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HPLC determination of Ursolic acid in flowers of an Iranian Pomegranate (*Punica granatum* L.) Cultivar

Research Article

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Abstract

Ursolic acid (UA), is a major bioactive compound in several traditional medicinal plants including pomegranate (*Punica granatum* L.) flower. This study presents the HPLC analysis of UA content in flowers of an Iranian pomegranate cultivar. The analytical method's accuracy and the repeatability were satisfactory. The Linear ranges were 100-400 µg/ml for UA with a good correlation. On the average, the recovery rate of UA was 100%. Here, the extraction of UA from an Iranian pomegranate flower cultivar by employing an ultrasound-assisted extraction method was reported, and several extraction solvents were studied to improve extraction yield of UA. Remarkable quantities of UA were found in cultivar of pomegranate flower evaluated. Our results showed that Iranian pomegranate flower cv. Ghorj Tafti Torsh is a good source for extraction and isolation of UA.

Keywords: HPLC, Pomegranate flowers, Ursolic acid.

Introduction

Punica granatum Linn. (Punicaceae), commonly known as pomegranate, is an important commercial fruit crop, extensively cultivated in the Middle East, North Africa, the Mediterranean and in parts of Asia (Tehranifar et al. 2010). The plant possesses an immense therapeutic value. Pomegranate flowers have been widely used in Ayurvedic, Unani and Chinese medicine systems (Mirjalili 2015). The flowers are strongly astringent and a unique traditional antidiabetic medicine. A decoction of pomegranate flowers stops bleeding and treats tympanitis. The flowers are also used in traditional Chinese medicine to treat injuries from falls, to cure graying hair in young men and to treat chronic diarrhea, especially in children (Kaur et al. 2006; Amjad et al. 2013). Pomegranate flowers contain multiple secondary metabolites, the most abundant of which are polyphenols such as ellagic acid, gallic acid and ethyl brevifolin-carboxylate; and triterpenes including ursolic, oleanolic, maslinic and asiatic acids. These compounds have shown strong medicinal values and biological activities (zhang et al. 2011). As an ursane-type pentacyclic triterpene, Ursolic acid (UA, 3β-hydroxy-12-urs-12-ene-28-oic acid) (Fig. 1) is a constituent of some medicinal plants. UA also forms the main part of protective coatings of different fruits including apple, pear, olive, prune, cranberry and fig (Rao et al. 2011; Chen et al. 2015). For a long time, UA was thought not to be active biologically, while in recent years, because of its pharmacological effects and low toxicity, it has attracted a lot of attention (Ikeda et al. 2008). UA possesses considerable pharmacological effects including hepatoprotective (Saravanan et al. 2006; Jin et al. 2012), immunomodulatory (Saaby et al. 2011), anti-inflammatory (Ali et al. 2007; Zhang et al. 2013), antidiabetic (Wang et al. 2008; Perez Gutierrez et al. 2009), antitumor (suhagia et al. 2013), antibacterial (Kurek et al. 2012; do Nascimento et al. 2013), antiviral (Wu et al. 2011; Kong et al. 2013), antiulcer (Ishikawa et al. 2008) and anticancer activities (Shanmugam et al. 2013). UA has recently attracted increasing attention due to its multifunctional anticancer activities (Shanmugam et al. 2013; Yang et al. 2013). Anti-inflammatory and anti-proliferative, proapoptotic, anti-metastatic and anti-angiogenic ability of UA have been reported in both *in vitro* and *in vivo* models of cancer (Shao et al. 2011; Shanmugam et al. 2013).

This study aimed to identify and quantify UA in Iranian pomegranate flowers cv. Ghorj Tafti Torsh by HPLC, for better nutritive evaluation and medicinal utilization in the future. Here, an ultrasound-assisted extraction method was employed to extract UA from pomegranate flowers and several extraction solvents were studied in an attempt to improve extraction of UA from pomegranate flowers.

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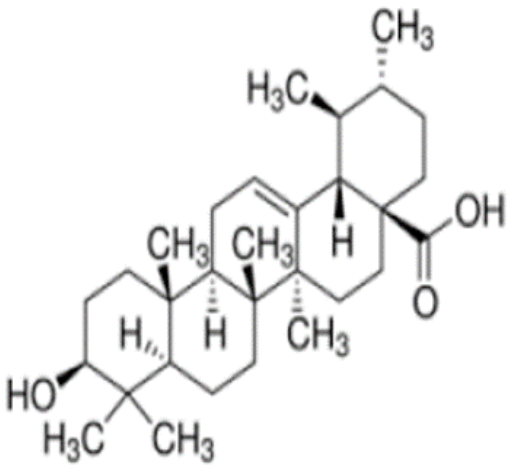


Fig.1 Structure of Ursolic acid (UA, 3β-hydroxy-12-urs -12-ene-28-oic acid)

Materials and Methods

Chemicals

Ursolic acid standard (European Pharmacopoeia Reference Standard) was purchased from Sigma-Aldrich to control biological and pharmaceutical products. Methanol (HPLC grade) was bought from Merck (Germany) and pure water was utilized to HPLC analysis. Analytical grade Ethanol and Ethyl acetate were purchased from Merck (Germany).

Preparation of standard solution

Stock standard solution of UA was prepared by solving an adequate amount of UA in methanol to obtain an ultimate concentration of 1mg/mL. A serial dilution was made with methanol to prepare standard solutions at concentrations of 100, 200, 300 and 400 µg/mL, from each of which 20 µL was utilized to plot a standard curve for UA.

High-performance liquid chromatography system

HPLC was performed on a SY-8100 system equipped with SY-8100 HPLC pump, a 7725i manual sample injector, a variable-wavelength UV detector, and SY-8000 HPLC software. The analytical column, which was used, was Venusil MP C18 (250mm×4.6 mm, 5 µm). The 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 and elute was monitored at 210 nm. The column temperature was kept fixed at 21±1°C.

Preparation of sample solution

Pomegranate flowers were collected in May 2016 from cv. Ghorj Tafti Torsh pomegranate trees growing in the Pomegranate Genetical Garden Collection in Isfahan Province, Iran. The perfect flowers were dried at 105°C for 15 min, and then at 65°C in a hot-air oven for 2 days.

Extracts from pomegranate flowers were prepared using 90% ethanol, ethyl acetate and ethyl acetate followed by 90% ethanol as solvents in order to determine the optimum extraction solvents of UA.

One gram of pomegranate flower powder was dissolved in 20mL solvent followed by 50min ultrasonic extraction at 40°C by MH S3 ultrasonic machine (Soltec Co. Milan, Italy). For HPLC analysis, the extracts were passed through a 0.45µm membrane filter.

Recovery

To test the extraction recovery, dried plant powder (1g) was added with 2mg UA standard before extraction. Follow-up extraction along with HPLC analysis was performed as mentioned above in detail. The assessment of the recovery was as follows:

Recovery (%) = (A-B)/C × 100

Where, A is the result after adding standard, B denotes the amount of sample before adding standard, and C is the amount of added standard.

Repeatability and precision

To assess the intra-day precision of the method, 200, 300 and 400 µg/mL standard solutions of UA were injected several times (n=5) during the same day. These studies were repeated on different days (n=5) in order to assess the inter-day precision.

Results and discussion

HPLC conditions

Because there are no chromophore moieties in Triterpenoids' chemical structures, they indicate poor UV absorption, which is the main limitation in analyzing this group of compounds by employing UV detection. In some cases, in order to detect at higher wavelengths, derivation has been employed (Maurya et al. 2012); however, this extra step may lead to significant errors in the method. In this study, the detection wavelength was chosen at 210 nm for UA because at this wavelength, it has better absorption and sensitivity.

Based on methanol, acetonitrile, phosphate buffer and phosphoric acid several mobile phases were carefully tested in order to determine better separation and peak shapes. At last, the mobile phase consisting of methanol (A) and phosphate buffer (PH=3)(B) with a ratio of 90:10 (A:B, v/v) was chosen. It was found that simply by using methanol separation was unsatisfactory, but as an organic modifier with a phosphate buffer solution (pH=3), methanol performed well. In this regard, adding phosphate buffer improved the separation and peak shapes by controlling pH with no ion pairing for acidic compounds. 0.9 mL min-1 of flow rate was found appropriate to shorten the run time with no compromise for the peak resolution. 21±1°C of controlled column temperature was needed to get reproducible results.

Under these HPLC conditions, the retention time was 9/20 min for UA. HPLC chromatograms of UA standard and the pomegranate flower extract are shown in Figs. 2 and 3, indicating that UA in the pomegranate flower was successfully separated and identified.

Fig.2 HPLC chromatogram of UA standard

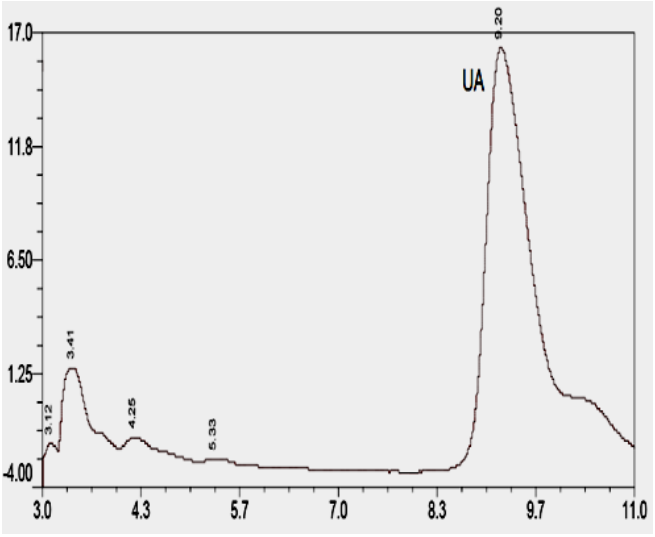
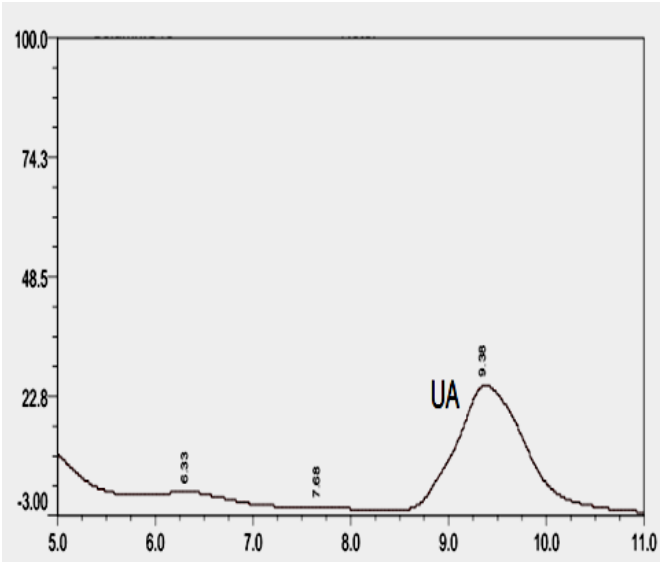


Fig.3 HPLC chromatogram of the pomegranate flower extract



In this part, the effect of different solvents including 90% ethanol, ethyl acetate and ethyl acetate followed by 90% ethanol on the extraction yield of UA was evaluated. Fu et al. (2014) showed that ethanol and chloroform are the most effective solvents for the extraction yield of UA in pomegranate flowers. Afterwards, the highest yield of UA was obtained with acetone and ethyl acetate. Most organic solvents (methanol, chloroform and acetone) are toxic (Bernatoniene et al. 2016); therefore, for the extraction of UA, ethanol and ethyl acetate were chosen as solvents. However, Fan et al. (2012) in their study reported that the solubility of UA in pure ethanol solvent is higher than that in mixed solvents (ethanol + water). but, the existence of some water in ethanol could increase the process of mass transfer with an increase in the polarity of the solvent; thus, improving solubilizing capacity of the solvent and also increase the contact surface for solute solvent interaction by efficient swelling in the plant material (Mandal et al. 2010). Therefore, the water content in the mixed solvents should be as low as possible in order to increase the solubility of UA (in our study 90% ethanol).

Other extraction parameters were as follows: liquid: material ratio, 20:1(ml/g) followed by 50min ultrasonic extraction at 40°C. Ultrasound, On the one hand, can produce mechanical action and cavitation in the extraction process, which is efficient in the breakdown of plant cell walls so that the active ingredients are present in a free state and are dissolved in the extraction solvent. On the other hand, Ultrasound can accelerate the movement of molecules, and cause the extraction solvent and active ingredients of plants to contact quickly and combine with each other (Mandal et al., 2010).

The content of UA in various extractions of pomegranate flowers cv. Ghorj Tafti Torsh determined by HPLC has been shown in table 1. The results showed that higher yields were obtained when 90% Ethanol or Ethyl acetate was used individually as solvents. Statistically, there were no significant differences in the yields of UA when 90% ethanol, compared to that when ethyl acetate was used, as the solvent (P>0.05).

Table 1: Effect of different solvents on the

Solvent	Extraction Yield of
90%Ethanol	19.044±0.231
Ethyl acetate	19.813±0.953
Ethyl acetate + 90%	16.886±1.332
Data are means (n = 3) ± standard deviation	

The pomegranate flowers cv. Ghorj Tafti Torsh had higher UA contents compared to yields from *Ligustrum lucidum* Ait. (9.8 mg/g) (Xia et al. 2011), *Eriobotrya japonica* Lindl.(5.6 mg/g) (Xu et al. 2012), *Rosmarinus officinalis* leaves(15.8 mg/g) (Bernatoniene et al. 2016) and *Ziziphora clinopodioides* Lam.(1.176 mg/g) (Tian et al. 2010). Jager et al. (2009) quantified the triterpene content of 39 plant materials. They determined maximum concentration of UA in *Malus domestica* peels (14.3 mg/g), *Lavandula angustif*olia leaves (15.9 mg/g), *Coffea arabica* leaves (18 mg/g), *Salvia officinalis* leaves (18 mg/g) and *Rosmarinus officinalis* leaves (29.5 mg/g). Although UA is present in a wide variety of plants, our results showed that Iranian pomegranate flower cv. Ghorj Tafti Torsh is also a good source of UA.

The extraction yield of UA by the proposed method was also higher than the yields that Fu et al. (2014) obtained from pomegranate flowers (12.59 mg/ g). This difference could be due to the nature of cultivar of Iranian pomegranate used, and is probably related to environmental effects. The chemical composition and the concentration of active ingredients of a plant could vary considerably depending on soil, water supply, light, the time when it is harvested and environmental cultivations.

Linearity

The linearity of the responses from the detector was studied for standard substance by plotting peak areas against the amounts, which were injected. There was good agreement between the peak area and the standard amounts at the range of 100-400µg/ml for UA. The regression equation and coefficient of determination was [y = 0.0006x + 18.142] (R2 = 0.9955) for UA.

Repeatability and precision

The inter-day and intra-day variations for the determination of UA were less than 3% at concentrations of 200,300 and 400 µg/ml (Table 2). The low value of %RSD shows the high accuracy of the method.

Table 2: Inter-day and intra-day precision for UA.

Concentration RSD% (µg/ml)	RSD%	
	Intra-day (n=5)	Inter-day (n=5)
200	2.21	1.56
300	1.99	1.63
400	1.51	2.13

Recovery

By mixing an appropriate amount of quantified samples with the standard compound, recovery experiment was conducted in order to confirm that the method was accurate. The average recovery of UA was 100%.

Conclusion

In the present study, a simple, accurate and precise HPLC method was developed for the quantification of ursolic acid in flowers of pomegranate. This method is isocratic, and it has an uncomplicated mobile phase; moreover, the preparation of the samples and evaluation procedures are quick and simple.

This study was the first report on the UA content of an Iranian pomegranate flower cultivar. In addition to plant materials supplying a high concentration of UA, the determination of UA in Iranian pomegranate flower cv. Ghorj Tafti Torsh has resulted in the identification of another plant material rich in UA (with concentrations over 1.9% in the dry plant material).

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Disclosure statement

The authors declare that there are no conflicts of interests.

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