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Original article

Determination of Hispidulin in the flowers of *Inula viscosa* (L.) Aiton Using HPLC and HPTLC Methods

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The flavone hispidulin (4′,5,7-trihydroxy-6-methoxyflavone) in the flowers of *Inula viscosa* (L.) Aiton was quantified by using High Performance Liquid Chromatography (HPLC), and also High Performance Thin-Layer Chromatography (HPTLC) fingerprint of the methanolic *I. viscosa* flower extract was presented. Both methods provided a rapid and easy approach for determination of the biomarker hispidulin. In the present study HPTLC and HPLC profiles of Inula viscosa flowers were presented to detect and quantify the small flavonoid hispidulin. The results reveal that *I. viscosa* flowers contain serious amount (0.151± 0.007 g/100 g dry weight) of hispidulin.

Key words: Hispidulin, *Inula viscosa,* HPLC, HPTLC

Inula viscosa (L.) Aiton Çiçeklerinde YPSK ve YPİTK Yöntemleri ile Hispidulin Analizi

Inula viscosa (L.) Aiton çiçeklerinde bir flavon bileşiği olan hispidulin (4′,5,7-trihidroksi-6-metoksiflavon) miktarı Yüksek Performanslı Sıvı Kromatografisi (YPSK) ile tayin edilmiş, ayrıca I. *viscosa* çiçeklerinin metanolik ekstresinin Yüksek Performanslı İnce Tabaka Kromatografisi (YPİTK) parmakizi kromatogramı da verilmiştir. Her iki yöntem de biyo-işaretleyici bileşen olan hispidulinin belirlenmesi için hızlı ve kolay yaklaşımlar sağlamaktadır. Bu çalışmada küçük bir flavonoit bileşiği olan hispidulinin teşhis ve miktar tayinini gerçekleştirmek amacıyla *I. viscosa* çiçeklerinin YPİTK ve YPSK profilleri ortaya konmuştur. Sonuçlar, *I. viscosa* çiçeklerinin önemli ölçüde (0.151±0.007 g/100 g kuru ağırlık) hispidulin içerdiğini göstermektedir.

Anahtar kelimeler: Hispidulin, *Inula viscosa,* YPSK, YPİTK

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INTRODUCTION

Inula viscosa (L.) Aiton (Asteraceae) is a perennial herb that is found in most of the Mediterranean territories (1-3). It has been used for years in traditional medicine for its antiinflammatory, antimicrobial, antidiabetic, antihelmintic and antipyretic activities (4-14). Pharmacologicaly active compounds of *I. viscosa* are phenolic acids, terpenes (mono-, sesqui-, di-and triterpenes), flavonoids and glycolipids (15-24). In Turkey, *I. viscosa* is known as "yapışkan andız" and its fresh leaves are used for wound healing (25).

Hispidulin (4′,5,7-trihydroxy-6-methoxyflavone) is a naturally occuring flavone in a number of traditional Chinese medicinal herbs including *I. viscosa* (21, 26, 27). Several studies have indicated its antioxidant, antimicrobial, anticonvulsant, antithrombosis, antiosteoporotic, anti-inflammatory, antimutagenic and anticancer activities (26,27).

Herein, the qualitative and quantitative analyses of hispidulin which was previously reported to be isolated from *I. viscosa* flowers were carried out (21). Hispidulin should be used as a marker compound for especially standardization of *I. viscosa* preparations.

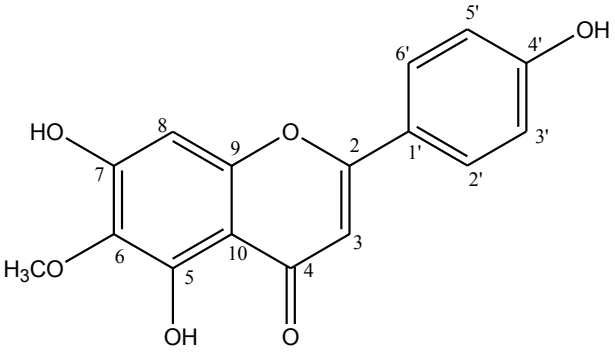

Figure 1. Structure of hispidulin

EXPERIMENTAL

Materials

Inula viscosa (L.) Aiton was collected from Isparta in October 2014. The flowers 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 26700).

Chemicals and standarts

Hispidulin (SML0582) was supplied from Sigma. All other chemicals were supplied from either Sigma or Merck.

Extraction

200 mg of dried and powdered flowers of *I. viscosa* were extracted with methanol (10 mL) by magnetic stirrer for 6 h (50°C, 250 rpm). The extract was then filtered using filter paper (Schleicher & Schuell MicroScience, Germany) and completed to 10.0 mL in a volumetric flask with methanol, passed through a 0.45 µm filter, and applied to the HPLC and HPTLC system as 10 µL and 5 µL, respectively.

HPLC Analysis

Chromatographic conditions and procedure

The qualitative and quantitative analysis of hispidulin in the methanol extract of *I. viscosa* flowers were performed according to the following procedure. The analysis was performed with an HPLC system consisting of an HP Agilent 1260 series quaternary pump, degasser, autosampler and diode array detector (DAD). The separation was achieved on ACE C18 column (5 □m, 250 mm; 4.6 mm). 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. Gradient elution was applied with a flow rate of 1 mL/min and column temperature was set to 30°C. The mobile phase was a mixture of trifluoroacetic acid 0.1% in water (solution A), trifluoroacetic acid 0.1% in methanol (solution B), trifluoroacetic acid 0.1% in acetonitrile (solution C). The composition of the gradient was (A:B:C), 80:10:10 at 0 min, 60:25:15 at 5 min, 50:30:20 at 10 min, 40:40:20 at 15 min and 0:75:25 at 20 min. The duration between runs was 5 min. All solvents were filtered through a 0.45 □m Milipore filter before use and degassed in an ultrasonic bath. From each solution and sample 10 □L were injected into the column and the chromatograms were recorded from 200 to 400 nm. Quantification was performed by measuring at 330 nm for hispidulin using

DAD. The chromatographic run time was set to 20 minutes.

Stock and standart solution

Hispidulin was prepared at the concentration of 3 mg/mL in methanol. Based on this, five different concentration of hispidulin (0.3 mg/mL, 0.15 mg/mL, 0.06 mg/mL, 0.015 mg/mL and 0.0015 mg/mL) were prepared for the establishment of calibration curves.

Calibration

Five different concentration of hispidulin as 0.0015 mg/mL, 0.015 mg/mL, 0.06 mg/mL, 0.15 mg/mL, 0.3 mg/mL were prepared and triplicate 10 □L 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 injection of hispidulin nine times at LOQ concentrations.

Precision

The precision of the method (intra-day and inter-day variations of replicate determinations) was checked by injecting hispidulin standard solution nine times at the LOQ levels in the same day and in two different days. The area values were recorded and RSD% values were calculated as RSD% = (SD / Mean) X 100.

Recovery

The spike recovery was carried out by the standard addition method. For the determination of the recovery, three different concentration of hispidulin standard solution (0.06 mg/mL, 0.015 mg/mL and 0.0015 mg/mL) were added prior to the extraction. In each additional level, six determinations were carried out and the mean value of recovery percentage was calculated.

HPTLC Analysis

Chromatographic conditions and procedure

HPTLC analysis was done using a Camag HPTLC system. Five microliters of samples were sprayed in the form of 8 mm bands with an Automatic TLC Sampler 4 (ATS 4, CAMAG, Switzerland) onto pre-coated HPTLC glass plates (20 x 10 cm, Si 60 F_{254}, Merck). The distance from the left side and the lower edge were 15 mm and 8 mm, respectively. Linear ascending development was carried out with an Automatic Developing Chamber 2 (ADC 2, CAMAG, Switzerland). Toluene-ethyl acetate-formic acid (5:4:1, v/v/v) was used as mobile phase. Relative humidity was not controlled. The twin through chamber was saturated for 20 minutes with 25 mL of the mobile phase before development. Ten mililitres of the same mixture were used for development. Plates were air dried before and after development by ADC2. Documentation of the plates was performed by Reprostar 3 (CAMAG, Switzerland) at 254 nm. Densitometric scanning was carried out at 254 nm with a TLC Scanner 3 (CAMAG, Switzerland) in absorption mode, and operated with WinCATS software.

RESULTS AND DISCUSSION

Herbal preparations are medicinal products consisting of single, two or more medicinal plants or plant extracts. Standardization of herbal preparations and botanicals are of great importance to the pharmaceutical and cosmetic industries. Several analytical methodologies including modern chromatographic techniques are being used for the chemical standardization of marker compounds or active constituents in herbal preparations. For this purpose, HPLC combined with different types of detectors (UV/Vis, MS, MS/MS) are preferred with priority (28-30).

In this study, reverse phase HPLC-DAD method was used for quantification of hispidulin in the methanol extract of *I. viscosa* flowers. Agilent 1260 series HPLC system was used and ACE C18 column was chosen for the best separation. To calculate the regression equation, five different concentration of the standard hispidulin were used to establish calibration curve. The equation was determined as y= 14051x-28.424. The correlation coefficient of hispidulin showed good linearity (R^2 = 0.9999). LOD and LOQ values were 0.0046 mg/mL and 0.0141 mg/mL, respectively. The RSD values for intra-day (repeatability) and inter-day (intermediate precision) variations were 2.13% and 2.72%, respectively. The recovery values were in the range of 94.6-98.2%. The chromatograms of standard hispidulin and the methanol extract of *I. viscosa* flowers were given in Figure 2 and 3. The peak of hispidulin was confirmed by comparing the retention time and UV spectrum of analyzed compound with authentic one (Figure 4).

Moreover, authentic hispidulin was added to the extract and the increase in the peak was observed. Retention time for hispidulin was 17.4 min. Consequently, the hispidulin amount of *I. viscosa* flowers was calculated as 0.151±0.007 g/100 g dry weight.

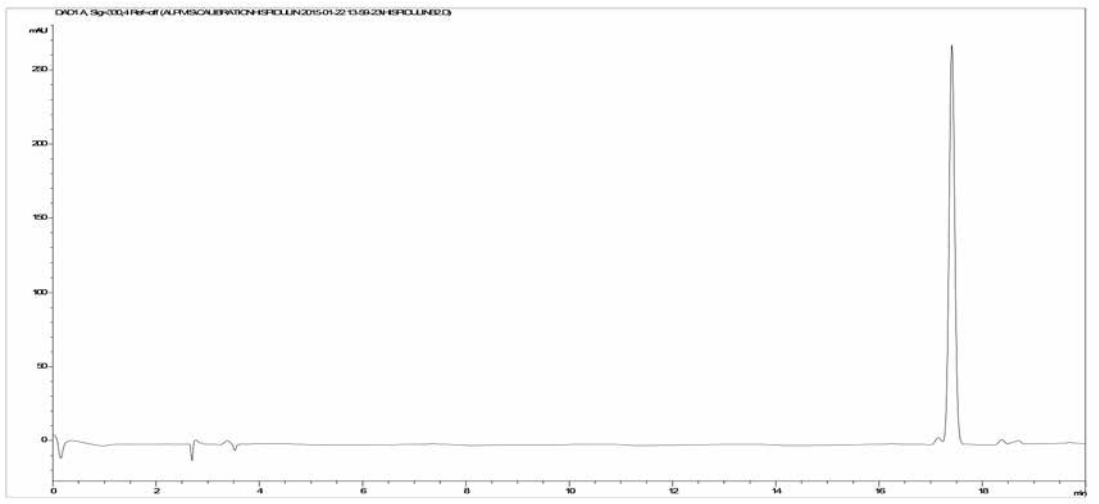

Figure 2. Chromatogram of the standard hispidulin

For the qualitative determination of hispidulin in *I. viscosa* flowers, HPTLC was also used. HPTLC system (CAMAG) equipped with ATS4, ADC2, Reprostar 3 and TLC Scanner 3 was used for the analysis. Various mobile phases were tried and toluene-ethyl acetate-formic acid (5:4:1, v/v/v) was chosen and used for the optimum separation. Three different concentration of *I. viscosa* flower extract (IV1-IV3) and six different concentration of standard hispidulin (H1-H6) were applied to the plate. The HPTLC chromatogram (fingerprint) was given in Figure 5. Hispidulin was clearly observed in the diluted and concentrated *I. viscosa* flower extracts with the same R_f value (0.59).

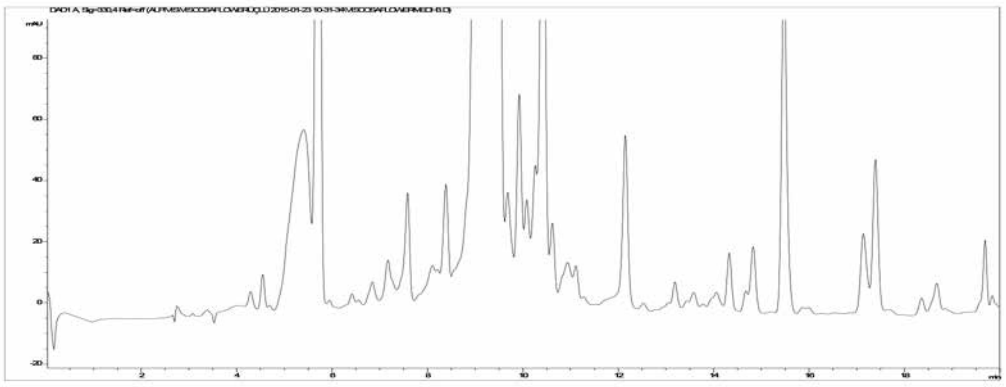

Figure 3. HPLC Chromatogram of the methanol extract of *I. viscosa* flowers

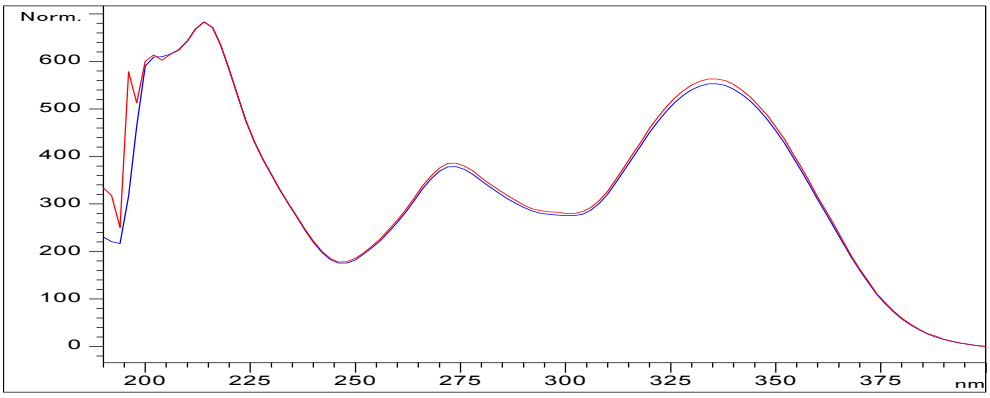

Figure 4. Overlaid UV spectra of standard hispidulin and hispidulin in the extract

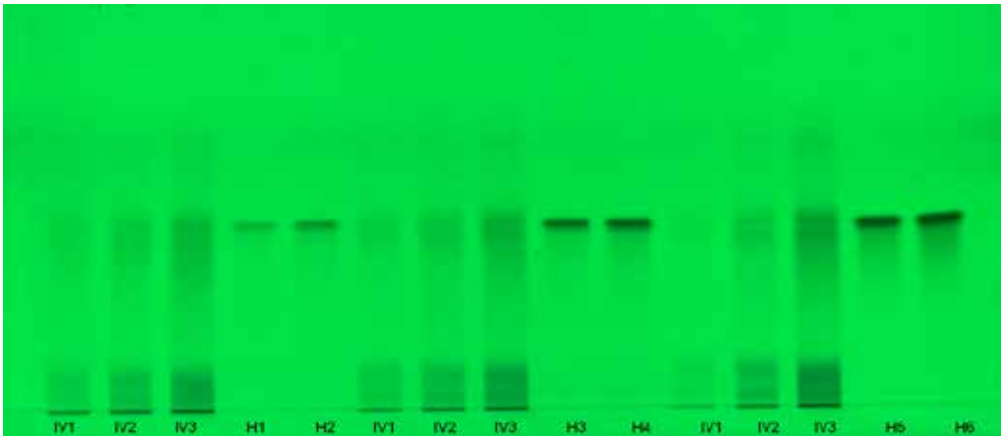

Figure 5. HPTLC chromatogram of *I. viscosa* flower extracts (IV1-IV3) and standard hispidulin (H1-H6) in different concentrations (without derivatization, 254 nm)

In conclusion, hispidulin which was isolated previously from *I. viscosa* flowers was chosen as marker compound, and standardization of the methanol extract of *I. viscosa* flowers was achieved for the first time in this study using a simple and efficient HPLC-DAD method.

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