FABAD J. Pharm. Sci., 36, 149-154, 2011

RESEARCH ARTICLE

Quantitative analysis of isoorientin in several Turkish *Gentiana* species by High Performance Liquid Chromatography

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Summary

Gentiana olivieri Griseb. (Afat, Gentianaceae) is used in Turkish folk medicine to reduce high blood glucose level in Southeastern Turkey. Through in vivo bioassay-guided fractionation procedures, the methanolic extract of G. olivieri yielded isoorientin as the active antidiabetic component. Same molecule has also exerted potent anti-inflammatory, antinociceptive, antioxidant and antihepatotoxic activities in rats and mice. Due to the wide range bioactivity profile of isoorientin, it has become important to find alternative herbal sources for this compound to analyze several Gentiana species (G. olivieri Griseb., G. asclepiadea L., G. cruciata L., G. gelida Bieb. and G. septemfida Pallas) for their isoorientin contents. In this study, an HPLC-UV method was used for determination of isoorientin contents in five Gentiana species. The precision of the analytical method was determined by applying the standard and test samples for five times. Standard deviation and relative standard deviation were determined for each sample by using "Instat" as the statistical program. The leaves of all Gentiana species, except G. gelida, were found to have isoorientin at higher concentration levels compared to their flowers and stems, whereas the isoorientin content of G. gelida flowers was higher than the other parts of this species. According to the results of current HPLC analysis, the highest isoorientin content was found in the leaves (3.70%) and flowers of G. olivieri (3.28%), the flowers of G. gelida (3.52%), and the leaves of G. asclepiadea (3.07%), while in lesser amounts in the flowers of G. asclepiadea (1.84%) and the leaves of G. cruciata (2.27%).

Key Words: *Gentiana, G. asclepiadea, G. cruciata, G. gelida, G. olivieri, G. septemfida, HPLC, isoorientin*

Türkiye'de yetişen çeşitli Gentiana türlerinin izoorientin miktarlarının Yüksek Basınçlı Sıvı Kromatografisi ile analizi

Özet

Gentiana olivieri Griseb. (Afat, Gentianaceae) Güneydoğu Anadoluda kan şekerini düşürmek amacıyla, halk ilacı olarak kullanılır. Anabilim dalımızda, G. olivieri metanollü ekstreleri üzerinde yapılan aktivite yönlendirmeli fraksiyonlama çalışmaları sonucunda, antidiyabetik etkili maddenin izoorientin olduğu tespit edilmiştir. Aynı maddenin sıçan ve fareler üzerinde yapılan çalışmalarda, antienflamatuvar, antinosiseptif, antioksidan ve antihepatotoksik etkilere sahip olduğu bulunmuştur. Bu geniş aktivite profilinden dolayı alternative kaynak olarak çeşitli Gentiana türlerinin (G. olivieri Griseb., G. asclepiadea L., G. cruciata L., G. gelida Bieb. and G. septemfida Pallas) izoorientin miktarlarını tespit etmek önemli olmaktadır. Bu çalışmada, beş Gentiana türünün izoorientin miktarlarını tayin etmek amacıyla bir HPLC- UV metodu kullanılmıştır. Analitik metodun doğrulaması için standart ve numunelerden en az beş tatbik yapılmıştır. Her numune için, standart hata ve bağıl standart hata değerleri "Instat" istatistik programı kullanılarak hesaplanmıştır. G. gelida dışındaki tüm Gentiana türlerinde yaprakların, çiçek ve gövdeye oranla daha fazla izoorientin ihtiva ettikleri bulunmuştur. HPLC çalışmalarımızın sonucuna göre; En yüksek izoorientin içeriği G. olivieri yaprak (%3.70) ve çiçeklerinde (%3.28), G. gelida çiçeklerinde (%3.52) ve G. asclepiadea yapraklarında (%3.07) bulunmuştur. Ayrıca G. asclepiadea çiçeklerinde (%1.84) ve G. cruciata yapraklarında (%2.27) ise bunlara göre daha az miktarda izoorientin tespit edilmiştir.

Anahtar Kelimeler: *Gentiana, G. asclepiadea, G. cruciata, G. gelida, G. olivieri, G. septemfida, HPLC, isoorientin*

Received: 12.12.2012
Revised: 20.02.2013
Accepted: 26.03.2013

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INTRODUCTION

Ethnobotanical field surveys have reported that the flowering aerial parts of *Gentiana olivieri* (Gentianaceae) is used as stomachic, stimulant for appetite, to reduce blood sugar in diabetics, against feverish symptoms, and to treat the symptoms of various mental illnesses in southern parts of Turkey (1-3). In a previous *in vivo* bioassay-guided fractionation study, isoorientin was isolated as the main active component responsible for the control of blood sugar in Type II diabetes (4). Isoorientin is a C-glucosylflavonoid, and the earlier investigations have revealed that isoorientin also displayed *in vivo* and dose-dependent analgesic, anti-inflammatory, antihyperlipidemic, antioxidant and hepatoprotective activities, in addition to its hypoglycemic effects (5-6).

Due to this wide range of activity profile of isoorientin, it has attracted the attention of the scientific community in drug discovery, to develop new and potent therapeutic agents. Although Kumazawa et al. (7) have recently developed a method for the bench-top synthetic production of isoorientin; its discovery from new natural sources is still an important task.

There are twelve *Gentiana* species grown in Turkey (8). The purpose of this investigation was to analyze the isoorientin content of several *Gentiana* species, to find out possible new source/sources for this compound. In this study, the flowers, leaves, and stems of the selected *Gentiana* species of Turkish origin were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) technique,s for their isoorientin contents. We hereby report the first HPLC study on quantification of isoorientin in *G.gelida, G. asclepiadae, G. cruciata* and *G.septemfida.*

MATERIALS AND METHODS

Chemicals

Isoorientin, as an authentic sample was purely isolated from *G. olivieri* by Dr. Mustafa Aslan (Faculty of Pharmacy, Gazi University, Ankara, Turkey) (6). HPLC grade methanol (Merck) and bidistilled water were used for the chromatographic studies.

Plant Materials

The *Gentiana* species were collected from various locations in Turkey. *G. olivieri* was collected in June; other species in July of 2002 and the analysis were performed in the same year. Collection sites of the plants and altitudes are given below. All the samples were identified by Dr. Osman Ketenoğlu (Faculty of Science, Ankara University, Ankara, Turkey). The specimens were stored in the Herbarium at the Faculty of Pharmacy of Gazi University (GÜEF).

G. asclepiadea L., Trabzon; Çaykara, Uzungöl Plateau, 1090 m (GÜEF 2319)
G. cruciata L., Kastamonu; Mount Ilgaz, 2000 m (GÜEF 2317)
G. gelida Bieb., Erzurum; Ilıca- Ispir Road, Gölyurt Pass, 2350 m (GÜEF 2316)
G. olivieri Griseb., Gaziantep- Araban Road, 600 m (GÜEF 99E001)
G. septemfida Pallas, Trabzon-Zigana Pass, 2010 m (GÜEF 2318).

Preparation of the Samples

The air-dried aerial parts of the mentioned species were divided into the flowers, leaves, and stem and ground to powder. Powdered (1 g) samples were extracted with 50 ml methanol twice, at 40ºC, for 24 hours, at room temperature. Combined methanolic extracts were evaporated to dryness in *vacuo*. The crude extracts were dissolved in HPLC grade methanol; suspended particles were removed through a membrane filter (Alltech, 0.45 µm). Appropriate dilutions were prepared and analyzed by chromatographic methods (TLC and HPLC).

TLC Analysis

TLC analysis was carried out on Kieselgel 60 F254 (precoated 0.2 mm thickness ready to use TLC plates, Merck 5554). The solvent systems consisting of either chloroform- methanol- water (61:32:7) (S1) or ethyl acetate -glacial acetic acid–formic acid–water (100:11:11:27) (S2) were used. Isoorientin (purple) was first detected under UV light at 366 nm, when the chromatogram was sprayed over with Naturstoff Reagent (NA, UV366) (Diphenylboric acid ethanolamine complex in methanol), the zones were observed to change to orange color.

HPLC Analysis

HPLC system consisted of a Hewlet-Packard High Performance Liquid Chromatography-1050 pump. Rheodyne 7125 injection valve, fitted with 20 µ loop, added to model HP-1050 UV detector and HP-3996A integrator. Separations were achieved with a reverse phase column LiChrospher 100 RP-18 (5 µm particle size, 4.6 × 250mm). An isocratical solvent system of water-methanol-glacial acetic acid (65:35:5, v/v/v) was employed as mobile phase at the flow rate of 0.8 ml/min. This solvent system described by Wulf et al (9) was modified by us. The column pressure was kept at 179-181 bars and the peaks were monitored at 354 nm wavelength with the paper speed of 0.3 cm/min. 10 µl of the samples were analyzed and each analysis was repeated for 5 times. The peaks were identified by comparison of the retention times with those of the authentic sample of isoorientin.

Quantitative determination

For quantification, the external standard method was applied. Calibration curves were established by repeated injections (n = 5) of the standard solutions. In order to prepare the standard solutions, 1.6 mg of isoorientin was dissolved in methanol and added to 10 ml in a volumetric flask. From this solution, 0.2, 0.4, 0.6, 0.8, and 1.0 ml were taken respectively and then, adjusted to 2.0 ml volume. The concentration of the standard solutions ranged from 0.016 to 0.080 mg/ml. The area of the individual target peaks and the corresponding concentrations were utilized to obtain the calibration curve.

Limits of detection and quantification

Limits of detection (LOD) were established at a signal to noise ratio (S/N) of 3 (LOD: 0.0286 mg/ml). Limits of quantification (LOQ) were established at a signal to noise ratio (S/N) of 10 (LOQ: 0.086 mg/ml). LOD and LOQ were experimentally verified by five injections of the standard solution at LOD and LOQ concentrations.

Precision

The precision of the method was checked by injection for five times of the standard solution at the LOQ level.

RESULTS AND DISCUSSIONS

In the present study, the methanolic extracts obtained from the flowers, leaves and stems of the five *Gentiana* species; *G. olivieri* Griseb., *G. gelida* Bieb., *G. septemfida* Pallas, *G. asclepiadea* L., and *G. cruciata* L. were comparatively analyzed for their isoorientin content using two chromatographic methods.

The methanol extracts of the studied species were first applied on silica gel plates in two different solvent systems (S1 and S2). The R_f value of standard isoorientin was calculated as 0.4 and 0.5 in S1 and S2, respectively. TLC results revealed that isoorientin was the major component in the flowers and leaves all of the species. Isoorientin high intensivity spots were observed in the extracts belonging to the *G. septemfida* flowers (R_f 0.2) and *G. asclepiadea* leaves (R_f 0.4) in comparison to those in *G. olivieri* plant parts. TLC data also showed that the extracts from the other two species *G. gelida* flowers and *G. cruciata* flowers also contained considerable amount of isoorientin.

For the quantitative analysis of isoorientin in samples, HPLC method was used on a LiChrospher RP 18 column and water-methanol-glacial acetic acid (65:35:5) as mobile phase and isocratic elution. The peaks were identified by comparison to the retention time of standard isoorientin, which was found to be 10.6 min. HPLC chromatograms of the extracts are given in Figure 1.

The detector response was linearity correlated with concentration in the ranges 0.016 -0.080 mg/ml for isoorientin. The regression equation and correlation coefficient were determined as $y = 736750\times+1304.4$ ($r^2 = 0.9992$) and employed to calculate the percentage of isoorientin. The precision of the analytical method was determined by assaying standard and test samples for five times. Isoorientin content in each species, standard deviation (SD) and relative standard deviation (RSD %) values are given in Table 1 and Figure 2.

Our results indicated that the leaves of all *Gentiana* species, except *G. gelida*, contained isoorientin at higher concentration levels, compared to their flowers and stems. In contrary, the isoorientin

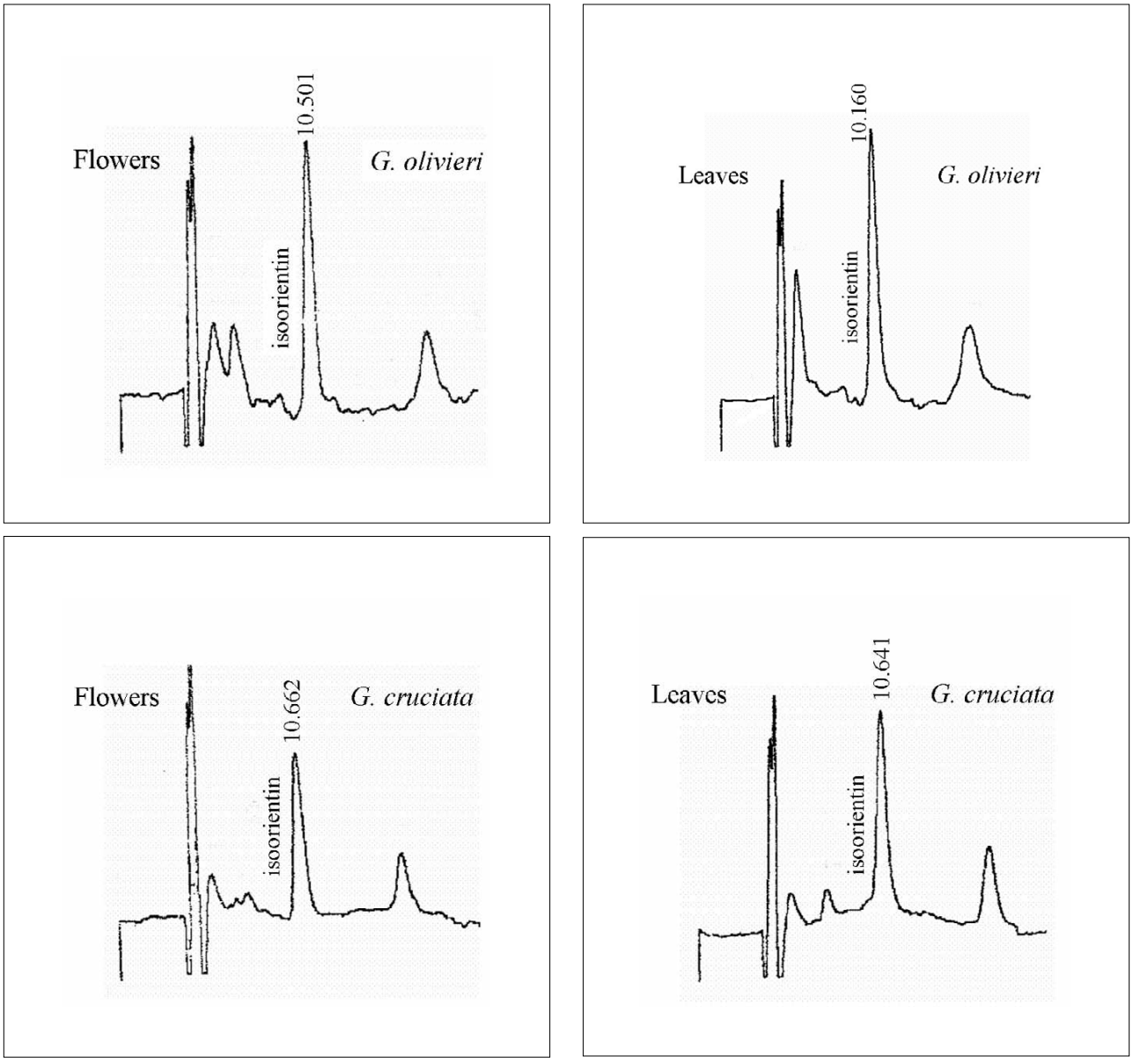

Figure 1. Representative HPLC chromatograms of the methanol extracts of *G. olivieri* and *G. cruciata*.

Table 1. Isoorientin content (%), standard deviation (SD) and relative standard deviation (RSD%) of *Gentiana* species.

Isoorientin (%) ±Standard Deviation						
Plant Species	**Flowers**	**RSD%**	**Leaves**	**RSD%**	**Stems**	**RSD%**
G. gelida	**3.516 ±0.081**	2.3037	0.883 ±0.021	2.3782	0.264 ±0.008	3.0303
G. olivieri	**3.280 ±0.149**	4.5426	**3.704 ±0.071**	1.9168	1.362 ±0.041	3.0102
G. septemfida	0.887 ±0.012	1.3528	1.519 ±0.056	3.6866	0.117 ±0.001	0.8547
G. asclepiadea	1.846 ±0.024	1.3001	**3.072 ±0.064**	2.0833	0.100 ±0.005	5.0000
G. cruciata	1.796 ±0.029	1.6146	**2.278 ±0.099**	4.3459	0.241 ±0.011	4.5643

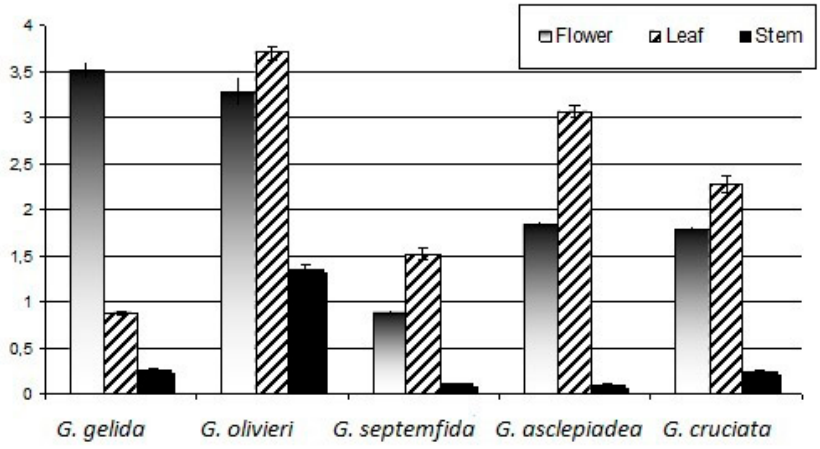

Figure 2. Isoorientin contents in different parts of five *Gentiana* species

content of *G. gelida* flowers was higher than the other parts of this species. Besides, isoorientin levels were observed as rather low in all stem samples of the analyzed species.

According to the results of HPLC analyses, the highest level of isoorientin was observed in the *G. olivieri* leaves (3.70%) and flowers (3.28%). The isoorientin concentration of other species was also found comparable to *G. olivieri, i.e.* the flowers of *G. gelida* (3.50%) and *G. asclepiadea* (1.84%); the leaves of *G. asclepiadea* (3.07%) and *G. cruciata* (2.27%) (Table 1 and Figure 2). HPLC chromatograms of *G. olivieri* and *G. cruciata* plant parts are shown in Figure 1.

The chemical content in various *Gentiana* species, isoorientin in particular, has been previously reported by different research groups. Zhang et al. (10) reported the isoorientin content of between 0.563% and 0.937% in the flowers of *Gentiana straminea* Maxim, collected from ten different locations in China. They also concluded that the isoorientin content of the flowers were affected by the ecological environment. In another study, seasonal variations in the chemical composition of *G. lutea* L. were investigated. HPLC analysis of this plant revealed that isoorientin level reached its highest concentration in June and July and the leaves contained higher isoorientin (0.51-0.93%) than the flowers (0.32-0.43%) (11).

Isoorientin has been reported to be present in many different plant species such as *Passiflora*, *Patrinia*, *Rumex*, *Swertia*, *Bambusa* and *Vitex species* (12,13). In the first study, the quantification of isoorientin has been determined in passion leaves using HPLC, UV method. The contents of isoorientin in *Passiflora alata*, *P. edulis*, *P. caerulea* and *P. incarnata* have been found to be 1.115, 1.095, 1.590 and 1.584 %, respectively (14). In the second study, the content of isoorientin has been calculated 0.123 % in healthy rinds of *P. edulis* by HPTLC with densitometric analysis (15). Wang et al. (16) reported the isoorientin content between 0.1% and 0.278% in three *Bambusa* leaves, using HPLC, UV/DAD method. Our results revealed that the leaves and flowers of all *Gentiana* species contained higher concentrations of isoorientin compared to other sources of plants mentioned above.

Conclusion

In all *Gentiana* species studied in the current study, the highest isoorientin contents were determined in the leaves (3.70%) and the flowers (3.28%) of *G. olivieri*, the flowers (3.52%) of *G. gelida* and the leaves (3.07%) of *G. asclepiadea*. When compared to those of the previous reports, *G. olivieri* seems to be the richest source of isoorientin, among the *Gentiana* species, which have been investigated so far. Therefore, *G. olivieri* is a potential candidate for further investigations for the production of isoorientin by agricultural or plant tissue culture techniques.

ACKNOWLEDGEMENTS

This study was supported by Gazi University Research Fund under project number: 02/2002/14.

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