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EXTRACTION AND CHARACTERIZATION OF 4-HYDROXYISOLEUCINE FROM *TRIGONELLA FOENUM GRAECUM* SEEDS

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Abstract:

The objective of the present investigation is to extract, quantify and characterize the active constituent, 4-hydroxyisoleucine in Trigonella foenum graecum seeds using HPLC and HPTLC methods. Aqueous extract of crushed seeds of Trigonella foenum graecum was prepared and subjected to HPLC and HPTLC analysis. HPLC analysis was performed using C18 reverse phase column and Fluorescence detector with an elution gradient composed of 65 mmol Sodium acetate, l.5% tetrahydrofurane (pH 5.7) and methanol. HPTLC analysis was performed using semiautomatic TLC sampler Linomat V (CAMAG) controlled by WinCATS software. A constant application rate of 80 nLs was employed with a band width of 3.0mm and distance between two bands was 6.2 mm. The plates were developed in 20 × 10 cm twin trough glass chamber containing 25mL of mobile phase as mixture of butanol: acetic acid: water (4: 1: 1, v/v/v) and detected at λ 254nm and 570 nm. HPLC analysis of Standard concentrations of 4-hydroxyisoleucine exhibited a linear plot with average retention time of 8.13 min and sample analysis revealed a retention time of 8.19 min. HPTLC analysis revealed Rf value of 0.45 for both standard and the seed extract at λ 254nm and 570 nm.

Keywords: *Trigonella, 4-hydroxyisoleucine, HPLC, HPTLC, Fluorescence detector, Retention time, Rf value.*

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INTRODUCTION:

4-hydroxyisoleucine, an unusual aminoacid found in seeds of *Trigonella foenum graecum* Linn. belonging to family Fabaceae, commonly known as Fenugreek seeds. The antidiabetic properties of fenugreek seeds have been known for a long time. 4-hydroxyisoleucine is reported as a bioactive compound responsible for antidiabetic activity of Fenugreek [1]. The amino acid 4-hydroxyisoleucine found to be insulinotropic compound. This amino acid is not present in mammalian tissues but only found in plants, especially in Trigonella species [2].

Present study is undertaken to develop rapid sensitive analytical methods of estimation of this constituent amino acid. Not much of work is done on extraction and quantification of 4-hydroxyisoleucine from *Trigonella foenum graecum* seeds Therefore there is a need to develop and validate methods for its estimation.

MATERIALS AND METHODS:

Materials

Seeds of *Trigonella foenum graecum* was procured from National Institute of Siddha, Chennai, Tamilnadu India.Seeds are authenticated by Taxonomist, bearing Voucher No.NISMB2192016.

Standard 4-hydroxyisoleucine were procured from Sigma Aldrich Pvt Ltd. All Analytical and HPLC grade chemicals were purchased from Merck Pvt.Ltd.

Processing of Plant material

Trigonella foenum graecum seeds 2.5 kg of seeds were air dried and ground in a grinder so that the powder could pass through a 0.8 mm mesh sieve and stored in a closed vessel for further use.

Extraction

***Preparation of aqueous extract from seeds of Trigonella foenum graceum seeds* [3]**

Dry seeds of Trigonella foenum graecum seeds are crushed into Coarse powder and 2500g powder was suspended in 5000 ml of water, kept in an incubator at 37ºC for 78 hrs, the slurry is stirred continuously for 2 days and left overnight. The mixture is filtered and to the filtrate, ethyl acetate is added to remove the complete water content under low pressure using Rotary evaporator (Superfit™ model) and the yellow gummy extract (23.895g) is stored at -20°C and used for further analysis.

HPLC analysis of 4-hydroxyisoleucine and seed extract

We adopted Yves Sauvaire et al method [2] which was based on precolumn fluorescence derivatization with O-phthtaldialdehyde .HPLC analysis of 4-hydroxyisoleucine was carried out on a Shimadzu HPLC LC-20AT (Dual pump system) equipped with fluorescence detector RF – 10AXL. The method is based on precolumn formation of a derivative with O-phthtaldialdehyde. Separation was performed on a reverse phase C18 column (250mm x 4.6mm, 5A° particle size) with an elution gradient composed of 65 mmol/L Sodium acetate, 5% tetrahydrofuran (pH 5.7) and methanol. The flow rate was adjusted to 1 mL/min and Sample Filtration was done by 0.22µm syringe filters. Detection was carried out by Fluorescence analysis (λ Excitation,355 nm; λ Emission,410nm).

HPTLC analysis of 4-hydroxyisoleucine and seed extract

Before use, precoated silica gel TLC (E. Merck, Darmstadt, Germany) plates 60F254 (20 cm × 10 cm with 0.2mm thickness) were prewashed by dipping the plates in methanol and the solvent was allowed to overrun the plate followed by drying in fume hood. The plates were activated at 60°C for 5min prior to chromatography. Sample solutions were applied onto the plates with semiautomatic TLC sampler Linomat V (CAMAG, Muttenz, Switzerland) controlled by WinCATS software version 1.4.4. A constant application rate of 80 nLs was employed with a band width of 3.0mm and distance between two bands was 6.2 mm. The plates were developed in 20 × 10 cm twin trough glass chamber containing 25mL of mobile phase as mixture of butanol: acetic acid: water (4: 1: 1, v/v/v). The optimized chamber saturation time for mobile phase was 15 minutes at room temperature (25 ± 2∘C) and 60 ± 5% relative humidity. The length of chromatogram run was up to 80mm from the point of application (10mm). After development, chromatographic plates were dried for 5 minutes in a current of air with the help of a hair dryer in normal mode. Quantitative evaluation of plate was performed with slit dimension of 3.0 × 0.45mm and scanning speed of 20mms^{-}1. The plates were directly scanned within 10 minutes using densitometry scanner III with WinCATS software (Camag) in the UV mode with the D2 light source set at 254 nm. The spots corresponding to 4-hydroxyisoleucine were observed at Rf = 0.45. After spraying the ninhydrin solution the spots corresponding to 4-hydroxyisoleucine were observed at Rf = 0.45 showing in the chromatogram at 570nm using Tungsten light source.

RESULTS AND DISCUSSION:

HPLC method

With HPLC method different concentrations of Standard 4-hydroxyisoleucine were found to be linear in the range of 1- 4 µg ml^{-1} .The chromatograms results revealed well separated peaks. It is a highly sensitive method with derivatization. Various HPLC parameters are given in the table no.1. The average

Retention time value of the different standard concentrations were found to be 8 min13 sec and the retention time of the 4-hydroxyisoleucine in the extract was compared with the reference standard, and sample exhibited retention time 8 min 19 sec which is closer to reference standard. Chromatograms of standard 4-hydroxyisoleucine and fenugreek seed extract are shown in figure 1 and 2. These retention times are closer to that reported by Yves Sauvaire et al.[2] i.e., 8 min 14 sec. whereas Hajimehdipoor H et al.[4],has reported the retention times of two isomers of aminoacid between 22 to 24 minutes following gradient elution.

Table 1: Parameters of the developed HPLC method for identification and quantification of 4-hydroxyisoleucine

Parameters	**Results**
Linearity range (µg ml^{-1})	*1-4*
Correlation coefficient (r^2)	0.997
Retention time(min)	8.133
Curve fit type	Linear
Regression equation	C=0 .009 A – 1.1044 (a = 3.632258e-006; b = -1.104439)
Mean Rf	$3.369x10^{-6}$
%RSD of Rf	***5.6139***

A = Peak Area, C = Concentration (µg ml^{-1})

Table 2: HPLC parameters of Trigonella *foenum graecum* seeds extract.

Sample	**Retention time of 4-hydroxyisoleucine(minutes)**	**Area**	**Theoretical Plates**	**Concentration(µg ml^{-1})**
Trigonella seeds extract	***8.19***	**3842**	**639211**	**1.3511**

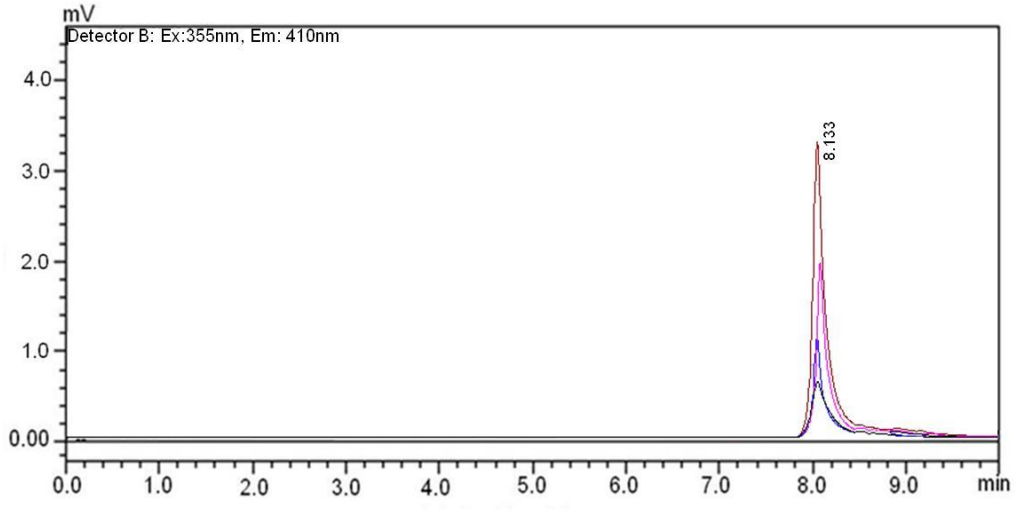

Fig 1: HPLC chromatogram of different concentrations of Standard 4-hydroxyisoleucine (Retention Time=8.13)

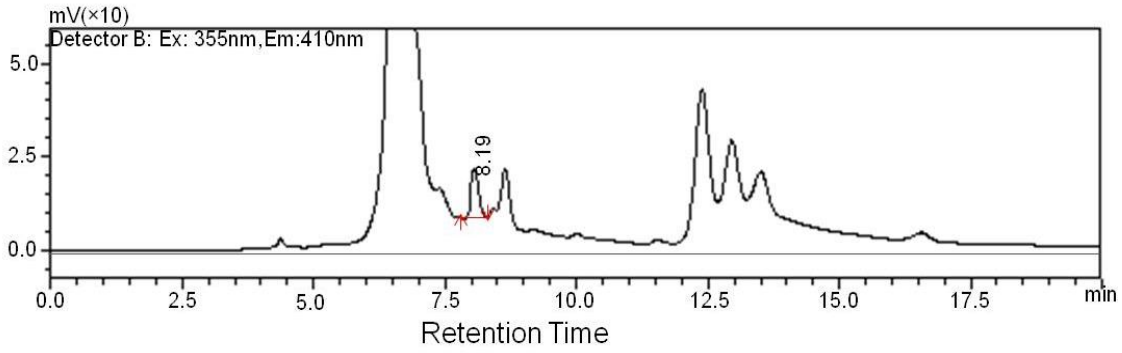

Fig 2: HPLC chromatogram of seed extract of Trigonella (RT of 4-hydroxyisoleucine=8.19)

HPTLC method

In the UV mode with the D2 light source set at 254 nm, the spots corresponding to 4-hydroxyisoleucine were observed at Rf = 0.45. After spraying the Ninhydrin solution the spots corresponding to 4-hydroxyisoleucine were observed at Rf = 0.45 showing in the chromatogram at 570nm using Tungsten light source. HPTLC bands and chromatograms are shown in the figures 3, 4 & 5. Atul N. Bedekar et al.[1], reported a closer Rf value for 4-hydroxyisoleucine i.e., 0.43 at 570nm using same solvent system.

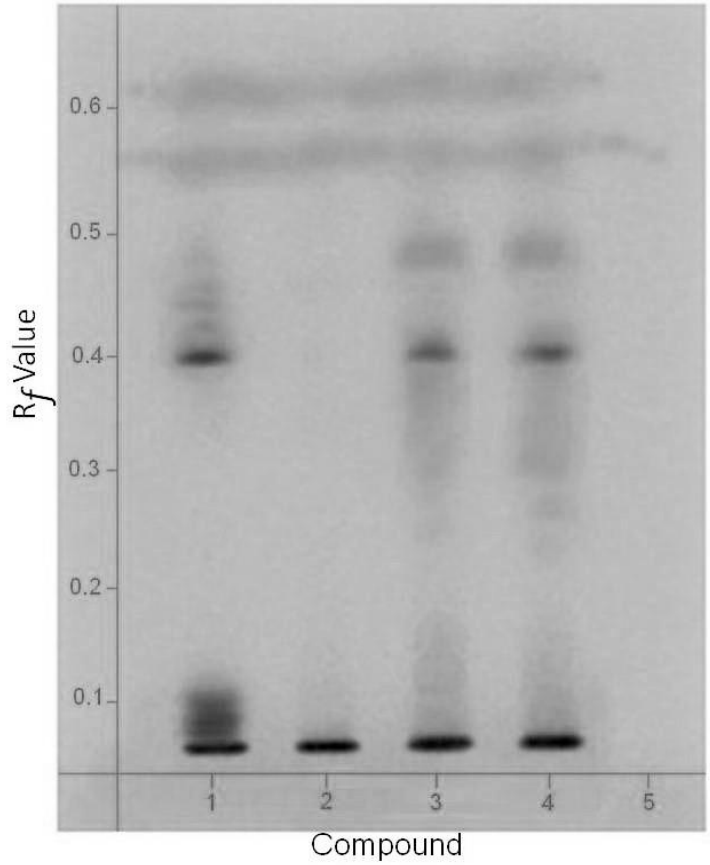

Fig 3: HPTLC chromatogram showing bands for standard 4-hydroxyisoleucine and Seed extract (1.Standard 4-hydroxy isoleucine, 2.Blank, 3.Seed Extract with high concentration, 4. Seed Extract with low concentration)

Table 3: HPTLC parameters of the *Trigonella foenum graecum* seeds extract.

Sample	RF value of 4-hydroxyisoleucine	Area under 570nm	% of Area	Concentration (µg ml^{-1})
Trigonella seeds extract	**0.45**	**248.75**	**3.55**	**1.3511**

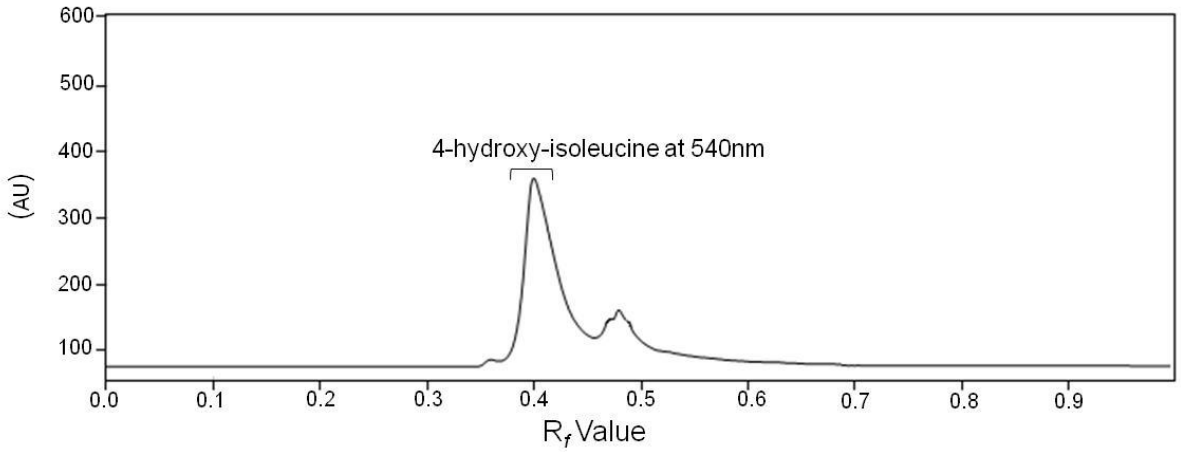

Fig 4: HPTLC analysis of standard 4 –hydroxy isoleucine with Rf value 0.45

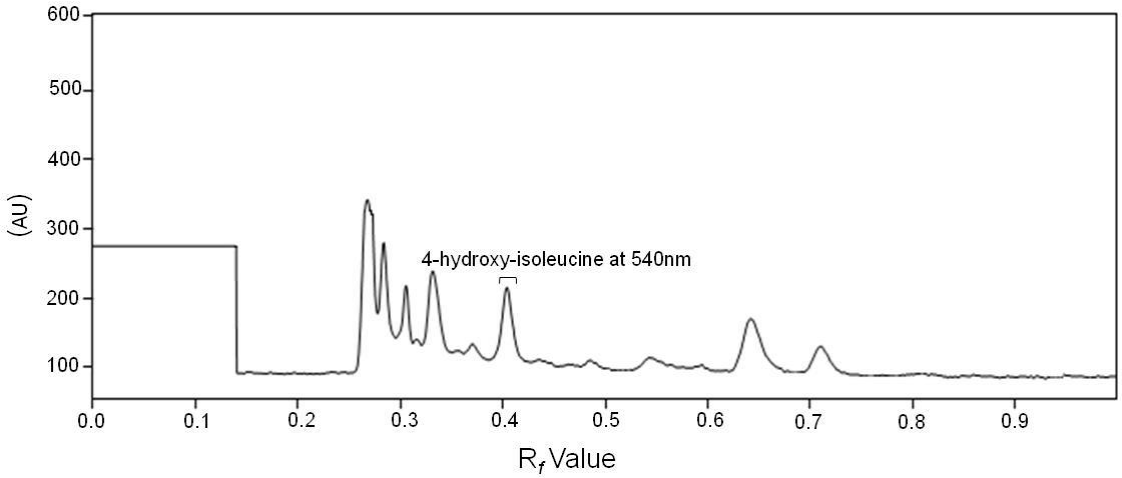

Fig 5: HPTLC analysis of seed extract (4-hydroxy isoleucine) with Rf value 0.45

CONCLUSION:

Aqueous extract of *Trigonella foenum graecum* seeds was analyzed for the presence of 4-hydroxyisoleucine which is detected and confirmed by HPLC and HPTLC methods. The methods were found to be accurate and precise, can be used for analysis of 4-hydroxyisoleucine in Trigonella seeds extracts.

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Conflict of Interest: The authors declare no competing financial interest .

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