



## Quantification of 20-hydroxyecdysone, a Major Phytoecdysteroid, in *Ajuga Chamaecistus* ssp. *Tomentella* Using High-Performance Liquid Chromatography

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### Abstract

The genus *Ajuga* is used for the treatment of joint pain, gout, jaundice, and wound healing in Iranian traditional medicine. *Ajuga chamaecistus* ssp. *tomentella* is an exclusive subspecies of *Ajuga chamaecistus* in the flora of Iran. Plants belong to *Ajuga* species are advantageous sources of phytoecdysteroids. 20-hydroxyecdysone (20E) is an important phytoecdysteroid with anabolic property. This study aimed to determine and quantify 20E in methanolic extract of aerial parts of *Ajuga chamaecistus* ssp. *tomentella*. The standard reference of 20E was isolated from n-butanolic fraction of aerial parts of *Ajuga chamaecistus* ssp. *tomentella* using high-performance liquid chromatography (HPLC). The analysis was carried out on an ODSA (C18) column with isocratic elution using water–acetonitrile (75:25). The standard calibration curve represented good linearity ( $r^2 = 0.9997$ ). The limit of quantification (S/N = 10) and detection (S/N = 3) were determined as 7.93 and 2.38  $\mu\text{g/ml}$ , respectively. The content of 20E in methanolic extract of *Ajuga chamaecistus* ssp. *tomentella* was determined to be 2.58% (w/w) (0.46% in dry plant). The quantitative proportion of the 20E found in *Ajuga chamaecistus* ssp. *tomentella* extract proposes the possible uses of this plant in commercial formulations or as a source of 20E.

**Keywords:** *Ajuga chamaecistus* ssp. *tomentella*, 20-hydroxyecdysone, Quantification, High-performance liquid chromatography (HPLC)

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## 1. INTRODUCTION

Some species belonging to genus *Ajuga* are used in traditional medicine of different countries in the world. In Iranian traditional medicine, *Ajuga* [Kamaphytus] has been used for treatment of joints pains, gout, jaundice and wound healing [1], [2]. *Ajuga chamaecistus* comprises five exclusive subspecies, including *Ajuga* (*A.*) *chamaecistus* ssp. *tomentella* [3].

Several biological effects have been reported from different species of *Ajuga* such as treatment of joint disease [4], inflammation [5], hyperglycemia [6], and malaria [7], as well as antioxidant, antimicrobial [8], and antinociceptive [9] effects. In our previous study, five ecdysteroids, 20-hydroxyecdysone (20E), cyasterone, ajugalactone, makisterone A, and 24-dehydroprecyasterone were isolated from *A. chamaecistus* ssp. *tomentella* [10], [11].

The ecdysteroids are a large class of polyhydroxysteroids isolated from both the animal and plant kingdom. 20E and cyasterone, in addition to ajugalactone, are the most common compounds in *Ajuga* species [12]. This group of natural products produces a wide range of pharmacological activities including adaptogenic and anabolic [13], hypoglycemic [14], [15], wound-healing, hepatoprotective, immunoprotective [16], antioxidant, and free radical scavenging [17] effects. Chemical structure of 20-hydroxyecdysone is shown in figure 1.

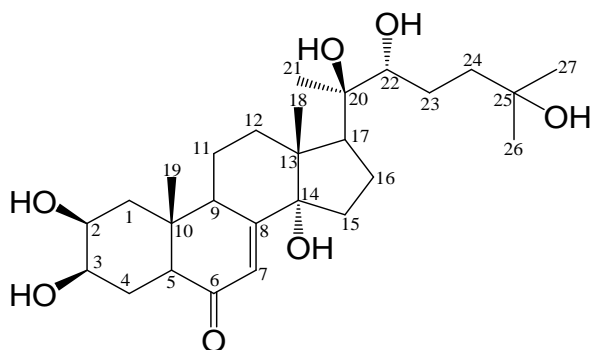


Figure 1. Chemical structure of 20-hydroxyecdysone

This study aimed to use (HPLC) method

for determination and quantification of 20E in methanolic extract of aerial parts of *Ajuga chamaecistus* ssp. *tomentella*.

## 2. METHODS

### Chemicals and materials

Acetonitrile (Merck, Germany) was used for mobile phase of HPLC. 20E was isolated previously from n-butanol fraction of *A. chamaecistus* ssp. *tomentella* using preparative HPLC.

### Instruments

Analytical HPLC separations were performed on YL9100 pumping system (South Korea) with a YL 9100 photodiode array (PDA) detector equipped with a Tracer Excel 120 ODSA (C18)–6  $\mu\text{m}$  TechnoChroma column. The detection wavelength was set at 254 nm. The flow rate was 1 ml/minute. The mobile phase included isocratic elution with water-acetonitrile (75-25) for 20 minutes. The injection volume was 60  $\mu\text{l}$ .

### Plant material

Aerial parts of *A. chamaecistus* Ging. ssp. *tomentella* (Boiss.) Rech. f. were collected from Tehran, Iran, in June 2013 and verified by Prof. G. Amin. A voucher specimen (THE-6697) had been deposited in the herbarium of the Department of Pharmacognosy, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

### Extraction

The air-dried and ground plants of *A. chamaecistus* ssp. *tomentella* (100 g) were extracted with 80% methanol (Merck, Germany) at room temperature and concentrated under reduced pressure to give a dark brown extract (18 g). Sample solution of the extract was obtained via dissolving in methanol (500  $\mu\text{g/ml}$ ) and then was filtered through a 0.45  $\mu\text{m}$  syringe-driven filter (Merck, Germany) before injecting to HPLC.

### Validation methods

a. Calibration curves: A stock solution of standard 20E (1000  $\mu\text{g/ml}$ ) was prepared via

dissolving in methanol. Standard solutions at five concentrations (100, 50, 25, 5, and 1  $\mu\text{g/ml}$ ) were obtained via appropriate dilution. Each concentration was injected three times. Calibration curves for 20E were constructed via plotting peak areas vs. concentrations of 20E ( $\mu\text{g/ml}$ ).

b. Precision and repeatability: The intra-day precision was determined via analyzing the standard solution in triplicate within a single day, and the inter-day precision was determined in triplicate on three successive days. The relative standard deviations (RSD %) were calculated as measures of precision, and repeatability.

c. Limit of detection (LOD) and quantification (LOQ): LOD and LOQ were determined using the signal-to-noise ratio = 3/1 and 10/1 criterion, respectively.

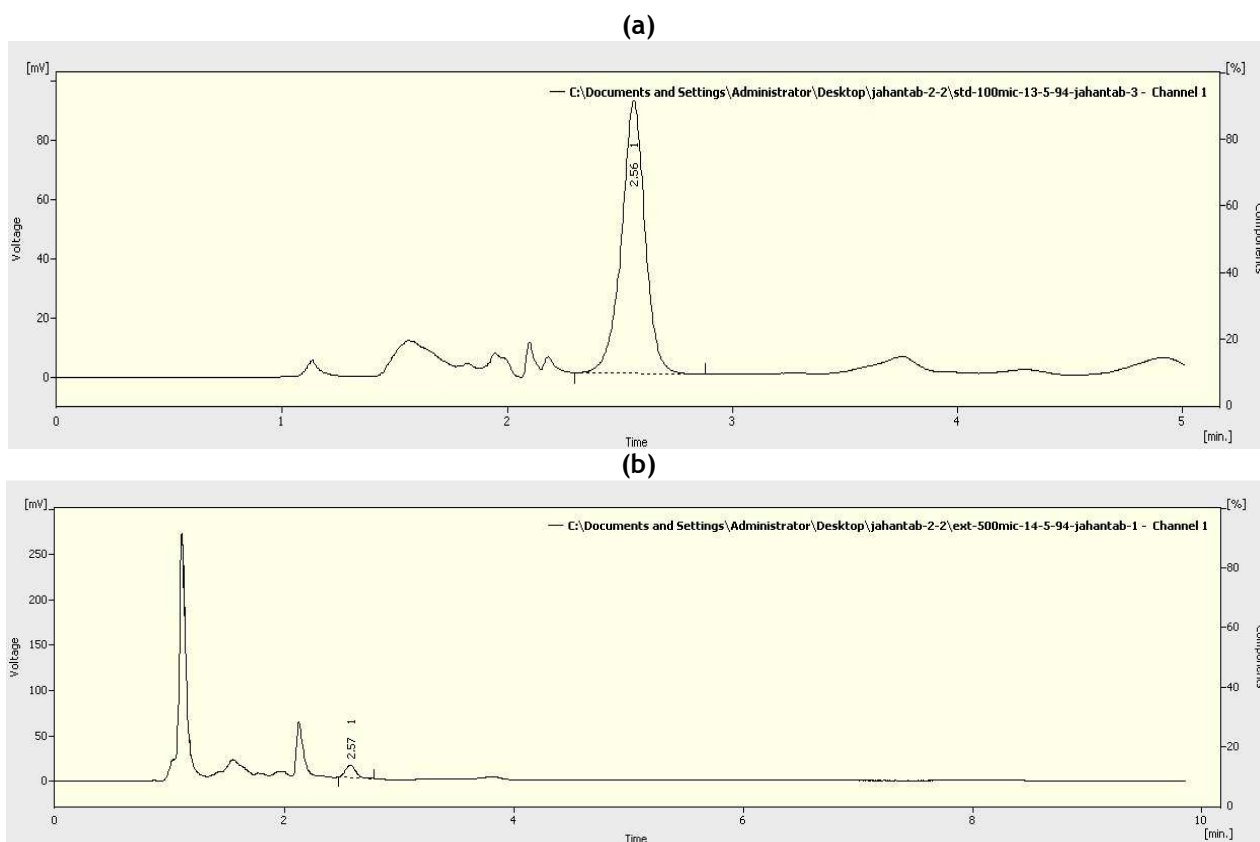
d. Accuracy: The accuracy of the method was determined via application of the standard addition method. 1 ml of standard

solution (5  $\mu\text{g/ml}$ ) was added to 1 ml of methanol extract (100  $\mu\text{g/ml}$ ) and then was analyzed in triplicate. The total amount of 20E was calculated from the corresponding calibration curve and the recovery of 20E was obtained according to the amount found and amount contained.

### 3. RESULTS

Optimum condition for analysis of 20E in the methanolic extract of *A. chamaecistus* ssp. *tomentella* was performed using a 6  $\mu\text{m}$  phenyl-hexyl silica column (ODSA 120 mm) and a isocratic elution of water-acetonitril (75-25) in 20 minutes. Chromatograms were recorded at 254 nm (Figure 2). The calibration curve for 20E showed good linear regressions with correlation coefficient of  $r^2 > 0.999$  (Figure 3).

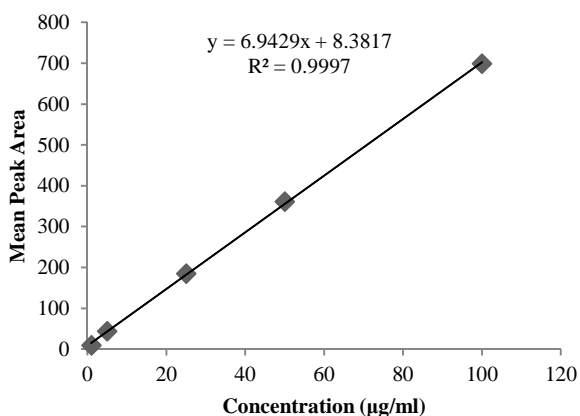
The intra- and inter-day precisions were expressed as RSD (%). Table 1 shows the results.



**Figure 2.** Chromatograms of 20-hydroxyecdysone (Rt 2.56, 2.57 minutes) in methanolic extract of *Ajuja chamaecistus* ssp. *tomentella*: (a) standard (100  $\mu\text{g/ml}$ ) and (b) crude extract (500  $\mu\text{g/ml}$ )

**Table 1.** The relative standard deviations (RSD) of intra- and inter-day precisions of 20-hydroxyecdysone

Concentration (µg/ml)	RSD (%)			
	1 <sup>st</sup> day	2 <sup>nd</sup> day	3 <sup>rd</sup> day	3 days
1	1.37	2.80	1.96	3.07
5	1.08	1.09	1.48	3.82
25	0.58	0.39	0.40	1.51
50	3.26	1.06	1.42	3.30
100	1.11	2.85	0.65	1.86

**Figure 3.** Mean calibration curves for 20-hydroxyecdysone (mean, n = 9)

The content of 20E in methanolic extract of *Ajuga chamaecistus* ssp. *tomentella* was determined to be 2.58% (w/w).

The mean of recovery percent was  $101.05 \pm 5.15$  (n = 3). The LOD and LOQ for 20E were determined at 2.38 and 7.93 µg/ml, respectively.

In addition, the content of 20E in methanolic extract of *A. chamaecistus* ssp. *tomentella* was equivalent to 0.46% (w/w) dry plant.

#### 4. DISCUSSION

In this study, the method development was carried out according to some quantitative determination studies for phytoecdysteroids in plants. HPLC has been commonly used in the analysis of 20E [18], [19], [20].

According to our results, RSD percent of

intra- and inter-day precision were less than 3.83%, representing good precision and reproducibility of this method for quantification of 20E in the extract.

The calculated mean of recovery percent indicated good accuracy of this analysis. Good linearity, precision, and accuracy of HPLC analysis showed suitability of this method for quantifying 20E in methanolic extract of *A. chamaecistus* ssp. *tomentella*.

Furthermore, the content of 20E in methanolic extract of *A. chamaecistus* ssp. *tomentella* was equivalent to 0.46% (w/w) dry plant. The level of phytoecdysteroids in most containing species is typically 0.01-0.10% of the dry weight; however, in particular plant species, the level of phytoecdysteroids can reach higher concentration (more than 1-2 percent) such as in *Rhaponticum carthamoides* (2% in the seed) and *Diploclisia glaucescens* (3.2% in the stems) [16].

Our analysis displayed that the content of 20E in methanolic extract of *A. chamaecistus* ssp. *tomentella* aerial parts is proportionally high in comparison with other plants which can suggest the possible uses of this plant as a source of 20E in commercial formulas.

#### 5. CONFLICT OF INTERESTS

Authors have no conflict of interests.

#### 6. ACKNOWLEDGMENTS

None.

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