet), q (quadruplet), dd (double doublet) and m (multiplet). The chemical shifts (δ) are given in parts per million relative to TMS (δ=0.00).

Purity determination

An accurately weighed quantity of UA standard (94%) and isolated UA from pomegranate flowers (after recrystallization) was dissolved in methanol to obtain solutions having a known concentration of 400 µg/ml. The prepared solutions were analyzedby HPLC and their peak areas were compared. The purity of isolated UA was calculated based on this comparison [36].

Statistical Analysis

Statistical analysis was performed with the Statistical Analysis System (SAS Institute Inc., Cary, NC) [41]. A least significant difference (LSD) procedure was used to detect statistical differences. Significance level of $P<0.05$ was used for all statistical analyses.

Results and discussion

Extraction of UA from pomegranate flowers

The effect of different solvents, including 90% ethanol, ethyl acetate and ethyl acetate followed by 90% ethanol,on UA extraction from pomegranate flowers was evaluated. Fu et al. proved that chloroform and ethanol are the most effective solvents for UA extraction from pomegranate flowers [38]. Then, the highest yield of UA was obtained with ethyl acetate and acetone. Since most organic solvents (methanol, chloroform and acetone) are toxic [42], for UA extraction, ethanol and ethyl acetate were selected as solvents. However, Fan et al. in their study reported that the solubility of UA in pure ethanol is higher than that in mixed solvents (ethanol + water) [43], but the existence of some water in ethanol could enhance the process of mass transfer with an increase in solvent polarity; thus, improving the solubilizing capacity of the solvent and increasing the contact surface for solute solvent interaction by efficient swelling in plant materials [44]. Hence, the lower the water content in the mixed solvents, the higher the solubility of UA will be (in our study 90% ethanol).Other extraction parameters were solvent: material ratio, 20:1(ml/g) followed by 50min ultrasonic extraction at 40°C. Ultrasound, on the one hand, can provide mechanical action and cavitation in the extraction process, which is so useful in the breakdown of plant cell walls that the active ingredients are released and dissolved in the extraction solvent. On the other hand, it can accelerate the movement of molecules, causing the extraction solvent and active ingredients to combine with each other quickly.The content of UA in various extractions of pomegranate flowers of Shirin Siyah Saveh cultivar determined by HPLC has been shown in Table 1.

Table 1. Effect of different solvents on the extraction yield of UA.

Solvent	Extraction Yield Of UA(mg/g)
90%Ethanol	20.517 ± 0.855^{c}
Ethyl acetate	21.182 ± 0.314^{c}
Ethyl acetate +90% Ethanol	18.256 ± 0.932^{ab}

Data are means (n = 3) ± standard deviation
Statistically significant:[a]compared with the 90%Ethanol ($P<0.05$); [b] Compared with the Ethyl acetate($P<0.05$); [c] Compared with the Ethyl acetate+90%Ethanol ($P<0.05$).

The results revealed that when 90% Ethanol or Ethyl acetate was used individually as a solvent, higher yields were obtained. Statistically, there were no significant differences in UA yields when 90% ethanol was used, compared to that when ethyl acetate was used as the solvent (P>0.05). Hence 90% ethanol was selected as an optimum solvent for the extraction of UA. Subsequently the powder of pomegranate flowers was ultrasonically extracted with 90% ethanol and concentrated under reduced pressure. The crude extract was used for column chromatography separation on silica gel.

Comparison of silica gel column chromatography and flash chromatography

A comparison of the SGCC and FCC methods was made to determine a rapid and efficient procedure for UA isolation (Table 2).

Table 2. Comparison of silica gel column chromatography and flash chromatography.

Method	Yield of UA (mg)	Purity of UA (%)	Isolation time	Volume of eluent (ml)
SGCC method	29.8±0.414	97.2±0.407	24.16±0.518 h	750±0.337
FCC method	62.0±0.368[a]	97.3±0.210	25±0.355 min[a]	500±0.149[a]

Data are means (n = 3) ± standard deviation.
[a]Statistically significant (P<0.05).

Silica gel column chromatography was traditionally used for the separation of bioactive compounds. The main disadvantages of this method are that it is time-consuming and laborious, and it requires a large amount of solvents. In a preliminary experiment, silica gel column chromatography was used for the separation of UA from pomegranate flowers. 29.8 mg Ursolic acid was obtained by one column chromatography run. The purity of UA was determined by HPLC more than 97%. The time taken for the separation of UA was 24 hrs. However, the SGCC method led to the isolation of UA with high yields and high-purity, but this method was very time-consuming and required a large volume of solvents (750 ml for elution). To save time and solvent, a modified version of column was designed from the literatures, and it was tested. Flash column chromatography (FCC), also known as medium pressure chromatography, was popularized several years ago as an alternative to slow and often inefficient gravity-fed chromatography. It can provide proper separation in a short time under suitable chromatographic conditions with a relatively high

flow rate with low pressure. It is also a fast, inexpensive and efficient separation technique for the isolation of natural products from plant extracts.

It is obvious that a crucial starting point in column chromatography is to find a solvent system which can be used efficiently with good separation for target compounds. TLC is the most suitable approach for selecting mobile phase of silica gel flash chromatography. Several binary solvent systems (ethyl acetate/methanol (30:2 v/v),(30:5 v/v), chloroform/methanol (30:1 v/v),(30:2 v/v), dichloromethane/methanol (30:2 v/v),(30:5 v/v), ethyl acetate/hexane (1:5 v/v),(1:3 v/v),(2:4 v/v),(2:3 v/v),(3:3 v/v), ethanol/hexane (1:3 v/v),(2:3 v/v),(2:4 v/v)) were tested in TLC separation of UA. Detection was done with iodine or ρ-anisaldehyde or vanillin in H_2SO_4, and Retention factor (R_f value) of Ursolic acid standard was used to identify UA in analyzed extracts. The solvent was selected which gave a good separation and moved the desired compound to R_f=0.35 on analytical TLC [45-46]. Better separation of UA on TLC was achieved with dichloromethane/methanol (30:2 v/v) solvent system. In a study Still and co-workers provided a table recommending column diameter, typical fraction size and volume of eluent to separate a given mass of sample by silica gel flash chromatography [45]. They found that one of the most popular grades of silica gel (63-200 μm) gives the poorest resolution, and particle sizes less than 40 μm offer no improvement in resolution in flash chromatographic method. On this basis, a50 mm diameter column was used and it was packed with 40-63 μm silica gel to a depth of 10 in. (170g) and fractions with a volume of 50ml were collected. Under the optimized conditions, 62 mg Ursolic acid (0.1% of dried plant powder) was obtained by one flash chromatography run. The purity of UA was more than 97% by HPLC analysis. In this process, 500 ml of solvent were consumed which were lower than those in SGCC method, and the time taken for the separation of UA was 25 min.

It should be considered that yield and purity are related to time; hence, in order to obtain higher degrees of yield and purity, more time is needed to separate the compound with the column chromatography method. However, using the developed flash chromatography technique, high-purity Ursolic acid was successfully separated from pomegranate flowers. Compared to the SGCC method, this method provides more yields (more than double) with lower solvent consumption and less time and effort. The flash chromatography method has been used before to purify many classes of compounds [36, 47-48], but it has not been reported for Ursolic acid purification to date. The highly pure Ursolic acid, provided by this method, indicates a new technique for refining this valuable triterpenoid.

Confirmation of UA

The chemical structure of isolated UAwas characterized by IR, MS and ^{1}H-NMR spectra and in comparison with literature data. Ursolic acid was obtained as a white amorphous solid with the following properties: IR (KBr, cm^{-1}): 3420 (OH alcohol), 2923 (OH acid), 2850, 1649 (CO), 1465, 1385, 1099, 1038; Mass spectra: m/z: 456, 239, 197, 183, 43; ^{1}HNMR (400MHz, $CDCL_3$): δ5.40 (m, 1H, H-12), δ3.63 (dd, 1H, H-3), δ2.2 (d, 1H, H-18), δ2.15-1.17 (m, 22H), δ0.79-1.12 (m, 21H, 7Me).

The IR spectrum of isolated UA showed characteristic absorption bands of one hydroxyl group at 3420 cm^{-1} and a carbonyl group at 1649 cm^{-1}. The mass spectrum showed a molecular ion at m/z 456 corresponding to a molecular formula C30H48O3. Fragment ion at *m/z* 239 is formed by a retro-Diels-Alder fragmentation between C-9 and C-11, and between C-8 and C-14. Such cleavage is characteristic of pentacyclic triterpenoids having a double bond at C-12 like an urs-12-en skeleton. Other peaks of different fragments were obtained at 197, 183 and 43. The ^{1}H-NMR spectrum displayed signals for seven methyl groups (δ0.79-1.12).Furthermore, the doublet at δ2.2 and the olefinic proton at δ5.40 showed the urs-12-en type in the structure. The doublet of doublets of proton with oxygenated carbon at δ3.63 showed a 3β-hydroxyl functionality.

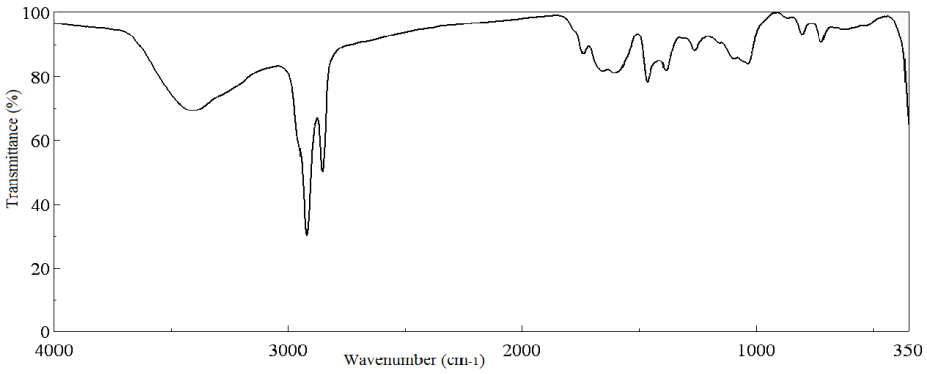

Figure 2.IR spectrum of Ursolic acid isolated from pomegranate flowers.

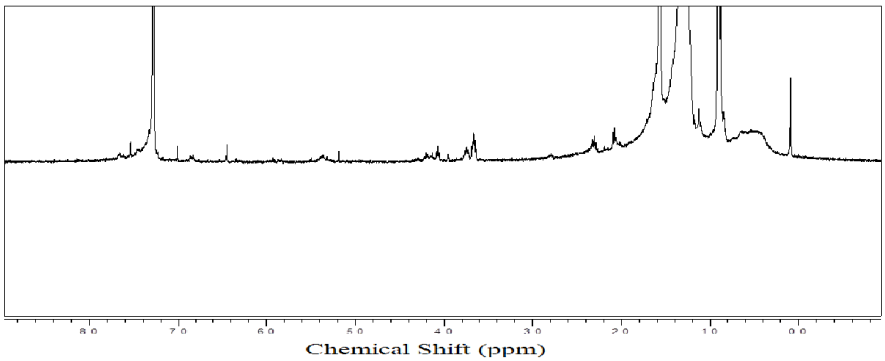

Figure 3.MS spectrum of Ursolic acid isolated from pomegranate flowers.

The IR, MS and ^{1}H-NMR spectra of isolated UA are shown in Figures 2, 3 and 4 respectively. These spectral data were consistent with previous literature data [23, 49-50].

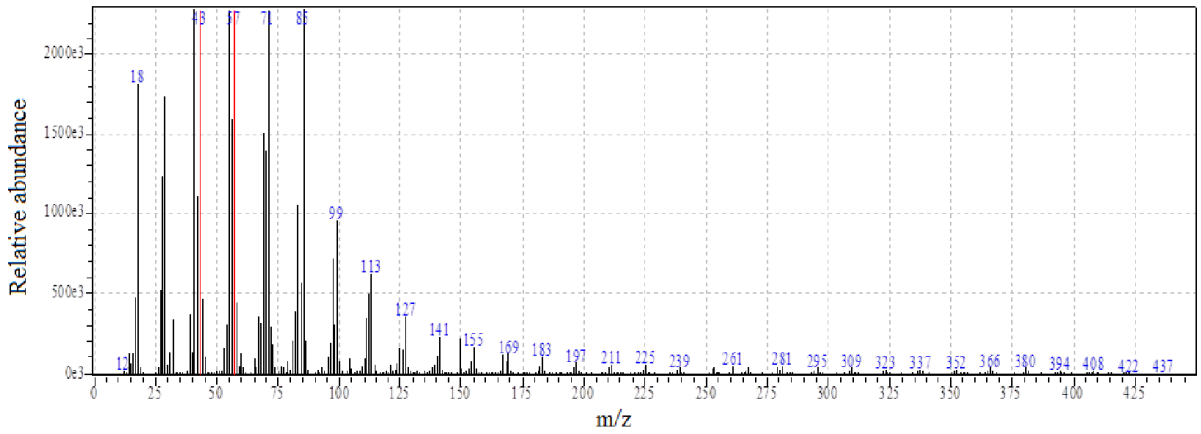

Figure 4. ^{1}H-NMR spectrum of Ursolic acid isolated from pomegranate flowers.

Conclusion

A rapid and efficient procedure was developed for the separation of UA from pomegranate flowers using ultrasound-assisted extraction followed by normal-phase flash chromatography. After one flash chromatography run, UA was successfully separated with high purity. The purified UA was characterized and identified by spectroscopic studies such as IR, MS and NMR.Compared to the SGCC method, this method provided more yields with less solvent consumption and in a much shorter time. Hence, this method is viable for rapid and large scale separation of this valuable triterpenoid, for use in a wide variety of bioactive compounds such as food supplements and health products.

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