



## Quantification of betulinic, oleanolic and ursolic acids as medicinally important triterpenoids in some *Thymus* species from Iran

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### Abstract

**Background and objectives:** Betulinic acid (BA), oleanolic acid (OA) and ursolic acid (UA) are well-known pentacyclic triterpenoids (PTs), which are produced by plants. They possess a variety of beneficial effects including anti-inflammatory, hepatoprotective, antitumor, anti-HIV, antimicrobial, gastroprotective, and antihyperlipidemic activities. In the present study, quantitative determination of these compounds was simultaneously carried out in some *Thymus* species native to Iran i.e. *T. daenensis*, *T. pubescens*, *T. persicus* and *T. caramanicus*. **Methods:** Lyophilized and powdered plant material (1.0 g) was drenched in MeOH and immediately sonicated at room temperature. The methanol extract was further separated into organic and aqueous layers. The organic layer was dissolved in HPLC grade methanol, filtered and analyzed by reverse-phase HPLC. **Results:** The maximum content of BA, OA and UA were determined in the aerial parts of *T. persicus* as 856.9, 480.6 and 941.7 mg per 100 g dry weight (DW) of plant, respectively while the other *Thymus* species had an almost negligible amount of these compounds. **Conclusion:** Results showed that the aerial parts of *T. persicus* could be a considerable source of these PTs which might be attractive for future phytochemical and biological investigations according to importance of their health benefits.

**Keywords:** betulinic acid, oleanolic acid, *Thymus*, ursolic acid

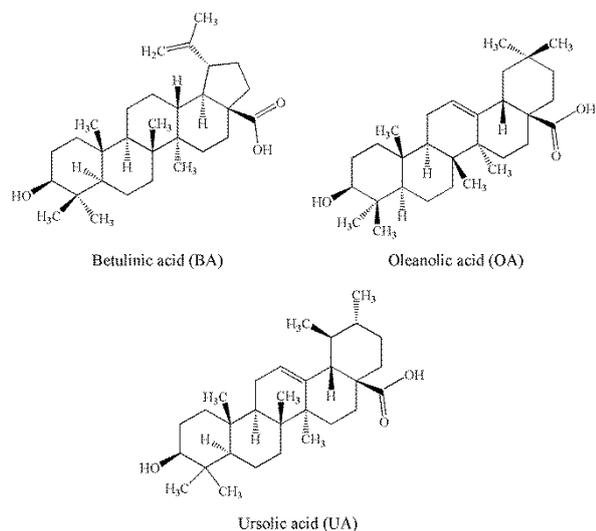
### Introduction

Standardization and characterization of herbal drugs are topics of continuous scientific interest in the herbal drug industry [1]. With the advent of modern chromatographic systems there is an ever increasing intent to use rapid and convenient methods for standardization of herbal drugs

based on their natural compounds. Terpenoids are the most structurally varied class of plant natural products, with over 20,000 known members [2]. They are commercially important due to their wide application in a vast number of industrial products such as flavoring agents,

pharmaceuticals, perfumes, insecticides and antimicrobial agents [3].

Betulinic acid (3 $\beta$ -hydroxylup-20(29)-en-28-oic acid, BA), oleanolic acid (3 $\beta$ -hydroxyolean-12-en-28-oic acid, OA), and ursolic acid (3 $\beta$ -hydroxyurs-12-en-28-oic acid, UA) are highly sought-after pentacyclic triterpenoids (PTs, figure 1) because of their wide spectrum of biological activities. They are most highly regarded for their anti-inflammatory, hepatoprotective, antimicrobial, anti-HIV-1, antiulcer, gastroprotective, hypoglycemic and antihyperlipidemic activities and also for specific cytotoxicity against a variety of tumor cell lines [4-7].



**Figure 1.** Chemical structures of the pentacyclic triterpenoids (PTs)

PTs naturally occur in the raw plant materials such as berries, leaves, flowers, and fruits. Their content has been previously assayed in *Sambucus chinensis* (Caprifoliaceae) [8], *Doliocarpus schottianus* (Dilleniaceae) [9], *Lycopodium cernuum* (Lycopodiaceae) [10], *Vaccinium* species (Ericaceae) [11], *Euphorbia microsciadia* (Euphorbiaceae) [12], *Sorbus cashmiriana* (Rosaceae) [13] and *Syzygium aromaticum* (Myrtaceae) [14]. Lamiaceae, one of the most important families among the medicinal plants, has been reported as a wide-ranging source for

isolation of free BA, OA and UA besides other compounds [15-17]. So far, isolation and quantitative determination of PTs have been performed in many members of Lamiaceae family such as *Leonurus cardiac* [18], *Ocimum* species [19], *Rosmarinus officinalis*, *Salvia officinalis*, *Satureja montana*, *Salvia sclarea*, *Salvia glutinosa* [20] and *Thymus persicus* [21]. Due to the importance of BA, OA and UA in clinical medicine [4,6], we attempted to simultaneously quantify these compounds in some *Thymus* species which are native to Iran [22]. In the present work, we have examined a previously reported method and have evaluated its specificity, linearity and accuracy for analysis of the three PTs in these *Thymus* species. It might be suggested as an easy, rapid and convenient procedure for characterization of herbal products based on these active important compounds.

## Experimental

### Chemicals

Standards of BA, OA, and UA were purchased from Sigma (Sigma-Aldrich Corporation, MO, USA). HPLC grade methanol and phosphoric acid of analytical grade were obtained from Merck (Darmstadt, Germany). HPLC grade water was used throughout the analysis.

### Plant material

Aerial parts of wild *T. daenensis*, *T. pubescens*, *T. persicus*, *T. caramanicus* and cultivated *T. vulgaris* were collected at full flowering stage from their natural habitats and cultivated fields in Iran, respectively (table 1). Voucher specimens of the plants were deposited at the Herbarium of Medicinal Plants and Drugs Research Institute (MPH), Shahid Beheshti University, Tehran, Iran.

### Extraction and HPLC analysis

The aerial parts of all tested *Thymus* species were extracted for the HPLC analyses as described before [23]. Lyophilized and powdered plant material (1.0 g) was drenched in MeOH (40 mL) and immediately sonicated at 30% amplitude for 40 min at room temperature.

**Table 1.** Geographic characteristics of the habitat of studied *Thymus* species

<i>Thymus</i> species	Collection site	Latitude (N)	Longitude (E)	Altitude (m)	Voucher number
<i>T. daenensis</i>	Semirom, Isfahan	31°13'	51°46'	2360	MPH-2000
<i>T. pubescens</i>	Mahneshan, Zanjan	36°36'	47°26'	2272	MPH-1999
<i>T. persicus</i>	Baderloo, Takab	36°28'	47°13'	2500	MPH-1673
<i>T. caramanicus</i>	Rabar, Kerman	29°22'	56°49'	3048	MPH-2001
<i>T. vulgaris</i>	Evin, Tehran	35°48'	51°23'	1785	MPH-247

The obtained methanol mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was pooled, filtered and concentrated in a rotary evaporator at 40 °C (Heidolph Instruments GmbH, Schwabach Germany). This methanol extract was further separated into organic (30 mL ethyl acetate) and aqueous (30 mL double distilled water) layers. The ethyl acetate layer was dried under reduced pressure in a rotary evaporator at 40 °C. The extract was dissolved in HPLC grade methanol (10 mL), filtered through a Millipore filter (0.45 mm) and stored in a refrigerator until analysis. A Knauer liquid chromatography apparatus consisting of a 1000 Smartline Pump, a 5000 Smartline Manager Solvent Organizer and a 2800 Smartline Photodiode Array Detector was used for the HPLC analysis. Injection was carried out through a 3900 Smartline Autosampler injector equipped with a 100 µL loop. The temperature of the column was controlled with a Jet Stream 2 Plus oven (Knauer, advanced scientific instrument, Berlin, Germany). Separation was achieved on a 25 cm×4.6 mm with a pre-column, Eurospher 100-5 C<sub>18</sub> analytical column provided by Knauer (Berlin, Germany). Data acquisition and integration was performed with EZchrom Elite software. MeOH-phosphoric acid-water (87:0.05:12.95, v/v/v, isocratically) was employed as the mobile phase with a flow-rate of 1 mL/min. Peaks were monitored at 210 nm wavelength. Injection volume was 20 µL and the temperature was maintained at 25 °C. All injections were repeated three times ( $n=3$ ). System suitability tests were performed according to the earlier reports [23,24] by

checking linearity, precision, and recovery of three triterpene acids in the quantification experiment which have been reported in our previous study [21].

### Results and Discussion

The simultaneous quantitative HPLC determination of BA, OA and UA in the aerial parts of *T. daenensis*, *T. pubescens*, *T. persicus*, and *T. caramanicus* has been reported here for the first time. According to Wang *et al.* [24], a mobile phase consisting MeOH-phosphoric acid-water (87:0.05:12.95, v/v/v, isocratically) gave symmetrical, sharp peaks at a retention time (RT) of 20.1, 21.9, and 22.9 min for BA, OA and UA, respectively (table 2).

**Table 2.** Standard curves and retention times of triterpene acids

Compound	Retention time	Standard equation	R <sup>2</sup>
Betulinic acid	20.1	$Y=6953.7416x+1074.3353$	0.9991
Oleanolic acid	21.9	$Y=9623.8718x-22522.2671$	0.9994
Ursolic acid	22.9	$Y=8365.6058x-16086.3008$	0.9994

$Y$  peak area,  $x$  concentration (mg),  $R^2$  correlation coefficient

Identification of these PTs in extracts was based on the comparison of their retention times with their authentic references, spike of standards and their UV spectra.

Complementary HPLC analysis data were presented in table 2. The maximum content of BA, OA and UA were observed in the aerial parts of *T. persicus* with 856.9, 480.6 and 941.7 mg per 100 g DW of plant, respectively (table 3). The concentration of all the tree triterpenic acids in *T. vulgaris* as a common *Thymus* species was detected much lower than the Iranian endemic *T. persicus*.

**Table 3.** Content and recovery percentage of triterpene acids in the aerial parts of some *Thymus* species

Name	Content (mg/100 g DW±SD)				
	<i>T. daenensis</i>	<i>T. pubescens</i>	<i>T. persicus</i>	<i>T. caramanicus</i>	<i>T. vulgaris</i>
Betulinic acid	13.73±1.48	2.37±0.62	856.89±10.15	0.88±0.28	6.56±0.34
Oleanolic acid	14.63±0.44	14.90±0.28	480.64±10.93	16.07±0.73	20.65±1.24
Ursolic acid	88.21±1.68	41.63±1.32	941.66±16.14	76.99±1.01	109.53±1.65

SD: standard deviation; n=3

Moreover, the high concentration of PTs in the plant material coupled with its consumption suggests *T. persicus* as an important and interesting medicinal plant. Detection and quantification of BA, OA and UA in some members of Lamiaceae by gas chromatography-mass spectrometry (GC-MS) have been reported as 0.6% BA, 0.09–0.9% OA, and 0.09–1.6% UA in dried plant materials [20]. Jäger *et al.* [25] studied PTs distribution in various plants and have found that the Lamiaceae family is an especially good source for BA, OA and UA, reaching the highest concentration measured within *Rosmarinus officinalis* leaves. Due to the importance of BA, OA and UA as natural compounds with potent antitumor activity [26,27], there is a need for further investigations on the variations of the compounds within and among wild populations of *T. persicus*. The variations in PTs content in the studied *Thymus* species suggest that the genetic factor plays an important role in the biosynthesis of these compounds. The chemical variation can also be attributed to the environmental factors. Hanover [28] provided evidence that terpene biosynthesis is strongly controlled by genetic factors; he also reported instances of environmental variations in terpene expression under extreme habitat conditions. In this respect, an intensive selection in wild plants is necessary to obtain PTs-rich cultivars for the extraction of these pharmaceutically interesting substances.

Our results showed that *T. persicus* is a valuable source of triterpene acids especially BA, OA and UA. It can provide an ample opportunity to take this plant for extensive research for mass cultivation of plants and enhanced antitumor compounds production through different

biotechnological strategies like cell suspension cultures and large scale cultivation in bioreactor system.

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### Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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