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Quantitative Assessment of Diosgenin from Different Ecotypes of Iranian Fenugreek (*Trigonella foenum-graecum* L.) by High-performance Liquid Chromatography

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Abstract

In this study, a sensitive, accurate and reproducible high performance liquid chromatographic method was developed for determination of diosgenin from fenugreek (*Trigonella foenum-graecum*) leaves in 22 ecotypes of Iranian fenugreek. The obtained results showed that the highest concentration of diosgenin with 23.8 mg g^{-1}dw was found in Boshruyeh ecotype. While, Mashhad ecotype with 3.51 mg g^{-1}dw diosgenin contained the lowest concentration of diosgenin. The method employed in current study confirmed significant variations in the diosgenin concentration in Iranian fenugreek. Therefore, Iranian fenugreek can be considered as a new potential crop and source of diosgenin in the pharmaceutical industry.

Keywords: Dioscin, HPLC, quantitative determination, secondary metabolites, steroidal saponin.

Introduction

Natural products available at plant tissues provide a diverse and unique source of bioactive compounds for medicine development. Despite significant progress in the pharmaceutical industry, substantial proportion of the medicines in the last 25 years, obtained directly from natural products or their semi-synthetic modification (Ressmann et al., 2012). Saponins are a structurally diverse class of natural compounds which are widely distributed in the plant kingdom and involved in plant defense systems against pathogens. Chemically, they are related to triterpenic or steroidal glycosides that structurally consist of nonpolar aglycones coupled to one or more polar saccharide. The structure of saponins is the main factor of their surface-active characteristics (Güçlü-Üstündağ and Mazza, 2007). Many kinds of saponins which isolated from different plant sources have wide range of physicochemical and biological functions such as foam production, sweetness and bitterness, antimicrobial and molluscicide that can be used in food industry, pharmaceutics, cosmetics and bioremediation (Kalinowska et al., 2005).

Diosgenin, is a steroidal sapogenin and the product of acids or enzymes hydrolysis process of dioscin and protodioscin mostly from *Dioscorea* and *trigonella* species

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(Fig. 1). This metabolite has considerable application as a bioinsecticide. In fact, in the pharmaceutical industries, it wildly used as a precursor in the synthesis of some essential steroidal drugs such as cortisone, pregnenolone and progesterone (Fernandes et al., 2003). Natural diosgenin is mainly obtained from some wild species of Mexican yam (*Dioscorea* sp.). Although this process is time-consuming and costly and also takes several years that tubers reach the proper size for profitable extraction of diosgenin (Rosser, 1985). With these reasons, fenugreek (*Trigonella foenum-graecum* L.) can be a suitable alternative for the natural source of diosgenin, because of its shorter growing period, cheaper production costs and more consistency in yield and quality.

Fenugreek (*Trigonella foenum-graecum* L.) is an annual, dicotyledonous plant, belonging to the *Fabaceae* family. Generally, the genus of *Trigonella* included 135 species which the most of them originated from southeastern Europe and Western Asia (Martin et al., 2011). De Candolle believes that the origin of fenugreek should be Asia rather than Southern Europe, because if a plant of fenugreek nature was indigenous in Southern Europe it would be far more common (De Candolle, 1886). Fazli and Hardman noticed that fenugreek grows wild in Punjab and Kashmir, in the desert of Mesopotamia and Persia, in Asia Minor and in some countries in Southern Europe such as Greece, Italy and Spain (Fazli and Hardman, 1968).

Fenugreek is one of the oldest medicinal plants which has many applications in traditional medicine to reduce blood glucose and cholesterol levels (Mathur and Mathur, 2005). The positive effect of fenugreek can be related to its bioactive compounds such as saponins, flavonoids, alkaloids, mucilaginous fiber, volatile oils and lysine-rich proteins (Mehrafarin, 2011). Profiling and discovery of different natural products from fenugreek including steroidal saponins is crucial for metabolomics and functional genomics studies (Huhman and Sumner, 2002). Previous chemical investigations of fenugreek also led to the identification of several new steroidal saponins (Zhang et al., 2012). It is reported that fenugreek contains a variety of steroidal saponins. Among them, dioscin as a significant water insoluble saponin, often used to obtain diosgenin through hydrolysis process (fig. 1).

Several methods have been reported for determination of diosgenin such as HPTLC, HPLC, LC ESI-MS and ELISA. Although, it has been reported that LC ESI-MS approach is the most sensitive method but it cannot be commonly used for analytical laboratories and institutes due to requirement of unique instruments and experienced experts (Hwang et al., 2011). Among them, spectrophotometry and high performance liquid chromatography are the common approaches for diosgenin analysis. HPLC method has also been extensively used for quantification of secondary metabolites and natural products (Li et al.,2012).

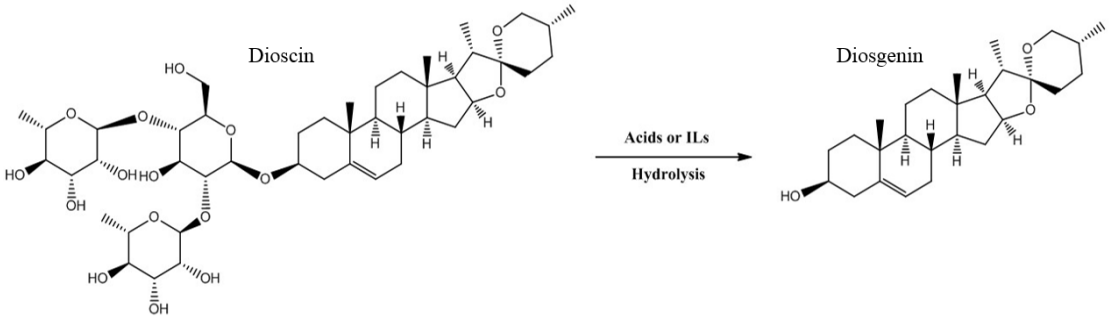

Fig. 1. Dioscin hydrolysis process which catalyzed by inorganic acids or ionic liquids aqueous solution

Since Iran is one of the largest worldwide producer of fenugreek, the development of simple and efficient methods for diosgenin analysis from Iranian fenugreek plants, could be a valuable contribution to the use of Iranian natural resources in green chemistry. To the best of our knowledge, there are no reports regarding diosgenin analysis of Iranian fenugreek.

The purpose of this study was to compare the content of diosgenin in different ecotypes of Iranian fenugreek by HPLC analysis. The results of this study provide valuable information about the content of diosgenins in many ecotypes of Iranian fenugreek; in addition provide a broad data set about this plant, which can be used as a source for the study of gene expression and transcriptome analyses.

Materials and Methods

Plant materials and growth conditions

The seeds of selected ecotypes including Arak, Shushtar, Yazd, Zanjan, Ardabil, Khash, Mashhad, Ardestan, Qaen, Shiraz, Damavand, Bushehr, Urmia, Shahr-e Kord, Hamadan, Mahallat, Sari, Boshruyeh, Varamin, Rafsanjan, Dezful and Kermanshah were requested from gene bank (National Botanical Garden of Iran, Research Institute of Forests and Rangelands) and then cultivated in a sterilized mixture of soil: perlite (1:1) under greenhouse conditions at 25 and 18°C day and night temperatures, respectively and a photoperiod of 16 and 8 hours light and dark, respectively. Sampling was conducted in the first peak of flowering (45 days after planting in research greenhouse of Tarbiat Modares University).

Chemicals

HPLC grade acetonitrile and all other solvents for extraction were purchased from Merck Company (Darmstadt, Germany). Diosgenin standard with 96% purity was acquired from Sigma-Aldrich. HPLC water was obtained from Millipore with SPE column.

Analytical method

Ultrasonic-CleanerXPS240-4L water baths (Sharpertek, USA) were used in the extraction step. The generators of these ultrasonic water baths have frequency of 60 kHz. The content of diosgenin was evaluated by high performance liquid chromatography system (Waters, USA) with an SPDM20A PDA detector and a Strategy C18 column (150 mm × 3 mm, 5 µm). During this process, 20 µl of the filtered extract was injected into the column at room temperature. The mobile phase composition was acetonitrile: water (90:10, v/v), at the flow rate of 0.4 ml min^{-1}, with ultraviolet (UV) detection at 210 nm (Li et al,. 2012). The peak identification was carried out by comparing its retention time with that of the corresponding peak in the standard diosgenin solution. All the experiments were performed with three replications. The content of diosgenin was determined based on the standard curve of diosgenin (Fig. 2). The concentration of diosgenin was used to evaluate each extraction method.

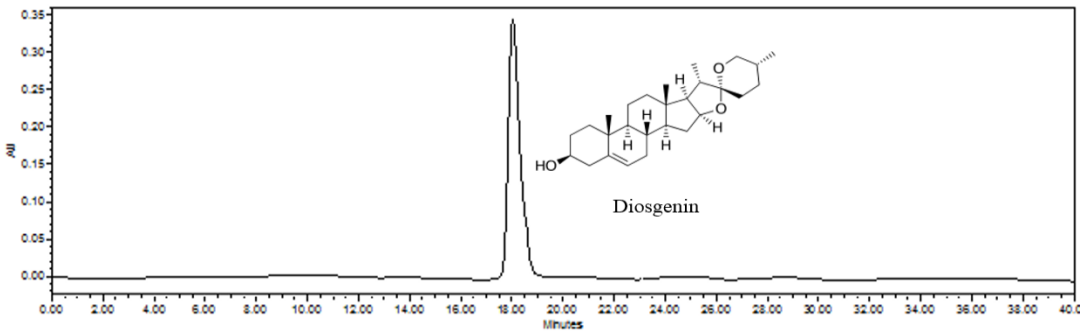

Fig. 2. The standard curve of diosgenin

Preparation of diosgenin from T. foenum-graecum leaves

Diosgenin extraction was performed as described previously with some modifications (Zhu et al., 2010). Briefly, 1.0 g of ground raw plant material was added into a tube with 20 ml of 96% ethanol and then subjected to ultrasonic extraction for 30 min. Ethanol is a solvent for preparation of total extract and extraction of glycoside saponins such as dioscin which in next step converts to diosgenin during the hydrolysis process. Then, sulfuric acid 2N (20 mL) was added and hydrolyzed under reflux conditions at 100°C for 120 min. The analyte was partitioned with *n*-Hexane for three times. The combined *n*-Hexane solution was washed twice with 1 mol L^{-1} of NaOH solution, and then twice with distilled water. After dehydration with anhydrous sodium sulfate, the *n*-Hexane solution was concentrated to dryness under vacuum conditions in a rotary evaporator. The extract was dissolved in acetonitrile and filtered with 0.22 μm before injection (Li et al.,. 2011). All the experiments were performed with three replications.

Results

Morphological analysis

To evaluate the morphological diversity of evaluated ecotypes of fenugreek, different factors such as days to first flowering, mean leaf number, height of stem, petiole length, leaf length and leaf width were measured. The results of statistical analysis showed a significant difference for morphological factors among ecotypes. Statistical analyzes were performed using SPSS 24.0 software.

Comparison of ecotypes in terms of days to first flowering showed that emergence of first flower in the Ardebil, Arak, Damavand, Shushtar, Urmia, Rafsanjan, Bushehr, Mahalat and Qaen ecotypes 38 days after planting. The results of data analysis showed that the Zanjan ecotype has a significant difference with other ecotypes at the level of 1%, which had the first flowers 60 days after planting (Table 1).

Table 1. Comparison of morphological factors measured in different ecotypes of Iranian fenugreek

Ecotype	Average values					
	Day to first flowering	Number of leaves	Length of stem	Length of petiole	Length of leaf	Width of leaf
Rafsanjan	38 days	7	16.6	2	1.8	1
Kermanshah	47 days	8	19.6	3.3	2.4	1.2
Hamadan	40 days	8	27.3	3.4	2.5	1.8
Mashhad	40 days	9	9	3.3	2.6	1.4
Zanjan	60 days	8	20.6	2.9	2	1.2
Arak	38 days	7	24.6	1.6	1.7	0.68
Shushtar	38 days	6	25	3	1.6	1
Damavand	38 days	7	18	3.4	1.6	1
Yazd	55 days	7	21	3.3	2.6	1.2
Shahr-e Kord	52 days	8	20.3	2.9	1.9	0.9
Mahallat	38 days	6	21.6	3	2.3	1.1
Sari	45 days	11	23.3	3.1	2.7	1.8
Shiraz	45 days	9	25.3	3.3	2.5	1.2
Ardestan	55 days	8	20.6	3	2.3	1.1
Ardabil	38 days	7	23.3	2.3	1.5	1.1
Bushehr	38 days	8	20.6	3.2	1.8	1
Dezful	40 days	8	20	2.5	2.3	1.2
Qaen	38 days	8	21.4	4.3	1.8	1.3
Khash	55 days	9	25	2.2	2	1.3
Varamin	52 days	8	28	2	1.8	1
Urmia	38 days	6	18.3	2.1	1.5	1
Boshruyeh	55 days	11	27.3	4.6	2.5	1.7

The number of leaves may be the most important indicator of performance from the consumer's point of view. Among the 22 ecotypes which evaluated in this study, Boshruyeh and Sari ecotypes had the highest and Mahalat, Shushtar and Urmia ecotypes had the lowest number of leaves (Table 1).

Generally, the length of stem is more sensitive to environmental factors and is less affected by genetic factors. Among the 22 ecotypes which evaluated in this study, the ecotypes of Qaen, Hamadan and Varamin had the highest and Rafsanjan, Urmia and Damavand had the lowest stem length (Table 1).

The results of data analysis showed that the studied ecotypes of fenugreek did not have a significant difference in terms of leaf length and width and the length of petiole.

Metabolic analysis

The highest concentrations of diosgenin (23.8 mg g^{-1} dw) were found in the extracts obtained from Boshruyeh ecotype. On the other hand, Rafsanjan ecotype showed the lowest concentration (3.51 mg g^{-1}dw) of diosgenin. The results showed the high diversity in the fenugreek samples and this method can be employed as a quantification method for determination of diosgenin in fenugreek leaves and pods. These results are shown in Figure 3 and 4.

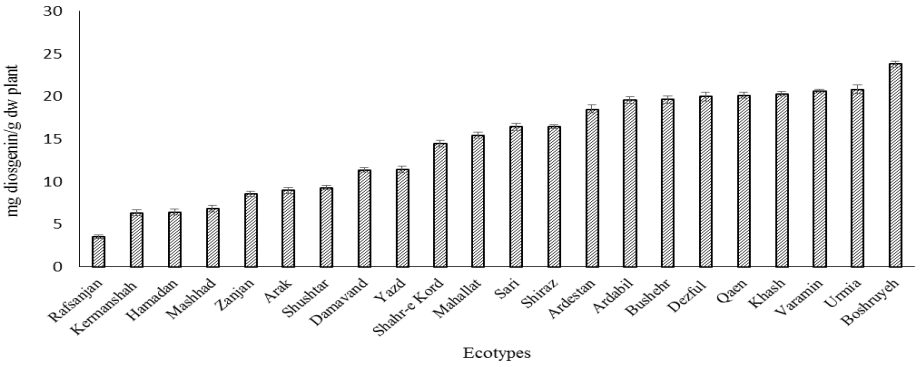

Fig. 3. HPLC analysis of diosgenin in different ecotypes of Iranian fenugreek.

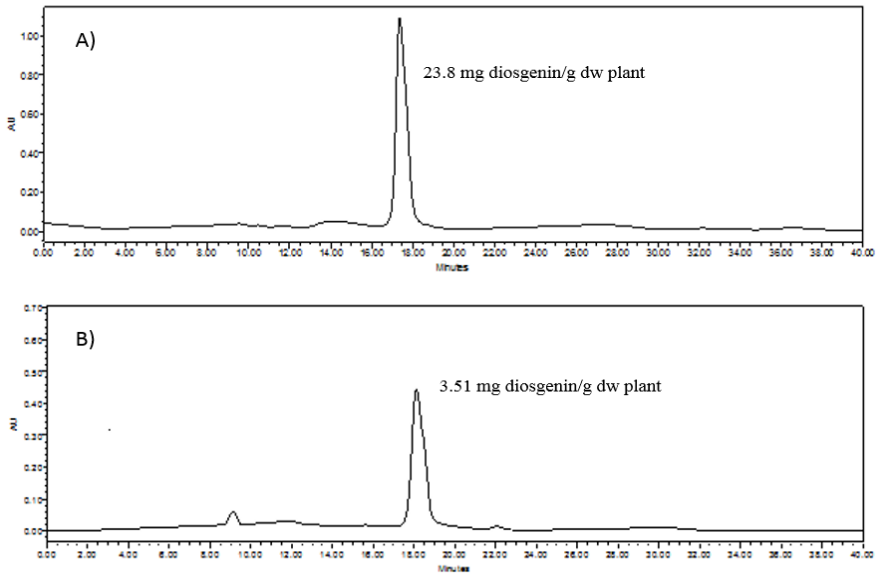

Fig. 4. Chromatograms of diosgenin (A) Boshruyeh ecotype with the highest content of diosgenin and B) Rafsanjan ecotype with the lowest concentration of diosgenin at 210nm. Column: HPLC C18. Mobile phase: Acetonitrile: water (90:10, v/v). Flow rate: 0.4 ml min^{-1}

Discussion

This is the first report to determine diosgenin content of Iranian fenugreek by HPLC analysis in the same time and the same cultivated location. The rapidity, accuracy, simplicity and economically of application of this proposed method make it an optimal analytical alternative approach for analyzing of diosgenin content. This study proved that the HPLC method have a high potential for selective quantification of diosgenin from fenugreek leaves. In this study, we managed to develop an effective strategy for separating of diosgenin from fenugreek leaves by using ultrasonic-assisted dissolution. This strategy allowed isolating diosgenin in acceptable yields. The results of this study can be used for improving the quality of this valuable vegetable, as well as providing a useful genomic source for further studies in order to produce cultivars with higher quality and more diosgenin content. Development of an efficient method for extraction and quantification of diosgenin from fenugreek leaves (*T. foenum-graecum* L.) gives a chance for effective exploitation of this metabolite and provides a chance for further studies on pharmacological activity of steroidal saponins. Therefore, the extracts of 22 ecotypes of Iranian fenugreek validated with sensitive HPLC method. Laila and Murtaza (2014) optimized two methods, HPLC and HPTLC, for quantification of steroidal saponins from fenugreek seeds. They reported that the HPLC method is an attractive simple, rapid, and selective method for quantitative determination of steroid saponins in fenugreek extract. They suggested that this method could be widely applied for direct routine analysis and quality assurance of related extracts and drugs (Laila et al., 2014).

Ariburnu and Fazli Uludag (2012) validated the content of diosgenin in seeds and leaves of 10 ecotypes of Indian fenugreek by HPLC method. The result showed that the amount of diosgenin in the leaves of the studied ecotypes was significantly higher than seeds (Ariburnu et al., 2012).

Dangi and Misar (2014) compared some species of *Trigonella* and *Dioscorea* in terms of the potential of diosgenin production. They concluded that although, the potential for the production of diosgenin in the species of *Dioscorea* is higher than *Trigonella* species, but *Trigonella* can be a suitable, alternative source for the production of diosgenin (Dangi et al., 2014). Hence, finding the ecotype of the fenugreek with the highest potential for the production of diosgenin can be of great importance.

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References

1. Ariburnu E, Uludag M.F, Yalcinkaya H, Yesilada E. 2012. Comparative determination of sibutramine as an adulterant in natural slimming products by HPLC and HPTLC densitometry. Journal of pharmaceutical and biomedical analysis 64, 77-81.
2. Dangi R, Misar A, Tamhankar S, Rao S. 2014. Diosgenin content in some Trigonella species. Indian Journal of Advances in plant Research 1, 47-51.
3. De Candolle A. 1886. Origin of cultivated plants (Hafner, New York, 1967). English translation of the second edition**.** 316-321.
4. Fazli F, Hardman R. 1968. The spice, fenugreek (*Trigonella foenum-graecum* L.): Its conmmercial varieties of seed as a source mehrafarin and authors of diosgenin. Tropical Science 10, 66-78.
5. Fernandes P, Cruz A, Angelova B, Pinheiro H.M, Cabral J.M.S. 2003. Microbial conversion of steroid compounds: recent developments. Enzyme and Microbial Technology 32, 688-705.
6. Güçlü-Üstündağ Ö, Mazza G. 2007. Saponins: properties, applications and processing. Critical reviews in food science and nutrition 47, 231-258.

7. Huhman D.V, Sumner L.W. 2002. Metabolic profiling of saponins in Medicago sativa and Medicago truncatula using HPLC coupled to an electrospray ion-trap mass spectrometer. Phytochemistry 59, 347-360.
8. Hwang W.T, Kim Y, Hong C, Lee J, Park D. 2011. Simple stability-indicating validated HPLC method for diosgenin in cosmeceutical formulations with long term stability application for light and heat. International Journal of Pharmacy and Pharmaceutical Sciences 3, 466-471.
9. Kalinowska M, Zimowski J, Pączkowski C, Wojciechowski Z.A. 2005. The formation of sugar chains in triterpenoid saponins and glycoalkaloids. Phytochemistry Reviews 4, 237-257.
10. Laila O, Murtaza I, Abdin M, Ahmad S, Ganai N.A, Jehangir M. 2014. Development and validation of HPTLC method for simultaneous estimation of diosgenin and quercetin in fenugreek seeds (Trigonella foenum-graceum). ISRN Chromatography.
11. Li P, Mao Z, Lou J, Li Y, Mou Y, Lu S, Peng Y, Zhou L. 2011. Enhancement of diosgenin production in Dioscorea zingiberensis cell cultures by oligosaccharides from its endophytic fungus Fusarium oxysporum Dzf17. Molecules 16, 10631-10644.
12. Li P, Mou Y, Lu S, Sun W, Lou J, Yin C, Zhou L. 2012. Quantitative determination of diosgenin in Dioscorea zingiberensis cell cultures by microplate-spectrophotometry and high-performance liquid chromatography. African journal of pharmacy and pharmacology 6, 1186-1193.
13. Martin E, Akan H, Ekici M, Aytac Z. 2011. New chromosome numbers in the genus Trigonella L.(Fabaceae) from Turkey. African journal of Biotechnology 10, 116.
14. Mathur V, Mathur N. 2005. Fenugreek and other lesser known legume galactomannan-polysaccharides: scope for developments. Journal of Scientific and Industrial research 64, 475.
15. Ressmann A.K, Strassl K, Gaertner P, Zhao B, Greiner L, Bica K. 2012. New aspects for biomass processing with ionic liquids: towards the isolation of pharmaceutically active betulin. Green Chemistry 14, 940-944.
16. Rosser A. 1985. The day of the yam. Nursing times 81, 47.
17. Zhang C.L, Gao J.M, Zhu W. 2012. Steroidal saponins from the rhizomes and roots of Smilax scobinicaulis. Phytochemistry Letters 5, 49-52.
18. Zhu Y, Huang W, Ni J. 2010. A promising clean process for production of diosgenin from Dioscorea zingiberensis CH Wright. Journal of Cleaner Production 18, 242-247.