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4-Hydroxyisoleucine from Fenugreek: Preparation of high pharmacological strength extract and assay method development

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Abstract
Our product marketed under the trade name FenuBet® was first standardized according to its trigonelline content but latest investigations showed that 4-Hydroxyisoleucine is more implicated in the antidiabetic effect of Fenugreek seeds.
In this study two main topics were covered: optimization of factors leading to the preparation of an extract with high pharmacological strength using multi-stage counter-current extraction and development and validation of a new, simple, and rapid HPTLC-Scanner densitometry method for quantitative determination of 4-Hydroxyisoleucine.
Extracts with plant to solvent ratio of 1/2.26 and 4-Hydroxyisoleucine concentration up to 1.28 mg/ml were obtained. The extraction yield showed that more than 82.5% of 4-Hydroxyisoleucine was recovered.
The quantification method was found to give compact spots (Rf = 0.36). The minimum detectable amount was found to be 22.5 ng/spot, whereas the limit of quantitation was found to be 160 ng/spot. The linear regression analysis data for the calibration plots showed good linear relationship with r^2 = 0.998±0.001 in the concentration range 22.5-160 ng/spot. %RSD for method repeatability was under 5.5% which is satisfactory.

Keywords: *Trigonella Foenum-Graecum*, diabetes, 4-Hydroxyisoleucine, multi-stage counter-current extraction, HPTLC-Scanner densitometry

Introduction
Increasing problems of drug resistance in various diseases and the risk of adverse drug reactions have prompted clinical scientists to seek for solutions from complementary and alternative medicine (CAM). CAM has gained popularity in last decades (WHO, 2005) and this trend was strengthened by the introduction of quality products.
Type 2 diabetes is a worldwide health problem and is ranked as one of the leading causes for severe morbidity and premature mortality in modern society.
Diabetes is rising all over the world and by 2030 diabetes prevalence is estimated to reach 578 million people worldwide [1].
The hypoglycemia effect of Fenugreek (*Trigonella Foenum-Graecum* L. Leguminosae) seed has been granted to the presence of the alkaloid trigonelline and an insulin-stimulating substance 4-hydroxy isoleucine (4-OHisoLeu) [2-4].
4-OHisoLeu is a natural, nonprotein, alpha amino acid that has been found in fenugreek seeds and has been shown to cause an increase in glucose-dependent insulin secretion [5-6] and to lower the elevated plasma triglycerides and total cholesterol levels [7].
Our study covered the following topics:

- Parameters optimization in order to get high pharmacological strength extracts including: Determination of solvent mix, stage number and solvent volume.
- Development and validation of a new, simple, rapid and precise high-performance thin-layer chromatographic-scanner (HPTLC-Scanner) method for quantitative determination of 4-OH isoLeu.

Materials and methods
Fenugreek seeds were purchased from local market (Tehran, Iran). After grinding the obtained seed powder was divided into five equal portions of 10 gr. Each portion was poured into a 500 ml Erlenmeyer flask. Counter current extraction was processed as follow: the minimum required volume of solvent was poured in flask I and after 20 min stirring, the filtered extract

was transferred into flask II and etc… till flask V. The resulting extract from flask V (V/1)was filtered and stored in flask T. Abbreviations used to name each extraction step at each stage were respectively I/1, II/1, … and V/1 for the first run, I/2, II/2, … V/2 and I/3, II/3, … V/3 for the second and third runs(Fig1). The resulting extract from V/2 and V/3 were added to V/1 into flask T. Due to the good solubility of 4-OHisoLeu in water (≥26 mg/ml) three solvent mixes were used: Ethanol – Water (50 – 50, 60 – 40 and 70 – 30). Ethanol 96% was purchased from Zakaria Co., Djahrom, Iran.

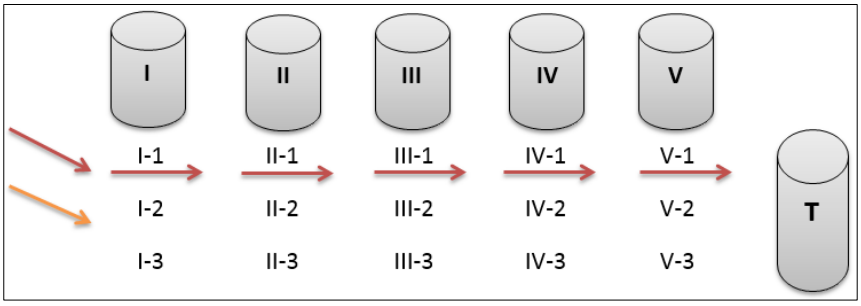


Fig 1: Schematic view of multi-stage counter-current extraction

Sampling for 4-OHisoLeu determination was done from flask III to V for all 3 extraction runs and also from flask T. Standard dilutions of 0.12 ,0.06 and 0.03 mg/ml of L-Leucine (Leu) (>99%, Merck), were used to plot the calibration curves for each TLC plate. 4-OHisoLeu and Leu are α-amino acids and they react similarly with ninhydrin to produce the same violet chromophore called Ruhemann's purple (fig2)

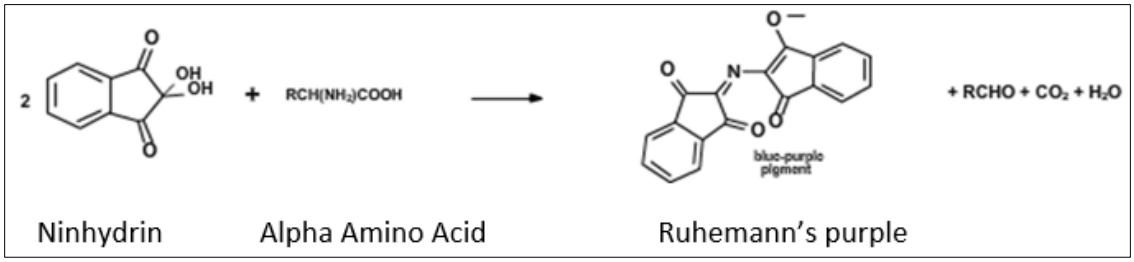


Fig 2: Reaction of alpha amino acids with ninhydrin and formation of Ruhemann's purple

1 µl from each extract sample was spotted on TLC plates (Silicagel 60 F_{254} TLC plates, Merck). The mobile phase was a mix of 1-butanol – glacial acetic acid and water (7 – 2 – 1) [8] (1-butanol, Merck), (Acetic acid, Carlo Erba). TLC was run over a path of 8 cm. After drying, the plates were sprayed with a mixture of ninhydrin, butanol and glacial acetic acid (30 mg – 10 ml – 0.3 ml), (Ninhydrin for analysis, Merck). Plates were kept in a laboratory oven (GCA Corporation, USA) at 110 °C for 3 – 4 min and 4-OHisoLeu concentrations were determined using a HPTLC- Scanner densitometry (Camag CE).

Results and discussion

1. Calibration plots and calculation of 4-OHisoLeu extracted amounts

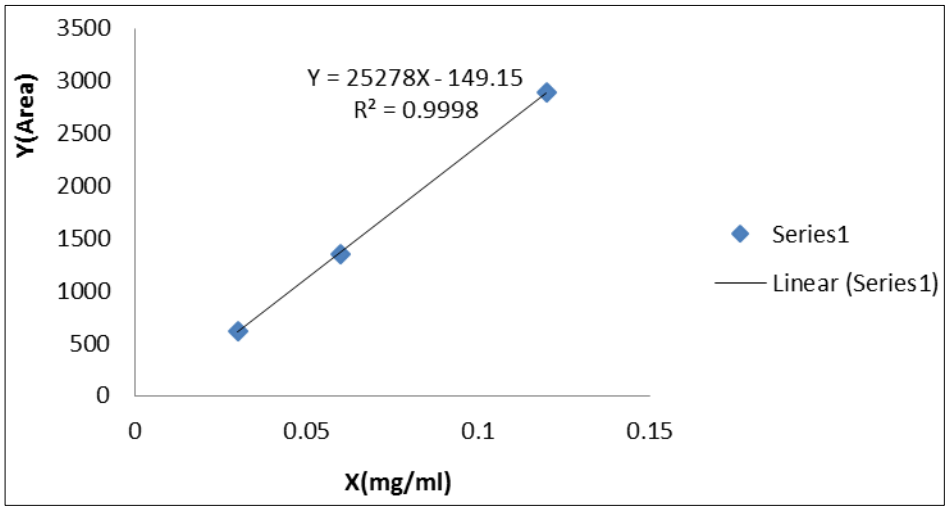


Fig 3: Calibration plot for extract obtained with solvent mix ethanol – water (50 – 50)

Y = Recorded AUC (area under the curve)
X = concentration of 4-OHisoLeu (mg/ml)
4-OHisoLeu (%) = (100.X.V.d) /M
4-OHisoLeu (%) = Amount of 4-OHisoLeu (%) in the Fenugreek seed sample
V= volume of extract (ml)

d= dilution
M= Fenugreek seed mass (mg)
Quantities of 4-OHisoLeu extracted from stage III, IV and V at steps 1, 2 and 3 are reported in tables 1 and 2. Using the obtained calibration curves the areas were converted into concentration then quantity of extracted 4-OHisoLeu.

Table 1: Amount of 4-OHisoLeu extracted from stages III, IV and V at steps 1, 2 and 3 using different ethanol/water mixes

Amount of 4-OHisoLeu (mg)									
Stage	**III**			**IV**			**V**		
Step	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Ethanol water mix %									
50	56.8	25.08	9	54.6	33.6	13.44	84	43.05	24.32
60	66.78	32.4	10.12	53.58	36.12	14.96	67.2	41.8	27.2
70	73.14	34.78	11.96	67.62	36.98	15.48	51.45	58.8	25.2

Table 2: Amount of 4-OHisoLeu assayed in final extract (T) using different ethanol/water mixes

4-OHisoLeu (mg) \ Ethanol Water Mix%	T
50	144.64
60	108.56
70	104.96

According to results in table 2 the 50% solvent mix extracted respectively 24% and 27% more 4-OHisoLeu than the 60 and 70% mixes. These results are in accordance with water solubility of 4-OHisoLeu (≥26 mg/ml). To increase 4-OHisoLeu extraction yield, we suggest to use ethanol/water 50% mix as solvent of extraction.

2. 4-OHisoLeu extraction yield

In order to compare extracted amounts of 4-OHisoLeu to its amount in the used seeds and calculate extraction yields, the following experiment was done. Three replicates of 500 mg Fenugreek seed powdered samples were poured in 100 ml Erlenmeyer flasks. 50 ml solvent (Ethanol-water, 70-30) was added and the mix was stirred for 90 min to be able to have an exhaustive extraction of 4-OHisoLeu [9]. After filtration the aliquot samples were processed for HPTLC-Scanner as described in paragraph "Material and methods". A wide range of 4-OHisoLeu amount in Fenugreek seeds has been reported in the literature (0.015%–0.4%) [10-11]. the method used in this paper showed an average amount of 0.35% which is in accordance with previous works [10].
The same method of calculation was used for each multi-stage counter-current extraction run (solvent mix 50, 60 and 70%) and the obtained results are reported in table 3.

Table 3: Calculated 4-OHisoLeu amount in Fenugreek seed samples and yield extraction

Ethanol/water mix %	Fenugreek sample mass (mg)	Solvent volume collected in flask T (ml)	Total amount of 4-OHisoLeu collected in flask T (mg)	Calculated 4-OHisoLeu amount in Fenugreek sample seed (%)	4-OHisoLeu extraction yield (%)
50	50000	113	144.64	0.28	82.5
60	50000	118	108.56	0.21	62
70	50000	128	104.96	0.20	60

As showed in Fig1, for each run the solvent mix was circulated through five extractors and after three runs the total extract was collected in flask T. According to table 3 the amount of 4-OHisoLeu extracted at stage 5 with solvent mix 50% consisted of more than 80% of the total 4-OHisoLeu present in the used raw plant material. Use of more stages (extractors) is not necessary because of cost production, time loss and pharmacological strength reduction due to extract dilution.

3. Determination of minimum solvent volume

The minimum solvent volume required to complete each multi-stage counter-current extraction for each solvent mix is shown in table 4.

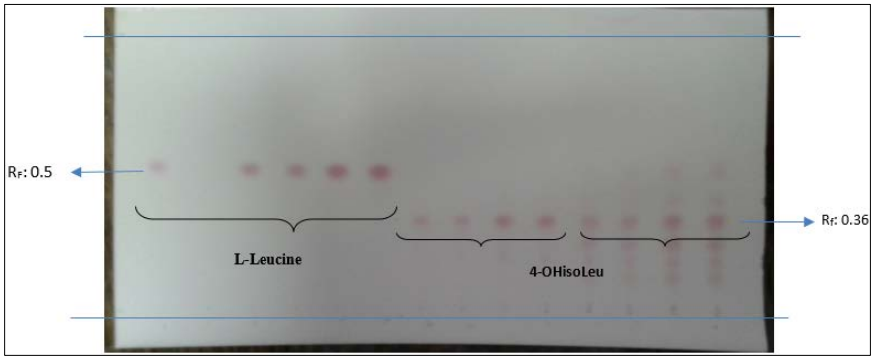
Table 4: Minimum solvent volume required to complete the five stage counter-current extraction with three runs of solvent.

Ethanol/water %	Minimum solvent volume
50%	ml113
60%	ml118
70%	ml128

With solvent mix 50% and five stages (extractors) the plant to solvent ratio was 1/2.26 which means a 8-10 fold more concentrated extract than the batch extraction mode and hence a higher pharmacological strength.

4. Method validation

The quantification method was found to give compact spots (Rf = 0.36). The minimum detectable amount (LOD) was found to be 22.5 ng/spot, whereas the limit of quantitation (LOQ) was found to be 160 ng/spot. The linear regression analysis data for the calibration plots showed good linear relationship with r^2 = 0.998±0.001 in the concentration range 22.5-160 ng/spot.



Repeatability of measurement of peak area was determined by analyzing different amount of 4-OHisoLeu samples covering low, medium, and higher ranges of the calibration curve six times without changing the position of plate. Obtained results are shown in table 5.

Table 5: Data obtained for repeatability measurements

Conc. (mg/ml)	Area (n=6)						%RSD
0.03	417.8	451.6	462.3	435.1	414.5	482.9	5.47
0.06	965.9	947.2	929.6	1052.6	1059.2	1054.9	5.49
0.12	2981.3	2889.6	2943.4	2719.6	2810.1	2943.9	3.13

Statistical analysis of the data showed that the method has a good repeatability; %RSD was consistently less than 5.5% (Table 7), which was well below the instrumental specifications, ensuring repeatability of developed method.

Table 6: Comparison of results from Tables 1 and 2 for solvent mix 50%

Ethanol/water mix %	V/1+V/2+V/3 (mg)	T (mg)	Difference %
50	151.37	144.64	4.4

Only 4.4% difference between amounts of 4-OHisoLeu assayed in extracts obtained from V/1, V/2, V/3 and final mixed extract in flask T was observed which is acceptable.

Conclusion

Parameters optimization for 4-Hydroxyisoleucine extraction allowed high pharmacological strength extracts preparation (1/2.26) (plant/solvent ratio) and high yields (>82%). Data obtained for this multi-stage counter-current extraction may be scaled-up for industrial production. During this study all assays for quantitative determination of 4-Hydroxyisoleucine were done using a new, simple, rapid and precise HPTLC-Scanner densitometry method. This low cost analytical method may be used easily in the site of production.

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