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Original article

ISOLATION AND QUANTIFICATION OF ALANTOLACTONE/ISOALANTOLACTONE FROM THE ROOTS OF *INULA HELENIUM* SUBSP. *TURCORACEMOSA*

Alper GÖKBULUT*, Engin ŞARER

Ankara University, Faculty of Pharmacy, Department of Pharmacognosy, 06100 Tandoğan-Ankara, TURKEY

Abstract

Alantolactone/isoalantolactone, a mixture of isomeric sesquiterpene compounds, was isolated from the roots of Inula helenium L. subsp. turcoracemosa Grierson and its amount was quantified by RP-HPLC. The structure of alantolactone/isoalantolactone was elucidated by using HPLC and ^{1}NMR data. Alantolactone/isoalantolactone was found in serious amount in the roots of the plant as 1.6338 ± 0.0198 % (w/w). The roots of Inula helenium subsp. turcoracemosa growing in Turkey were found rich in this isomeric pair known to be strong antiproliferative and antimicrobial agent.

Key words: *Alantolactone/isoalantolactone, Inula helenium subsp. turcoracemosa, RP-HPLC*

Inula helenium subsp. *turcoracemosa* Köklerinden Alantolakton/İzoalantolakton İzolasyonu ve Miktar Tayini

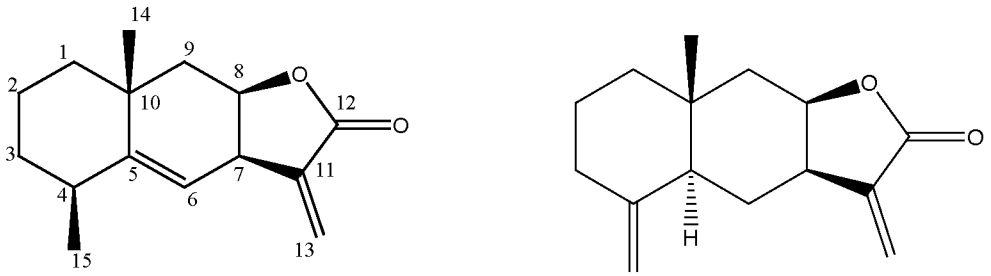
Inula helenium L. subsp. turcoracemosa Grierson köklerinden izomer seskiterpen bileşiklerin karışımı olan alantolakton/izoalantolakton izole edilmiş ve miktarı TF-YPSK ile tayin edilmiştir. Alantolakton/izoalantolakton'un yapısı YPSK ve ^{1}NMR verileri kullanılarak aydınlatılmıştır. Alantolakton/izoalantolakton bitkinin köklerinde % 1.6338 ± 0.0198(a/a) oranla önemli miktarda bulunmaktadır. Türkiye'de yetişen Inula helenium subsp. turcoracemosa köklerinin, kuvvetli bir antiproliferatif ve antimikrobiyal ajan olarak bilinen bu izomer çifti açısından zengin olduğu saptanmıştır.

Anahtar kelimeler: *Alantolakton/izoalantolakton, Inula helenium subsp. turcoracemosa, TF-YPSK*

***Correspondence**: E-mail:gokbulut@pharmacy.ankara.edu.tr; Tel: +903122033106;

INTRODUCTION

Inula helenium L. (Asteraceae) is a perennial herb up to 1-2 m with thick aromatic rhizomes and is widely distributed in Europe and East Asia. It's an important medicinal plant and roots of the plant have been traditionally used as antimicrobial, diuretic and expectorant agent to treat chronic enterogastritis, tuberculotic enterorrhea, bronchitis and to kill parasites. Its roots contain high amount of eudesmanolide type sesquiterpene lactones which possess antiproliferative, anticancer, antihelminthic and antimicrobial activities (1-8). *I. helenium* is represented by 4 subspecies in Turkey (9). In this study, one of those, *I. helenium* L. subsp. *turcoracemosa* Grierson collected from central Anatolia was investigated, and herein isolation, structure elucidation and quantification of alantolactone/isoalantolactone (Figure 1) from the roots of the plant were presented.



Alantolactone Isoalantolactone

Figure 1. Structure of sesquiterpene lactone compounds

EXPERIMENTAL

Materials

Inula helenium L. subsp. *turcoracemosa* Grierson was collected from Bala-Ankara. The roots of the plant were dried at room temperature away from sunlight. Voucher specimen of the plant has been deposited at the Herbarium of Ankara University, Faculty of Pharmacy, (AEF 25193).

Chemicals and standards

Chromatographic grade double distilled water, HPLC grade methanol (Hipersolv Chromanorm, 20864.320), n-hexane (Merck, 1.04391) and ethyl acetate (Sigma Aldrich, 27227) were used. Alantolactone/isoalantolactone (Helenin) (Roth 7677) was supplied from Roth. All other chemicals were supplied from either Sigma or Merck.

Extraction

For isolation studies, 15 g of dried and powdered roots of *I. helenium* subsp. *turcoracemosa* were extracted with methanol by magnetic stirrer for 6 h (50°C, 250 rpm). After filtration methanol was evaporated completely by rotary evaporator (Buchi R200) and the crude extract (3.11 g) was extracted with n-hexane by magnetic stirrer for 6 h (50°C, 250 rpm). After filtration, n-hexane was evaporated completely, and gained extract (370 mg) was applied to preparative TLC with a solvent system of n-hexane:ethyl acetate (9:1), and the compounds were detected by violet color after spraying vanilin-sulphuric acid reagent. After several applications of preparative TLC, 45 mg of alantolactone/isoalantolactone were isolated.

For HPLC analysis, 200 mg of dried and powdered roots of *I. helenium* subsp. *turcoracemosa* were extracted with methanol by magnetic stirrer for 6 h (50°C, 250 rpm). The

extract was then filtered and completed to 10.0 mL in a volumetric flask with methanol, passed through a 0.45 μm filter, and injected into the HPLC system.

Apparatus

HPLC analysis was performed with a LC system consisting of a HP Agilent 1100 series quaternary pump, degasser and photodiode array detector. The samples were injected to a HP Agilent 1100 Autosamplers with thermostatted column compartment on a Phenomenex-Luna C_{18} column (5 μ, 250 mm; 4.6 mm) at 30°C. The system was controlled and data analysis was performed with Agilent ChemStation software. All the calculations concerning the quantitative analysis were performed with external standardization by measurement of the peak areas.

^{1}H-NMR spectrum was recorded on a Varian Mercury 400BB Spectrometer in $CDCl_3$. Chemical shifts (δ) were given as ppm and the coupling constants (*J*) were reported as Hz.

HPLC Analysis

Stock and standart solutions

Alantolactone/isoalantolactone (10.3 mg) was accurately weighed into a 10 mL volumetric flask, dissolved in methanol and filled up to volume for preparing stock solutions. Based on this, five different concentrations of alantolactone/isoalantolactone were prepared for the establishment of calibration curves.

Chromatographic conditions and procedure

The analysis was performed by isocratic elution with a flow rate of 1 mL/min. Column temperature was set to 30 °C and the mobile phase was methanol:water (60:40). All solvents were filtered through a 0.45 μm Milipore filter before use and degassed in an ultrasonic bath. 10 μL volumes of the each solution and sample were injected into the system and the chromatograms were recorded from 200 to 400 nm. Quantification was performed by measuring at 205 nm for alantolactone/isoalantolactone using photo-diode array detector. The chromatographic run time was 45 minutes where the column void volume was 2 minutes.

Calibration

Five different concentrations of alantolactone/isoalantolactone as 0.0103 mg/mL, 0.02575 mg/mL, 0.0515 mg/mL, 0.103 mg/mL, 0.206 mg/mL were prepared and triplicate 10 μL injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak area of each drug was plotted against the concentration to obtain the calibration graph.

Limits of detection and quantification

Limit of detection (LOD) was established at a signal to noise ratio (S/N) of 3. Limit of quantification (LOQ) was established at a signal to noise ratio (S/N) of 10. LOD and LOQ were experimentally verified by nine injections of alantolactone/isoalantolactone at LOQ concentrations.

Precision

The precision of the method (within–day variations of replicate determinations) was checked by injecting nine times of alantolactone/isoalantolactone at the LOQ level. The precision of the method was expressed as RSD% at the LOQ level. RSD% = (SD / Mean) X 100.

RESULTS AND DISCUSSION

The methanol extract of the air-dried and powdered roots of *I. helenium* subsp. *turcoracemosa* was extracted with n-hexane. The extract gained was subjected to preparative TLC to obtain the sesquiterpene lactone compound. After preparative TLC applications, crystal

needles were obtained. The structure of isolated compound was elucidated to be alantolactone/isoalantolactone according to the ^{1}H-NMR spectral data of which is identical with the literature findings (10). ^{1}H-NMR spectral data was given in Table 1. In addition, TLC and HPLC results supported this finding with identical Rf, Rt and UV spectral values of authentic and isolated compounds.

Table 1. ^{1}H-NMR spectral data of alantolactone/isoalantolactone

	Alantolactone		**Isoalantolactone**	
Position	**Chemical shift (δ, ppm)**	**Coupling constant (Hz)**	**Chemical shift (δ, ppm)**	**Coupling constant (Hz)**
1	1.30-1.70 (2H)	-	1.96-2.29 (2H)	-
2	1.30-1.70 (2H)	-	1.40-1.60 (2H)	-
3	1.30-1.70 (2H)	-	1.34-1.81 (2H)	-
4	2.43 (m)	-	-	-
5		-	2.34 (m)	-
6	5.12 (d)	J:4.1	1.68 (ddd) 1.42 (d)	J_1:2.7 J_2:7.1 J_3:14.0 J:12.0
7	3.56 (m)	-	2.94 (m)	-
8	4.80 (m)	-	4.47 (m)	-
9	2.09 (dd) 1.52 (dd)	J_1:2.8 J_2:14.9 J_1:2.8 J_2:14.9	2.15 (dd) 1.46 (d)	J_1:1.6 J_2:15.6 J:15.5
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	6.18 (d) 5.60 (d)	J:1.9 J:1.7	6.10 (d) 5.56 (d)	J:1.1 J:1.0
14	1.17 (s, 3H)	-	0.80 (s, 3H)	-
15	1.07 (d, 3H)	J:7.6	4.75 (d) 4.41 (d)	J:1.6 J:1.5

After isolation and structure elucidation studies, we aimed to perform the quantification of alantolactone/isoalantolactone in the roots of *I. helenium* subsp. *turcoracemosa*. HPLC is the most frequently used chromatographic technique for quantification of plant secondary metabolites (11, 12). For this purpose, a simple and accurate RP-HPLC method was used. The quantification of alantolactone/isoalantolactone was performed by external standard method and was found as 1.6338 ± 0.0198%(w/w). An isocratic elution profile of methanol:water (60:40) was chosen among the different trials of solvent systems as the most convenient one and the retention time of alantolactone/isoalantolactone was determined as 37.3 min. The linear relationship between peak areas and concentrations can be expressed as y=23147x-16.493 with r=0.9997. The LOD was 1.872 µg/mL and the LOQ was 6.24 µg/mL for alantolactone/isoalantolactone. The mean value of the peak area obtained by injecting nine times of alantolactone/isoalantolactone at the LOQ concentration was 141.556 and the SD value was determined as 4.454. Hereunder, the precision of the method, expressed as RSD% at the LOQ level was 3.1463%. HPLC chromatograms were given in Figure 2-3.

Stojakowska et al. developed an RP-HPLC method for the quantification of alantolactone/isoalantolactone in the roots of *I. helenium* growing in Poland and the content of alantolactone/isoalantolactone was found as 3.598 ± 0.074% (13). The content of alantolactone/isoalantolactone in Poland sample is nearly twice more than Turkish sample but it should be better not to compare these results because of the application of different extraction techniques and usage of different solvents during extraction. However, it was clear that alantolactone/isoalantolactone was found as the major constituent of the roots of *I. helenium* in

both studies. Also, as can be understood from both sudies, the separation of this isomeric pair is not possible under standard RP-HPLC conditions. However, to separate alantolactone and isoalantolactone, a number of succeeded separation techniques have been achieved by using gas chromatography, capillary electrophoresis, microemulsion electrokinetic chromatography and HPLC with more efficient columns which were packed with special techniques (14-17).

Alantolactone and isoalantolactone are the main constituents of *I. helenium* roots. This isomeric pair constitutes over 90% of the sesquiterpene fraction in the roots of *I. helenium* (1, 13). Most of the biological activities as antimicrobial, antiproliferative and antihelminthic activities of *I. helenium* roots, depend on this sesquiterpene lactone compounds. So that, quantification of such compounds in the roots of the plant is very important. To the best of our knowledge, the amount of alantolactone/isoalantolactone in *I. helenium* subsp. *turcoracemosa* was determined for the first time in this study. Also, this study is very important for the standardization of raw material and preparations of *I. helenium* roots.

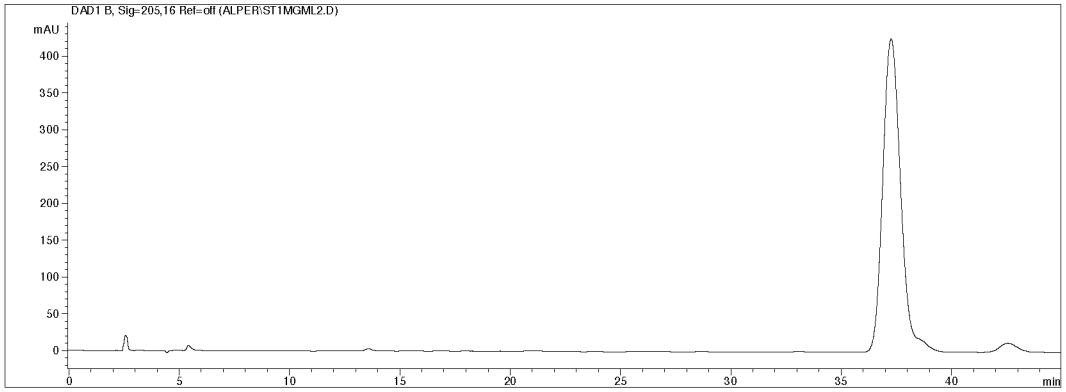


Figure 2. Chromatogram of standard alantolactone/isoalantolactone

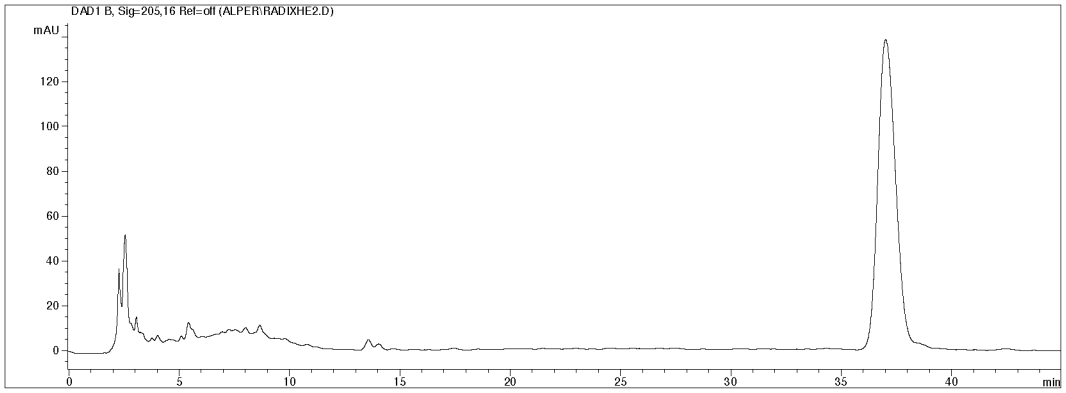


Figure 3. Chromatogram of *I. helenium* subsp. *turcoracemosa* roots

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