Identification and Quantitative Determination of 4-Hydroxyisoleucine in *Trigonella foenum-graecum* L. from Iran

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Abstract

Background: The seeds of *Trigonella foenum-graecum* L. (Fenugreek) are used in Iranian traditional medicine as blood sugar lowering agent. According to last investigations, hypoglycemic property is related to the amino acids of the plant specially 4-hydroxyisoleucine.

Objective: In this research, identification and quantitative determination of 4-hydroxyisoleucine in Fenugreek seeds from Shahreza, central part of Iran, has been investigated.

Methods: Powdered seeds of the plant were defatted and then extracted by using ethanol 50%. In order to isolation of 4-hydroxyisoleucine from the extract, ion exchange chromatography was used and the amino acid content was determined by using high performance liquid chromatography technique.

Results: The results showed the presence of 0.4% 4-hydroxyisoleucine in the plant seeds which is considerable amount of the amino acid in the plant collected from Iran.

Conclusion: According to the effects of 4-hydroxyisoleucine such as antihyperglycemic and antihyperlipidemic properties, fenugreek can be considered as an herbal remedy in supportive treatment of these disorders.

Keywords: ***Trigonella foenum-graecum*, Amino acid, Diabetes, 4-hydroxyisoleucine**

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Introduction

Trigonella foenum-graecum L. (Legominosae) is one of the oldest medicinal plants and is native to southeastern Europe, northern Africa and western Asia, but it widely cultivated in other parts of the world. It is known commonly fenugreek. Fenugreek seeds often have a pungent aroma and may have a bitter taste, which is said to be similar to celery. The seeds of the plant contain many active compounds such as iron, vitamin A, B, C, phosphates, flavonoids, saponins, alkaloids such as trigonelline and amino acids [1]. The leaves and seeds are used for medicinal purposes. Applications of fenugreek were documented in ancient Egypt, where it was used in incense and to embalm mummies. In modern Egypt, fenugreek is steel used as a supplement in wheat and maize flour for bread-making [2]. In ancient Rome, fenugreek was purportedly used to aid labor and delivery. In traditional Chinese medicine, fenugreek seeds are consumed as a tonic, as well as a treatment for weakness and edema of legs [3]. In India, fenugreek is commonly used as a condiment and used medicinally as a lactation stimulant [4]. In Iranian traditional medicine the seeds are used as tonic and blood sugar lowering [5]. The possible hypoglycemic properties of oral fenugreek seed powder have been suggested by the results of preliminary animal and human trials. The studies showed that defatted seeds are thought to be associated with the hypoglycemic effects of fenugreek. These effects have not been observed in studies of lipid extracts [3]. Other Clinical studies conducted on fenugreek have focused on investigating a specific sub-fractions of the fenugreek seeds or, in the alternative, have focused on the specific effect of 4-hydroxyisoleucine in animals and humans with diabetes or lipid disorder [1]. They proved 4-hydroxyisoleucine is a potent blood sugar lowering [6-8] and anti-dyslipidemic agent [8, 9].

4-Hydroxyisoleucine is a branch amino acid which has been extracted from seeds of fenugreek and it has not been found in mammalian. The studies have confirmed the presence of 4-hydroxyisoleucine in fenugreek seeds in two diastroisomers: the major one being the (2S, 3R, 4S) configuration, representing about 90% of the total content of 4-hydroxyisoleucine, and the minor one being the (2R, 3R, 4S) configuration [10]. The major isomer is presently interesting with respect to experimental evidence indicating its ability to stimulate glucose-induced insulin secretion in micromolar concentrations [7]. Some studies have also shown that the natural analogue of 4-hydroxyisoleucine is more effective as an antidiabetic agent than a synthetic version. There is, therefore, a suggestion that the therapeutic effects of 4-hydroxyisoleucine are best obtained from extracts of the fenugreek seeds [1]. But another study has shown synthetic and natural 4-hydroxyisoleucine are same in hypoglycemic properties [11].

Since, the seeds of the plant are used in Iranian traditional medicine as antihyperglycemic agent, we decided to extract and evaluate the amount of 4-hydroxyisoleucineas as the most blood sugar lowering agent in the plant growing in Iran.

Materials and Methods

General experimental procedures

Ion exchange column chromatography was performed using Amberlite cation exchange resin CG50 (Sigma chemicals CO., USA). HPLC was carried out with Spherimage 80 C_{18} column (5 μm, 4×250 mm) using a Knauer controller and RF-10 A×L Fluorescence

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detector. Paper chromatography was performed using cellulose paper (Watmann 1chr, Maidstone, UK). The standard of 4-hydroxyisoleucine was purchased from Toronto chemicals Co., Canada.

Plant Materials

The seeds of *Trigonella foenum-graecum* L. were collected in summer 2006 from Shahreza, Isfahan province and identified in herbarium of faculty of pharmacy, Tehran University of medical sciences.

Extraction and isolation of amino acids

Milled and powdered seeds of the plant (800 g) were macerated using petroleum ether (2 l, 24 h, 4 times) and ethanol 50% (1 l, 24 h, 4 times), respectively. In order to determination of free amino acids in each extract, spot test was performed using cellulose paper and ninhydrine as reagent [12]. The presence of amino acids was confirmed by purple spots on the paper. The results were obtained from spot test showed the presence of amino acids only in ethanol extract (total volume: 4 l). In order to separation of amino acids from ethanol extract, ion exchange chromatography method was used. At first, the column (2.5 × 40 cm) was washed with distilled water (0.5 L) and HCl 2 N (1 L), respectively, for 3 days. After acidifying the resin, distilled water was passed from the column until pH of elute became neutral. Then 100ml ethanol extract was subjected to the cation exchange column and eluted with distilled water (1 L) and ammonium hydroxide 1N (1 L), respectively. The fractions were collected (100 mL each one) when pH of elute became alkaline. Spot test was performed on each fraction and the fractions containing amino acids were mixed together (fraction A).

Identification and quantitative determination of 4-hydroxyisoleucine

In order to identification and quantitative determination of 4-hydroxyisoleucine in fraction A, the fraction (ammonium hydroxide extract) concentrated under reduced pressure. Amino acids in dried extract were analyzed using OPA derivatization [13-15] and HPLC technique. At first, different concentration of 4-hydroxyisoleucine in distilled water were prepared and 2µL of each solution was derivated with OPA and then 10µl of final solution injected to HPLC instrument (run time= 55 min, flow rate= 0.8 ml/min, λ_{ex}=330nm, λ_{em}=440nm). A linear gradient from solvent A (sodium acetate 0.1M, pH 6.95: methanol: tetrahydrofuran; 92.5:5:2.5) to solvent B (methanol: tetrahydrofuran; 97.5:2.5) was used as mobile phase. After injection of standard solutions, 2 µl of sample solution (5 mg/ml) was derivated with OPA and injected to HPLC as same as standard solutions. Three replicates of each sample were injected. 4-hydroxyisoleucine in sample chromatogram was identified by comparing the retention time with that of reference standard and quantitatively determined by calibration curve of 4-hydroxyisoleucine (y= 324.83x-27.975, R^2=0.9995).

Results

Chromatograms of 4-hydroxyisoleucine and fenugreek extract have been shown in figures 1 and 2. As it is observed in figure 1, there are two peaks in standard chromatogram at retention times 22.0 and 24.2 minutes that are related to major and minor isomers of 4-hydroxyisoleucine, respectively. These two peaks are observed in sample chromatogram as

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well. In different concentrations of standard solutions, major and minor isomers ratios are different, because these two isomers are converted to each other. Because of that sum of two peaks area has been considered as AUC in calibration curve and sample.

Percentage of two isomers in standards and sample solutions has been shown in table 1.

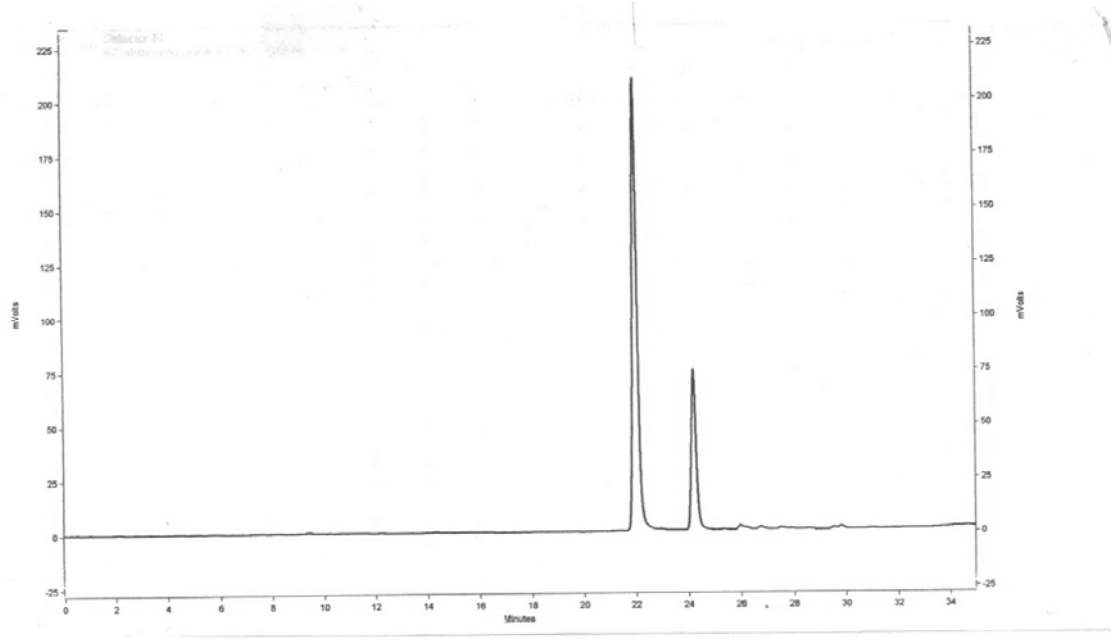

Fig. 1- HPLC chromatogram of 4-hydroxyisoleucine

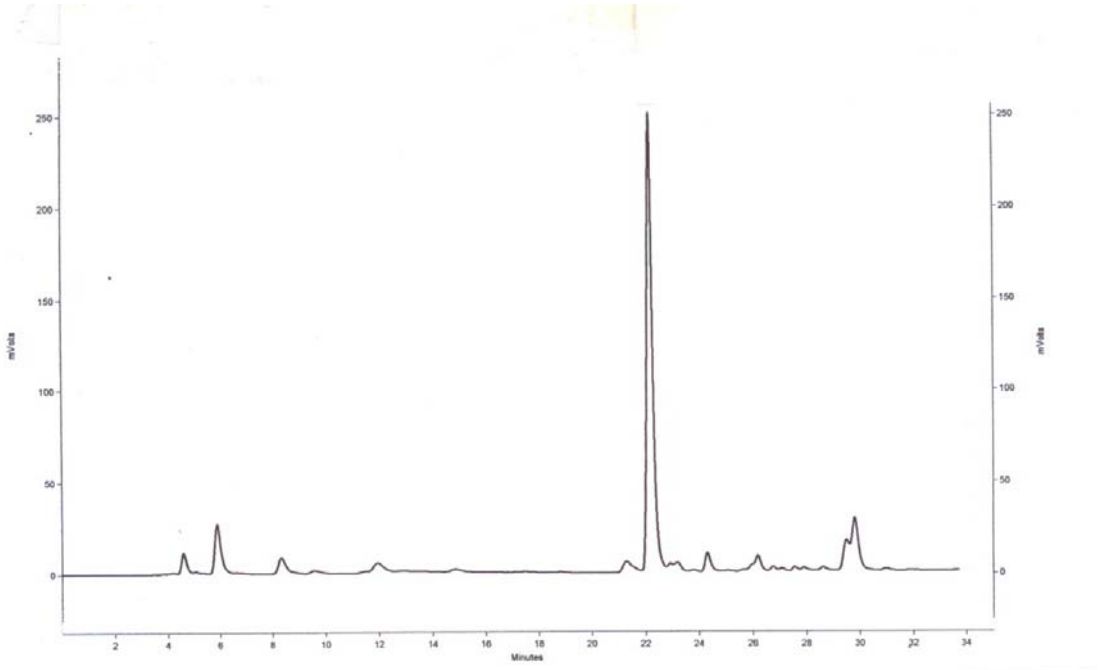

Fig. 2- HPLC chromatogram of Fenugreek seed extract

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Table 1- Percentage of major and minor isomers in standard and sample solutions

Standard Concentration (mg/ml)	% major isomer	% minor isomer
0.07	81.8	18.2
0.10	82.3	17.7
0.20	74.0	26.0
0.33	66.0	34.0
0.50	70.6	29.4
1.00	64.1	35.9
1.50	62.0	38.0
sample	96.0	4

Discussion

The results obtained from HPLC chromatogram of standard solutions show that percentage of major and minor 4-hydroxyisoleucine isomers are different in each solution. As it is observed in table 1, in standard solutions, two isomers of 4-hydroxyisoleucine are converted to each other more than in sample solution. It is may be because of the presence of other compound in the plant extract that prevent to change of major isomer to minor one. Therefore, in quantitative determination of 4-hydroxyisoleucine by HPLC technique, it is not possible to assay major isomer alone. So, it is necessary to use sum of AUC of isomers for plotting calibration curve.

The sample chromatogram shows that about 61% acidic amino acids in fenugreek seeds is 4-hydroxyisoleucine. In the other hand, 4-hydroxyisoleucine is the major acidic amino acid in the plant seeds. Moreover, the results obtained of quantitative determination of 4-hydroxyisoleucine showed the presence of 0.4% the amino acid in the dried plant seeds. It is considerable amount of the amino acid in the plant collected from Iran compared to those reported from India (0.015%) [8] and Greece (0.2%) [16]. According to the effects of 4-hydroxyisoleucine such as antihyperglycemic and antihyperlipidemic properties, fenugreek can be considered as a herbal remedy in supportive treatment of these disorders. But in order to obtain therapeutic effects of the plant seed, large amount of seed is usually needed that can cause some difficulties such as gastrointestinal upset [1]. Therefore, it is better to de-fat and de-bitterize the seeds and use extract containing amino acids, especially 4-hydroxyisoleucine.

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