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RESEARCH ARTICLE

# REVISED A Validated Trigonelline-Based Method for the Standardization and Quality Control of *Trigonella foenum-graecum* L.

[version 3; peer review: 2 approved]

Mohammed Aldholmi[1], Rizwan Ahmad[1], Salma Hago[2], Ali Alabduallah[1]

[1]Department of Natural Products, College of Clinical Pharmacy, Imam Abdulrahman Bin Faisal University, Dammam, Eastern Province, Saudi Arabia
[2]Faculty of Pharmacy, Department of Pharmacognosy, University of Gezira, Wad Madani, Gezira, Sudan

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## Abstract

### Background

Fenugreek, or *Trigonella foenum-graecum* L, is an edible and medicinal plant of the Fabaceae family. Fenugreek seeds are rich in proteins, lipids, and essential nutrients, and they also contain various phytochemicals, including flavonoids, steroidal saponins, coumarin, and alkaloids such as trigonelline. Trigonelline (TG) is a bioactive plant alkaloid initially extracted from fenugreek seeds. A substantial portion of fenugreek's health benefits may rely on the presence of TG. This study addresses the critical need for a fast, green, and economical method that overcomes inefficiencies, high solvent usage, and sensitivity limitations in the quantification of TG.

### Methods

Fenugreek seeds from various origins were extracted using three green solvents: acetone (ACt), ethanol (EtOH), and water (H2O). The UPLC-MS/MS method was developed and validated using a green mobile phase of H2O: EtOH, and an $r^2$-value of 0.999 in the linearity range of 0.1-500 ppb was adopted. The method was validated with an accuracy of 98.6% for trace analysis of TG using a small amount (10 mg) of fenugreek samples from five different origins.

**Open Peer Review**

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1. **Abdulrahman Koshak**, King Abdulaziz University, Jeddah, Saudi Arabia

2. **Abdul Sadiq**, University of Malakand, Chakdara, Pakistan

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**Results**

The average extract yield was 5.36 mg/100 mg with a standard deviation (SD) of 6.3, with the highest extract yield observed in H2O. The ESI (+ve) of the UPLC-MS/MS resulted in the fragmentation pattern (*m/z*) 138→94.10→92.05→78.20. The TG quantification revealed an average TG concentration of 181.4 ppb (SD = 176.4), with the highest amount of TG in H2O extract (mean = 392.7, SD = 132.4 ppb), followed by EtOH (mean = 91.9, SD = 83.3 ppb) and ACt (mean = 59.5, SD = 30.9 ppb). The TG amount observed in the validation step substantiated the efficiency and reproducibility of the developed method.

**Conclusions**

The method may be used as an effective tool for a green, rapid, economical, and eco-friendly extraction and quantification of TG in diverse matrices of pharmaceutical, cosmeceutical, herbal, and food products.

**Keywords**
Fenugreek, UPLCMSMS, green, extraction, analysis

**Corresponding author:** Salma Hago (salmahago124@gmail.com)

**Author roles: Aldholmi M**: Conceptualization, Formal Analysis, Methodology, Resources, Software, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Ahmad R**: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Resources, Software, Supervision, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Hago S**: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Alabduallah A**: Investigation, Writing – Original Draft Preparation, Writing – Review & Editing

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**REVISED** **Amendments from Version 2**

In the new version of the article No.157659 "A Validated Trigonelline-Based Method for the Standardization and Quality Control of *Trigonella foenum-graecum* L.", we have integrated revisions to address the reviewer' comments. Specifically, we have clarified the differences in TG (trigonelline) concentrations observed in previous studies and our current work.

The higher TG concentrations reported in earlier studies can be attributed to the preparation and crushing of fenugreek seeds into powder, which enhances solvent penetration and the extraction of bioactive compounds like TG. In contrast, our study used intact seeds to mimic traditional preparation methods, where crushing or grinding may not always be applied.

This approach highlights the potential limitations of traditional methods, such as making fenugreek tea or extracts from uncrushed seeds, which may result in lower TG extraction yields. Moreover, these findings underscore the critical role of seed preparation in maximizing the release of active constituents and emphasize the importance of considering such factors when comparing TG concentrations across studies and extraction methods. We also correct a typo error into UPLC-MS/MS.

Additionally, we would like to clarify that no updated figures, updates to the author list, or additional data or code have been added.

We hope these clarifications provide additional insights and address the reviewers' concerns effectively.

**Any further responses from the reviewers can be found at the end of the article**

## Introduction

*Trigonella foenum-graecum* L. (fenugreek) is a medicinal and food plant that belongs to the family *Fabaceae*, which includes common medicinal and food plants such as *Pisum sativum* (pea), *Cicer arietinum* (chickpeas), *Phaseolus* (beans), *Glycine max* (soybean), *Arachis hypogaea* (peanut), and *Glycyrrhiza glabra* (licorice).[1] Fenugreek is cultivated in several countries worldwide, including the Mediterranean region, North Africa, and India.[2] The seed is consumed as a condiment and seasoning in food preparations and is believed to possess nutritional and health benefits.[2] For several centuries, fenugreek has been consumed orally as a traditional treatment for a wide range of diseases, including diabetes, fever, and abdominal colic, and applied topically for abscesses, boils, and carbuncles.[3] Various pharmacological and clinical studies have confirmed significant medicinal properties of fenugreek seeds, including antidiabetic activity, anti-obesity activity, hypolipidemic activity, anticancer activity, antioxidant activity, anti-inflammatory activity, and anti-bacterial activity due to the presence of numerous bioactive components.[4] Fenugreek seeds contain several classes of compounds, including proteins, lipids, amino acids, vitamins, minerals, galactomannan fibre (a carbohydrate), flavonoids, steroidal saponins, coumarin, and alkaloids such as trigonelline (TG).[2]

TG is a bioactive plant alkaloid that was first isolated from fenugreek seeds but is also abundant in other plant-derived food products, such as coffee beans.[5] It is an N-methylated form of nicotinic acid or vitamin $B_3$ (Figure 1), hence chemically known as N-methylnicotinic acid ($C_7H_7NO_2$). This fenugreek alkaloid has been reported to have antidiabetic, antihyperlipidemic, antimigraine, sedative, antibacterial, antiviral, anticancer, and phytoestrogenic effects; also it inhibits platelet aggregation and improves memory function.[2,4] It has been reported to demonstrate its antidiabetic efficacy by enhancing the insulin signalling pathway, alleviating endoplasmic reticulum stress, and reducing oxidative damage in individuals with type 2 diabetes.[4] TG has been shown to enhance the axonal formation of neurons and recover memory function in Alzheimer's disease model mice.[6] It has also been demonstrated to incorporate into the nicotinamide adenine dinucleotide (NAD+) pool and increase NAD+ levels, improving muscle function during ageing.[5] Therefore, this fenugreek bioactive compound appears to be a significant contributor to the medicinal properties of fenugreek seeds.

Since a significant part of the fenugreek health benefits potentially depends on the presence of TG in the utilised product, there is a critical need to develop TG-based extraction and analysis methods for the standardization and quality control of fenugreek products to ensure their efficacy and safety. The optimal extraction and analysis methods should be fast,

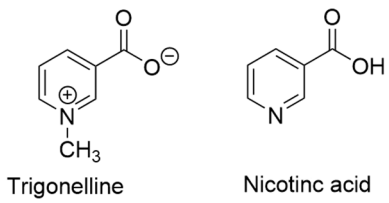



**Figure 1. Chemical structures of trigonelline and nicotinic acid.**

efficient, and accurate, using an economical, eco-, and human-friendly approach. Additionally, the developed methods should avoid the use of high temperatures, as it has been reported that trigonelline breaks down to niacin at high temperatures.[2] Previous studies reported the use of HPLC-UV-Vis and LC-MS techniques for TG quantification, but they have the disadvantage of involving non-green and toxic solvents in the mobile phase system.[7–12] Therefore, a loophole still exists for the extraction and determination of TG in fenugreek samples using a fast, green, economical, eco-friendly, and less toxic methodology. The current study aims to investigate the efficiency of three green solvents for extraction of TG from fenugreek using an ultrasonic dismembrator (20 kHz), develop an economical, eco-, and human-friendly analysis method, and practically apply the method to fenugreek samples from different origins.

## Methods

### Solvents, chemicals, and instruments

Analytical-grade acetone (ACt, Cat. 107021) and ethanol (EtOH, Cat. 107017), as well as HPLC-grade ethanol (EtOH, Cat. 111727), were purchased from Merck (Darmstadt, Germany). Water ($H_2O$) used for extraction and LCMSMS analysis was produced in-house using Millipore equipment (Millipore, Bedford, MA, USA). The standard chemical trigonelline (TG, Cat. 1686411) was obtained from Sigma Aldrich St Louis, MO, USA.

Fisher Scientific (2000 Park Lane Pittsburgh, PA, USA) ultrasonics dismembrator [power (50 Watt), frequency (20 kHz), transducer (Model CL-334), horn (220 A), titanium probe (420 A; 1 mm diameter)] was used for extraction. Ultra-pressure liquid chromatography-mass spectrometry (UPLC-MS/MS) instrument (Shimadzu, Japan, LCMS-8050) consisting of binary pumps (LC-30AD), thermostatted column compartment (CTO30A), UV-DAD detector (SPD-M20A), and triple-quadrupole mass detector with electrospray-ionisation-source (TQMS-ESI) was used for quantitative analysis. The data was processed and analysed with the help of LabSolutions software (Kyoto, Japan V 5.93). The evaporation and drying of the solvents from the samples were accomplished with the help of a miVac Quattro Concentration System (Genevac Ltd., Ipswich, United Kingdom). The analytical column used for the separation was a C18 Acquity UPLC column from Waters (2.1 × 100 mm; 1.7 mm).

### Fenugreek samples collection and preparation

Dried fenugreek seed samples originating from five different countries consisting of India, Egypt, Iran, Saudi Arabia, and Yemen were collected from local markets in the Kingdom of Saudi Arabia, as reported in our previous study.[13] Three different types of fenugreek seeds (based on color and size) were collected for each origin, resulting in fifteen fenugreek samples (3 × 5 = 15).

### Green Ultrasonics Extraction (USE) for TG

Fenugreek seed samples were extracted for the three accessions from each origin using three green solvents of ACt, EtOH, and $H_2O$, resulting in forty-five fenugreek extracts (3 × 5 × 3 = 45). The samples were properly coded from F1 to F15 plus A, B, and C letters standing for ACt, EtOH, and $H_2O$, respectively. The extraction procedure was adopted with slight modification from the previously developed in-house USE method.[14] Briefly, 100 mg of the fenugreek seeds from each accession were weighed, and 10 mL of the respective solvent was added. The amplitude and pulse were set at 30% and 30/10 s, respectively. The five-minute extraction was followed by the evaporation of solvents using the miVac Quattro Concentration System (Genevac Ltd., Ipswich, United Kingdom). The dried samples were weighed for extract yield calculation (mg/100 mg).

### Green UPLC-MS/MS analytical method for TG

**Method development for green UPLC-MS/MS analysis**

The standard drug TG was dissolved (1 mg/mL) in HPLC-grade EtOH to prepare a stock solution. For working standards, the stock solution was further diluted in the required solvent volume to construct six points in the linearity range of 0.1-500 ppb (0.1, 5, 10, 50, 100, and 500 ppb). The extracts, stock, and working standard solutions were prepared in HPLC-grade EtOH and filtered through a syringe filter (0.2 mm). For separation and quantitation of TG, the mobile phase of $H_2O$ (A) and EtOH (B) was used to run the TG standard solutions with a gradient of 5-95% B using the C18 column. Mobile phase at different gradient and isocratic elution compositions, flow rates (0.1, 0.2, 0.3 mL/min), and injection volumes (1, 2, 5 mL) were tested for the efficient separation of TG.

The conditions used for mass spectrometry were optimised to obtain the most appropriate ionisation and fragmentation pattern. The ESI (electrospray ionisation) interface was used for the mass analysis, and a positive MRM (multiple reaction monitoring) mode was applied to quantify and analyse the daughter fragments using an external standard calibration curve. Following the successful fragmentation via optimal collision energy (CE) selection, the fragment (m/z) with the highest intensity (base peak), along with the retention time (Rt) of the peak, were used to identify the target analyte in all samples. The final LC and MS conditions are presented in Section Chromatographic separation and MS optimisation.

**Method validation for green UPLC-MS/MS analysis**

The UPLC-MS/MS was validated in terms of accuracy, linearity, LOD (limit of detection), and LOQ (limit of quantification). The linearity of the method was evaluated using six different calibration points in the range of 0.1-500 ppb, where the regression equation and $r^2$-value were calculated. The accuracy, LOD, and LOQ were calculated via in-built LabSolutions software using the ICH guidelines[15] and previously validated parameters for the UPLC-MS/MS analytical method.[16]

### Applicability of the USE-UPLC-MS/MS for trace analysis

The developed USE-UPLC-MS/MS was further tested in terms of efficiency and applicability of the method for trace analysis by $H_2O$ extraction and analysis of lower amounts (10 mg) of the fenugreek samples containing the lowest TG quantity from each origin (F1, F5, F8, F10, and F15), as mentioned previously. The extracted samples were dried, diluted, and prepared for UPLC-MS/MS analysis to determine TG concentrations.

## Results

### Extracts yield

**Determination of the extract yield in different fenugreek samples**

The extract yield for the forty-five fenugreek extracts (*N=45*) exhibited a sum of 241.0 mg/4500 mg, with a mean of 5.36 (SD = 6.3). The range for the extract yield observed was (minimum to maximum) 1.1-30.6 mg (*N=45*). For the average extract yield/origin (*N=9:* mg/100 mg), the descending order observed was Egypt (mean = 7.1, SD = 8.5 mg)> Yemen (mean = 6.6. SD = 9.2 mg)> Saudi Arabia (mean = 6.0, SD = 6.0 mg)> Iran (mean = 3.7, SD = 2.9 mg)> India (mean = 3.4, SD = 2.0 mg). With regard to the highest extract yield in individual fenugreek samples of each origin (*N=9*), the following samples exhibited the highest extract yield in each origin: F3C in $H_2O$ (6.3 mg) for the Indian samples, F5C in $H_2O$ (21.5 mg) for the Saudi Arabian, F7A in ACt (9.6 mg) for the Iranian, F11C in $H_2O$ (28.1 mg) for the Egyptian, and F15C in $H_2O$ (30.6 mg) for the Yemeni origin. The fenugreek samples noted with the highest yield among the 45 samples were F15C (30.6 mg)> F11C (28.1 mg)> F5C (21.5 mg)> F11A (10.8 mg). The results of average and individual yields in different origins are summarized in Table 1.

**Table 1. Average and individual extract yields for fenugreek samples (F1-F15) extracted with ACt (A), EtOH (B), and $H_2O$ (C).**

| Country of origin | Sample code | Extract yield (mg/100 mg) | Average Yield/origin (mg/100 mg) | SD |
|---|---|---|---|---|
| India | F1A | 5.6 | 3.4 | 2.0 |
| | F1B | 1.3 | | |
| | F1C | 1.1 | | |
| | F2A | 5.3 | | |
| | F2B | 2.1 | | |
| | F2C | 4.2 | | |
| | F3A | 3.0 | | |
| | F3B | 1.8 | | |
| | F3C | 6.3 | | |
| Saudi Arabia | F4A | 4.3 | 6.0 | 6.0 |
| | F4B | 3.7 | | |
| | F4C | 6.0 | | |
| | F5A | 3.4 | | |
| | F5B | 1.8 | | |
| | F5C | 21.5 | | |
| | F6A | 5.9 | | |
| | F6B | 2.2 | | |
| | F6C | 5.5 | | |

**Table 1.** *Continued*

| Country of origin | Sample code | Extract yield (mg/100 mg) | Average Yield/origin (mg/100 mg) | SD |
|---|---|---|---|---|
| Iran | F7A | 9.6 | 3.7 | 2.9 |
| | F7B | 1.4 | | |
| | F7C | 2.9 | | |
| | F8A | 7.5 | | |
| | F8B | 1.7 | | |
| | F8C | 3.8 | | |
| | F9A | 3.1 | | |
| | F9B | 1.1 | | |
| | F9C | 2.1 | | |
| Egypt | F10A | 7.6 | 7.1 | 8.5 |
| | F10B | 1.4 | | |
| | F10C | 4.9 | | |
| | F11A | 10.8 | | |
| | F11B | 4.2 | | |
| | F11C | 28.1 | | |
| | F12A | 3.1 | | |
| | F12B | 1.1 | | |
| | F12C | 2.4 | | |
| Yemen | F13A | 3.2 | 6.6 | 9.2 |
| | F13B | 1.2 | | |
| | F13C | 3.9 | | |
| | F14A | 3.6 | | |
| | F14B | 1.4 | | |
| | F14C | 4.2 | | |
| | F15A | 7.5 | | |
| | F15B | 3.6 | | |
| | F15C | 30.6 | | |

**Variation of extract yield in green solvents**

For the green solvents, the sum and mean of extract yield in each solvent (*N=15*) were 83.5 and 5.6 mg for ACt, 30.0 mg and 2.0 mg for EtOH, and 127.5 and 8.5 for $H_2O$, respectively. The average extract yield for each individual origin in the extraction solvents resulted in the following descending order: India, mean = 4.6, SD = 1.4 mg (ACt) > mean = 3.9, SD = 2.6 mg ($H_2O$) > mean = 1.7, SD = 0.4 mg (EtOH); Saudi Arabia, mean = 11.0, SD = 9.1 ($H_2O$)> mean = 4.5, SD = 1.3mg (ACt)> mean = 2.6, SD = 1.0 mg (EtOH); Iran, mean = 6.7, SD = 3.3 mg (ACt)> mean = 2.9, SD = 0.9 mg ($H_2O$)> mean = 1.4, SD = 0.3 mg (EtOH); Egypt, mean =11.8, SD = 14.2 mg ($H_2O$)> mean = 7.2, SD = 3.9 mg (ACt)> mean 2.2, SD = 1.7 mg (EtOH)); and Yemen, mean = 12.9, SD = 15.3 mg ($H_2O$)> mean = 4.8, SD = 2.4 mg (ACt)> mean = 2.1, SD = 1.3 mg (EtOH).

On an individual basis, the highest extract yield was observed for $H_2O$ (30.6 mg/100 mg) in the Yemeni fenugreek sample, followed by ACt (10.8 mg/100 mg) in the Egyptian fenugreek sample, and EtOH (3.7 mg/100 mg) in the Saudi Arabian fenugreek sample. The total and average extract yield data for the fenugreek samples suggest a descending order of extract yield with the superior solvent to be $H_2O$>ACt> EtOH. The data for the extract yield in green solvents is shown in detail in Table 2.

**Table 2.** **Yield/Origin (mg) and total extract yield (mg) in green solvents for fenugreek samples with SD.**

| Origin | Code | Yield in Act (A) | | | Yield in EtOH (B) | | | Yield in $H_2O$ (C) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Yield/ sample | Average/ Origin | SD | Yield/ sample | Average/ Origin | SD | Yield/ sample | Average/ Origin | SD |
| India | F1 | 5.6 | 4.6 | 1.4 | 1.3 | 1.7 | 0.4 | 1.1 | 3.9 | 2.6 |
| | F2 | 5.3 | | | 2.1 | | | 4.2 | | |
| | F3 | 3.0 | | | 1.8 | | | 6.3 | | |
| Saudi Arabia | F4 | 4.3 | 4.5 | 1.3 | 3.7 | 2.6 | 1.0 | 6.0 | 11.0 | 9.1 |
| | F5 | 3.4 | | | 1.8 | | | 21.5 | | |
| | F6 | 5.9 | | | 2.2 | | | 5.5 | | |
| Iran | F7 | 9.6 | 6.7 | 3.3 | 1.4 | 1.4 | 0.3 | 2.9 | 2.9 | 0.9 |
| | F8 | 7.5 | | | 1.7 | | | 3.8 | | |
| | F9 | 3.1 | | | 1.1 | | | 2.1 | | |
| Egypt | F10 | 7.6 | 7.2 | 3.9 | 1.4 | 2.2 | 1.7 | 4.9 | 11.8 | 14.2 |
| | F11 | 10.8 | | | 4.2 | | | 28.1 | | |
| | F12 | 3.1 | | | 1.1 | | | 2.4 | | |
| Yemen | F13 | 3.2 | 4.8 | 2.4 | 1.2 | 2.1 | 1.3 | 3.9 | 12.9 | 15.3 |
| | F14 | 3.6 | | | 1.4 | | | 4.2 | | |
| | F15 | 7.5 | | | 3.6 | | | 30.6 | | |
| Sum | 83.5 | | | | 30.0 | | | 127.5 | | |
| Mean | 5.6 | | | | 2.0 | | | 8.5 | | |
| SD | 2.5 | | | | 1.0 | | | 9.7 | | |

## Chromatographic separation and MS optimisation

The chromatographic method development resulted in a mobile phase of $H_2O$ (A)+EtOH (B) with an isocratic elution of 30 (A):70 (B). The retention time for TG was 0.882 min with a runtime of 1.5 min at a flow rate of 0.3 mL/min. The method was validated using ICH guidelines in the linearity range of 0.1-500 ppb, where an accuracy of 98.6% with an $r^2$-value of 0.999 was obtained, showing the efficiency and reproducibility of the method. For MS optimisation, a positive (+ve) MRM mode with an ESI interface resulted in the daughter fragments (*m/z*) of 138 →94.10 →92.05 →78.20. The base peak of 94.10 was selected for the data analysis and calibration curve development using the six different concentrations of calibration points. The details regarding LC separation and MS optimisation are presented in Table 3. A representative chromatogram showing the Rt for the TG-peak is given in Figure 2 whereas the mass fragmentation pattern with the base peak for TG is shown in Figure 3. The details regarding the calibration curve, regression equation, and $r^2$-value with the linearity range points are presented in Figure 4.

**Table 3.** **LC and MS conditions for the analysis of TG in fenugreek samples.**

| LC and MS parameters | |
|---|---|
| **Mobile phase** | $H_2O$ (A)+EtOH (B) |
| **Mobile phase composition** | Isocratic at 30 (A):70 (B) |
| **Flow rate (FR)** | 0.3 mL/min |
| **Rt (retention time)** | 0.882 min |
| **Runtime** | 1.5 min |
| **LOD (limits of detection)** | 0.5 ppb |
| **LOQ (limits of quantification)** | 1.4 ppb |
| **Accuracy** | 98.6% |

**Table 3.** *Continued*

| LC and MS parameters | | | | | |
|---|---|---|---|---|---|
| **Regression equation** | | | Y=22846.9x+100685 | | |
| **$r^2$-value** | | | 0.999 | | |
| **Linearity range** | | | 0.1-500 ppb | | |
| **TG-fragmentation pattern** | **Precursor mass** | **Product m/z** | **Q1 pre-bias (V)** | **CE** | **Q3 pre-bias (V)** |
| | 138 | 94.10 | -12.0 | -22.0 | -17.0 |
| | 138 | 92.05 | -10.0 | -20.0 | -22.0 |
| | 138 | 78.20 | -13.0 | -25.0 | -14.0 |
| **MRM mode** | | | +ve | | |
| **Dwell time** | | | 100 msec | | |
| **Event time** | | | 0.309 | | |
| **Mass end time** | | | 1.5 | | |
| **Interface** | | | ESI | | |
| **Nebulizing gas flow** | | | 3 L/min | | |
| **Drying gas flow** | | | 10 L/min | | |
| **Heating gas flow** | | | 10 L/min | | |
| **Interface temperature** | | | 300°C | | |
| **DL temperature** | | | 250°C | | |
| **Heat block temperature** | | | 400°C | | |
| **Interface current** | | | 0.4 uA | | |

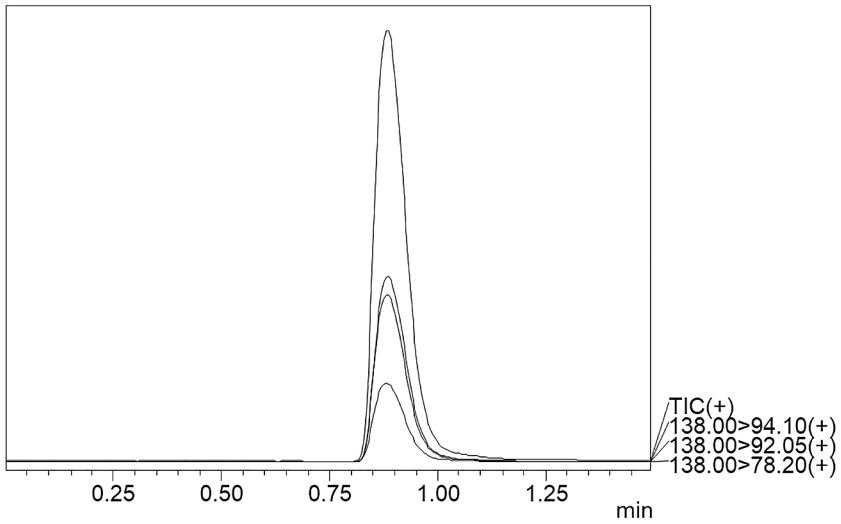



**Figure 2. UPLC-MS/MS chromatogram of TG standard.**

## TG quantification

**UPLC-MS/MS analysis of TG in different fenugreek samples**

The TG amount observed was within the range of (minimum to maximum) 22.2-535.0 ppb (N = 45). The sum, mean, and SD for the TG amount in the fenugreek samples (*N* = 45) were 8161 ppb, 181.4, and 176.4 ppb, respectively. The average TG amount/individual origin (*N=9;* ppb) created a descending order of Yemen (mean = 220.0, SD = 191.7)> India (mean = 211.3, SD = 198.1)> Saudi Arabia (mean = 190.8, SD = 210.7)> Egypt (mean = 189.5, SD = 165.2)> Iran (mean = 95.2, SD = 110.4). The greatest TG amounts were observed in the following fenugreek samples: 535.0 ppb for F3C (Indian)> 510.0 ppb for F6C (Saudi Arabian)> 491.0 ppb for F4C (Saudi Arabian)> 475.0 ppb for F14C (Yemeni).

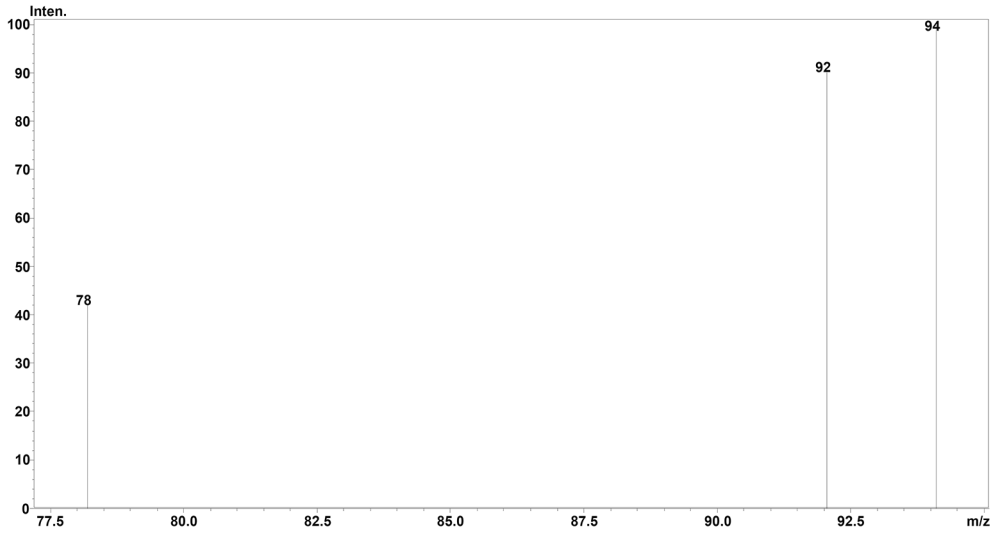



**Figure 3. MSMS fragmentation pattern of TG (*m/z*).**

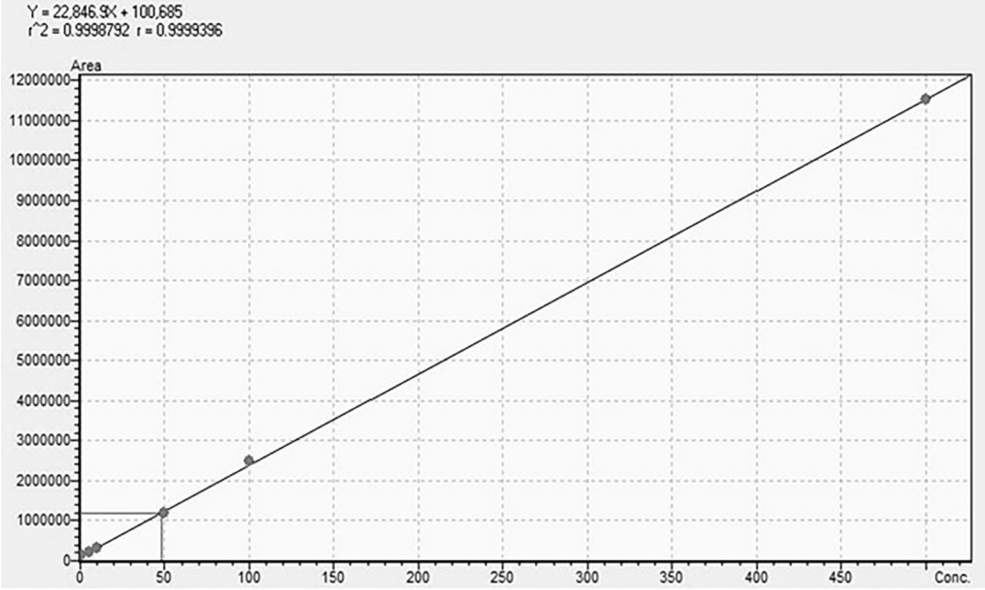



**Figure 4. Standard calibration curve of TG.**

For the highest TG amount in each individual origin (N = 9), an amount of 535.0 ppb in $H_2O$ was observed for F3C (India), 510.0 ppb in $H_2O$ for F6C (Saudi Arabia), 370.0 ppb in $H_2O$ for F9C (Iran), 423.0 ppb in $H_2O$ for F12C (Egypt), and 475.0 ppb in $H_2O$ for F14C (Yemen). Detailed data regarding the TG amount in 45 different samples of fenugreek seeds is provided in Table 4.

### Variation of TG amount in green solvents

The UPLC-MS/MS analysis of the green extracts of fenugreek seeds exhibited the highest average amount of TG (*N=15*) in $H_2O$ (mean = 392.7, SD = 132.4 ppb) followed by EtOH (mean = 91.9, SD = 83.3 ppb) and ACt (mean = 59.5, SD = 30.9 ppb). On an individual basis, the highest amount of TG was detected in $H_2O$ extracts of all fenugreek samples (*N = 45*) when compared to the EtOH and ACt extracts of the same samples.

**Table 4. Average and individual TG amounts in fenugreek samples (F1-F15) extracted with ACt (A), EtOH (B) and $H_2O$ (C).**

| Country of origin | Sample code | TG amount (ppb) | TG % | Average TG amount/origin (ppb) | SD |
|---|---|---|---|---|---|
| **India** | F1A | 56.6 | 0.00011 | 211.3 | 198.1 |
| | F1B | 105.5 | 0.00021 | | |
| | F1C | 434.0 | 0.00087 | | |
| | F2A | 43.2 | 0.00009 | | |
| | F2B | 146.0 | 0.00029 | | |
| | F2C | 437.0 | 0.00087 | | |
| | F3A | 36.7 | 0.00007 | | |
| | F3B | 108.1 | 0.00022 | | |
| | F3C | 535.0 | 0.00107 | | |
| **Saudi Arabia** | F4A | 37.0 | 0.00007 | 190.8 | 210.7 |
| | F4B | 46.7 | 0.00009 | | |
| | F4C | 491.0 | 0.00098 | | |
| | F5A | 80.4 | 0.00016 | | |
| | F5B | 62.3 | 0.00012 | | |
| | F5C | 405.0 | 0.00081 | | |
| | F6A | 34.3 | 0.00007 | | |
| | F6B | 50.1 | 0.00010 | | |
| | F6C | 510.0 | 0.00102 | | |
| **Iran** | F7A | 27.3 | 0.00005 | 95.2 | 110.4 |
| | F7B | 22.2 | 0.00004 | | |
| | F7C | 92.6 | 0.00019 | | |
| | F8A | 22.6 | 0.00005 | | |
| | F8B | 24.3 | 0.00005 | | |
| | F8C | 82.9 | 0.00017 | | |
| | F9A | 133.2 | 0.00027 | | |
| | F9B | 81.7 | 0.00016 | | |
| | F9C | 370.0 | 0.00074 | | |
| **Egypt** | F10A | 100.7 | 0.00020 | 189.5 | 165.2 |
| | F10B | 107.5 | 0.00022 | | |
| | F10C | 391.0 | 0.00078 | | |
| | F11A | 47.2 | 0.00009 | | |
| | F11B | 35.4 | 0.00007 | | |
| | F11C | 407.0 | 0.00081 | | |
| | F12A | 91.6 | 0.00018 | | |
| | F12B | 102.3 | 0.00020 | | |
| | F12C | 423.0 | 0.00085 | | |
| **Yemen** | F13A | 49.1 | 0.00010 | 220.0 | 191.7 |
| | F13B | 102.7 | 0.00021 | | |
| | F13C | 452.0 | 0.00090 | | |
| | F14A | 74.3 | 0.00015 | | |
| | F14B | 359.2 | 0.00072 | | |
| | F14C | 475.0 | 0.00095 | | |
| | F15A | 58.4 | 0.00012 | | |
| | F15B | 24.4 | 0.00005 | | |
| | F15C | 385.0 | 0.00077 | | |

**Table 5. TG amount/Origin (ppb) and total TG amount (ppb) quantified via UPLC-MS/MS in the green extracts of fenugreek.**

| Origin | Code | TG in Act (A) | | | TG in EtOH (B) | | | TG in $H_2O$ (C) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | TG/ sample | Average/ Origin | SD | TG/ sample | Average/ Origin | SD | TG/ sample | Average/ Origin | SD |
| India | F1 | 56.6 | 45.5 | 10.1 | 105.5 | 119.9 | 22.7 | 434.0 | 468.7 | 57.5 |
| | F2 | 43.2 | | | 146.0 | | | 437.0 | | |
| | F3 | 36.7 | | | 108.1 | | | 535.0 | | |
| Saudi Arabia | F4 | 37.0 | 50.6 | 25.9 | 46.7 | 53.0 | 8.2 | 491.0 | 468.7 | 55.9 |
| | F5 | 80.4 | | | 62.3 | | | 405.0 | | |
| | F6 | 34.3 | | | 50.1 | | | 510.0 | | |
| Iran | F7 | 27.3 | 61.0 | 62.6 | 22.2 | 42.7 | 33.8 | 92.6 | 181.8 | 163.0 |
| | F8 | 22.6 | | | 24.3 | | | 82.9 | | |
| | F9 | 133.2 | | | 81.7 | | | 370.0 | | |
| Egypt | F10 | 100.7 | 79.8 | 28.6 | 107.5 | 81.7 | 40.2 | 391.0 | 407.0 | 16.0 |
| | F11 | 47.2 | | | 35.4 | | | 407.0 | | |
| | F12 | 91.6 | | | 102.3 | | | 423.0 | | |
| Yemen | F13 | 49.1 | 60.6 | 12.8 | 102.7 | 162.1 | 175.1 | 452.0 | 437.3 | 46.8 |
| | F14 | 74.3 | | | 359.2 | | | 475.0 | | |
| | F15 | 58.4 | | | 24.4 | | | 385.0 | | |
| Sum | 892.6 | | | | 1378.4 | | | 5890.5 | | |
| Mean | 59.5 | | | | 91.9 | | | 392.7 | | |
| SD | 30.9 | | | | 83.3 | | | 132.4 | | |

For the average TG amount in the three solvent extracts of each individual origin, a descending order may be constructed (*N=9*) as follows: India, mean = 468.7, SD = 57.5 ppb ($H_2O$)> mean = 119.9, SD = 22.7 ppb (EtOH)> mean = 45.5, SD = 10.1 ppb (ACt); Saudi Arabia, mean 468.7, SD = 55.9 ppb ($H_2O$)> mean = 53.0, SD = 8.2 ppb (EtOH)> mean = 50.6, SD = 25.9 ppb (ACt); Iran, mean = 181.8, SD = 163.0 ppb ($H_2O$)> mean = 61.0, SD = 62.6 ppb (ACt)> mean = 42.7, SD = 33.8 ppb (EtOH); Egypt, mean = 407.0, SD = 16.0 ppb ($H_2O$)> mean = 81.7, SD = 40.2 ppb (EtOH)> mean = 9.8, SD = 28.6 ppb (ACt); and Yemen, mean = 437.3, SD = 46.8 ppb ($H_2O$)> mean = 162.1, SD = 175.1 ppb (EtOH)> mean = 60.6, SD = 12.8 ppb (ACt). The average and individual TG amounts suggest the descending order for solvents with optimal TG amount to be $H_2O$> EtOH> ACt. The data regarding the TG amount is provided in detail in Table 5.

## Applicability of USE-UPLC-MS/MS for trace analysis

The developed USE-UPLC-MS/MS was further tested in terms of the efficiency and applicability of the method for trace analysis by $H_2O$ extraction and analysis of lower amounts (10 mg) of fenugreek samples. A sample from each origin

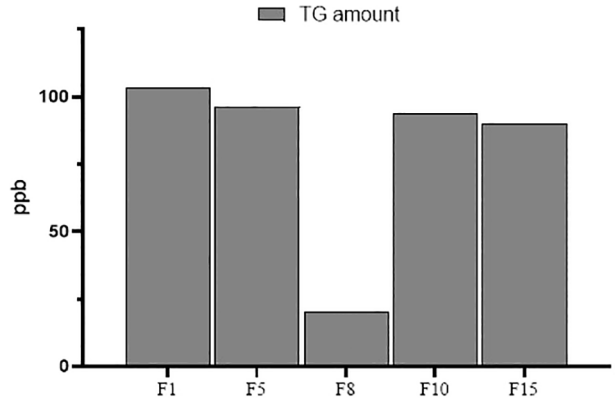



**Figure 5. Trace analysis of TG in 10 mg of five fenugreek samples.**

(F1 for India, F5 for Saudi Arabia, F8 for Iran, F10 for Egypt, and F15 for Yemen) was selected for this purpose. The developed USE-UPLC-MS/MS was able to extract and detect TG in the low-amount samples (10 mg) with a minimum amount of 20 ppb in F8 (Iran). The TG amount from the 10 mg extracts of fenugreek samples (*N=5*) showed a sum of 403.4 ppb with a mean of 80.68 (SD = 34.28 ppb). The TG amount ranged from a minimum of 20 ppb to a maximum amount of 103.5 ppb. The data for the TG amounts in all five samples is presented in Figure 5.

## Discussion

The USE protocol was selected for extraction due to its numerous advantages, including high extraction efficiency and the use of the least possible sample amount, solvent volume, and extraction time.[14,16] The USE method used three green solvents (ACt, EtOH, and $H_2O$) to assess the optimal solvent with the highest TG amount. Compared to toxic solvents, these green solvents significantly reduce health risks, environmental contamination, and regulatory challenges, while also addressing consumer demands for sustainable and safe extraction methods. The combined benefits of these solvents highlight their superiority as extraction solvents, particularly for food bioactive compounds like trigonelline in fenugreek. The extraction results, represented by the individual and total extract yield, suggested $H_2O$ as an appropriate solvent for extraction. This study reports a high aqueous extract yield and TG amount for $H_2O$ among the three green solvents, which agrees with previous studies reporting $H_2O$ as an efficient extraction medium for TG.[7,8] The total extract yield for the green solvents exhibited an order of $H_2O$>ACt>EtOH. Moreover, the highest yield on an individual basis was observed for $H_2O$ in the Yemeni fenugreek sample. The aim of this study was to quantify the TG amount in these fenugreek samples; hence, an advanced analytical instrument of UPLC-MS/MS was employed to develop and validate a green in-house method for TG trace quantification.

A rapid, sensitive, green, and reproducible method was developed using a green mobile phase of $H_2O$ and EtOH with a low flow rate of 0.3 mL/min in a short runtime of 1.5 min with equilibration. The developed method was validated for accuracy, detection and quantification limits, and coefficient value determination as per ICH guidelines, where all the results were obtained within the specified limits. To the best of our knowledge, this is the first green, sensitive, and rapid UPLC-MSMS analysis method for the quantification of TG in fenugreek samples. Previous studies reported the use of HPLC-UV-Vis and LC-MS techniques for TG quantification in coffee beans, fenugreek, and dietary supplements available in the market, but non-green and toxic solvents were used in the mobile phase system.[7–10] Therefore, the main strength of this study is the use of green solvents for extraction and analysis of TG in fenugreek samples. A successful green and rapid extraction and analysis method was developed in this study.

Following a successful UPLC-MS/MS, the extracted samples were analysed using the in-house method. A similar pattern was observed for the extracted samples, where the highest TG amounts were observed in the $H_2O$ samples. A descending order for the solvent with the highest quantities was constructed for all the forty-five extracted samples as $H_2O$> EtOH> ACt. Previous studies have reported trigonelline to be freely soluble in water.[7–12] Therefore, the solubility and extraction behaviour for TG in the aqueous medium complements the concept of TG aqueous solubility, which corroborates the outcomes.

This is the first time to report a comprehensive assessment of TG in fifteen samples from different origins marketed in Saudi Arabia using a green, rapid, efficient, and reliable method of USE-UPLC-MS/MS. The method assessed the quality of the fenugreek samples in terms of their TG concentration, where significant variation was observed among the samples from different origins. Previous TG quantification studies were conducted using coarsely powdered fenugreek samples, as demonstrated in a study by Bakhtiar et al. (2024) in Iran.[17] In that study, the fenugreek samples were analyzed using HPLC–PDA, and the average TG concentrations ranged between 0.426% and 0.678%. The variation in concentration was attributed to differences in geographical location and extraction procedures. In another study, the trigonelline content varied between 0.74% and 0.97%.[18] The higher TG concentrations observed in these previous studies can be explained by the preparation and crushing of fenugreek seeds into powder, which facilitates more efficient solvent penetration and extraction of bioactive compounds like TG. In contrast, the current study used intact seeds to mimic traditional preparation methods, where crushing or grinding may not always be performed. This approach highlights the potential limitations of traditional methods, such as making fenugreek tea or other extracts from uncrushed seeds, which could result in lower TG extraction yields. Furthermore, the findings emphasize the critical role of seed preparation in maximizing the release of active constituents and underscore the importance of considering these factors when comparing TG concentrations across different studies and extraction methods.

The real challenge is to validate the efficiency and reproducibility of the developed USE-UPLC-MS/MS method to be applied practically for the assessment and evaluation of low concentrations of target analytes in complex matrices. At times, the challenge of trace analysis for TG in samples of dietary supplements or marketed products may be encountered; hence, it becomes essential to authenticate the scope of the developed method for quantification of TG at low amounts in a

matrix. To prepare the method for the mentioned challenge, an extra validation step was undertaken for extraction and trace analysis of TG. A small amount (10x lower in concentration) of the fenugreek amount for the five different origin samples was weighed and extracted with USE as previously described. The extracted samples were diluted and analyzed via the UPLC-MS/MS analytical method. The quantification of TG was successfully accomplished at the lowest TG concentration present in the tested fenugreek samples, ensuring the applicability and reliability of the developed method.

The USE-UPLC-MS/MS method developed in this study extends the benefit of using TG as a marker for the quality control and standardization of any pharmaceutical, cosmeceutical, herbal, and dietary products containing TG. The method avoids the need for additional steps, such as seed crushing and sieving, by using an ultrasonic dismembrator. Furthermore, the method employs small sample amounts (10 mg) and low solvent volumes, ensuring cost-efficiency and eco-friendliness. Another add-on advantage is the use of $H_2O$ as a medium for extraction, which is usually produced within the labs using Millipore apparatus. Overall, this method offers high levels of convenience, reliability, sensitivity, and accuracy for the quality evaluation and standardization of any TG-containing samples.

## Conclusions

The study developed a green, fast, economical, and reliable method (USE-UHPLCMSMS) for the extraction and analysis of TG in different fenugreek seed extracts collected from different geographical origins. The method was further validated to ensure the efficiency in matrices containing trace amounts of TG. The use of water with the lowest possible amount of samples is considered a superior economical point of the developed extraction method, in addition to the green and short analysis time of the UPLC-MS/MS analytical method. The USE-UPLC-MS/MS method may serve as a tool in research labs and for the quality control and standardization of TG amounts in different commercial herbal products and dietary supplements.

## Ethics and consent

The present study did not involve the use of human or animals; therefore, ethical approval and consent were not required.

## Authors' contributions

Conceptualization, S.H., M.A., and R.A.; Methodology, M.A., R.A. and S.H.; Software, M.A. and R.A.; Validation, M.A., S.H. and R.A.; Formal Analysis, M.A. and R.A.; Investigation, A.A. and R.A.; Resources, M.A. and R.A.; Data Curation, S.H. and R.A.; Writing—Original Draft Preparation, M.A., R.A., A.A. and S.H.; Writing—Review and Editing, M.A., R.A., S.H. and A.A.; Visualization, M.A., S.H. and R.A.; Supervision, S.H. and R.A.; Project Administration, S.H. and R.A. All authors have read and agreed to the final version of the manuscript.

## Data and software availability

### Underlying data

Zenodo: A validated trigonelline-based method for the standardization and quality control of Trigonella foenum-graecum L., https://doi.org/10.5281/zenodo.13991270.[19]

This project contains the following underlying data:

- Raw data for extract yields and trigonelline amounts in fenugreek (extract yields and trigonelline amounts in 45 fenugreek samples).
- Trigonelline LC-MSMS data (mass spectrum of trigonelline).

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

## Software availability statement

LabSolutions is proprietary software used for the analysis of mass spectra and the calculation of method validation parameters, such as the regression equation, coefficient of determination ($R^2$), accuracy, linearity, limit of detection (LOD), and limit of quantification (LOQ). The mass spectral data analysis and method validation calculations presented in this article, originally performed with LabSolutions, can alternatively be conducted using the freely accessible software LibreOffice version 24.8.2.

License: OSI approved open license software is under the Mozilla Public License (MPL) 2.0.

## Acknowledgements

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# Open Peer Review

## Current Peer Review Status: ✔ ✔

**Version 3**

Reviewer Report 31 December 2024

https://doi.org/10.5256/f1000research.175958.r350252

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✔ **Abdul Sadiq**
University of Malakand, Chakdara, Khyber Pakhtunkhwa, Pakistan

I approve this manuscript

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Pharmaceutical Sciences

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 23 December 2024

https://doi.org/10.5256/f1000research.175958.r350251

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✔ **Abdulrahman Koshak**
King Abdulaziz University, Jeddah, Saudi Arabia

I confirm that the authors have addressed and resolved all comments on the manuscript, and I approve it for indexing.

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Pharmacognosy and natural products

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

**Version 2**

Reviewer Report 12 December 2024

https://doi.org/10.5256/f1000research.175377.r345766

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**Abdulrahman Koshak**
[1] King Abdulaziz University, Jeddah, Saudi Arabia
[2] King Abdulaziz University, Jeddah, Saudi Arabia

The manuscript is much clearer now; however, there are still two minor comments to address:

1. Under the section *Method Validation for Green UPLC-MS/MS Analysis*, what do you mean by "UPLCMSMS-MD"? Is it the same as UPLC-MS/MS? Please clarify.

2. It is evident that the percentages of TG differ significantly from those reported in the literature. You attributed this to the use of unprocessed seeds, which is a valuable observation. I suggest emphasizing the importance of seed crushing in releasing the active constituents of the plant. Additionally, you might want to highlight that certain traditional preparation methods, such as making Trigonella tea from uncrushed seeds, could result in a limited extraction of TG.

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Pharmacognosy and natural products

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 13 Dec 2024

**Salma Hago**

Response to the reviewer comment No. 1:
This typo error and we modified it in the manuscript into UPLC-MS/MS

Response to the reviewer comment No. 2
The higher TG concentration observed in these previous studies can be explained by the

preparation and crushing of the fenugreek sample into powder, which facilitates more efficient solvent penetration and extraction of bioactive compounds like TG. In contrast, the current study used intact seed to mimic traditional preparation methods where crushing and grinding may not be performed. This highlights the potential limitations of traditional methods such as making fenugreek tea, which result in lower TG extraction yield. Further, the findings emphasise the critical role of the seed preparation in maximising the release of active constituents and underscore the importance of considering these factors when comparing TG concentrations across different studies and extraction methods.

***Competing Interests:*** No competing interests

Reviewer Report 06 December 2024

https://doi.org/10.5256/f1000research.175377.r345767

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**Abdul Sadiq**
[1] University of Malakand, Chakdara, Khyber Pakhtunkhwa, Pakistan
[2] University of Malakand, Chakdara, Khyber Pakhtunkhwa, Pakistan

I approve the manuscript in the current form

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Pharmaceutical Sciences

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

**Version 1**

Reviewer Report 26 November 2024

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**Abdul Sadiq**
[1] University of Malakand, Chakdara, Khyber Pakhtunkhwa, Pakistan
[2] University of Malakand, Chakdara, Khyber Pakhtunkhwa, Pakistan
[3] University of Malakand, Chakdara, Khyber Pakhtunkhwa, Pakistan

The manuscript presents a scientifically sound and innovative method for the extraction and analysis of trigonelline in fenugreek seeds. The approach is environmentally sustainable, reproducible, and suitable for widespread application. Minor enhancements could be considered for future work, but the study meets the standards for publication. However, there are a few minor areas where clarity or wording could be slightly improved for better readability:

**Abstract:**
"Fenugreek seeds contain a variety of phytochemicals, including proteins, lipids, amino acids, vitamins, flavonoids, steroidal saponins, coumarin, and alkaloids."
Phytochemicals usually refer to biologically active compounds produced by plants, which usually include non-nutritive substances such as alkaloids, flavonoids, phenolic acids, tannins, terpenes, and saponins. If proteins or amino acids are being mentioned in the context of plant composition, it is more accurate to separate them from the term "phytochemicals." A clearer phrasing for the description of fenugreek seeds might be:
"Fenugreek seeds are rich in proteins, lipids, and essential nutrients, and they also contain various phytochemicals, including flavonoids, steroidal saponins, coumarin, and alkaloids such as trigonelline."

**Introduction:**
"Fenugreek is cultivated in several countries worldwide, including the Mediterranean region, North African areas, India, and other countries."
The phrase "and other countries" is unnecessary after "including." Consider revising to avoid redundancy.

**Methodology:**
"The USE protocol was selected due to the range of advantages USE presents, including high extraction efficiency and the use of the least possible sample amount, solvent volume, and extraction time."
The phrase "the range of advantages USE presents" could be simplified to "the numerous advantages of USE."

**Conclusion:**
"The method avoids the need for an extra step of seed crushing and sieving via the use of an ultrasonic dismembrator with small amounts of sample (10 mg) and low solvent volumes."
Consider breaking this into two sentences for clarity, as the crushing and sieving highlight the simplicity of the method, while the use of small sample amounts and solvent volumes emphasizes cost-efficiency and eco-friendliness.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Pharmaceutical Sciences

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 27 Nov 2024

**Salma Hago**

**Reviewer Comment 1:** Phytochemicals usually refer to biologically active compounds produced by plants, which typically include non-nutritive substances such as alkaloids, flavonoids, phenolic acids, tannins, terpenes, and saponins. If proteins or amino acids are mentioned in the context of plant composition, it is more accurate to separate them from the term "phytochemicals." A clearer phrasing for the description of fenugreek seeds might be: "Fenugreek seeds are rich in proteins, lipids, and essential nutrients, and they also contain various phytochemicals, including flavonoids, steroidal saponins, coumarin, and alkaloids such as trigonelline."
**Response:** We agree with the reviewer's comment regarding the definition of phytochemicals. The sentence in the abstract has been revised to separate proteins and amino acids from the phytochemical category for accuracy. The updated sentence now reads: "Fenugreek seeds are rich in proteins, lipids, and essential nutrients, and they also contain various phytochemicals, including flavonoids, steroidal saponins, coumarin, and alkaloids such as trigonelline."

**Reviewer Comment 2**: "Fenugreek is cultivated in several countries worldwide, including the Mediterranean region, North African areas, India, and other countries." The phrase "and other countries" is unnecessary after "including." Consider revising to avoid redundancy.
**Response:** We appreciate the reviewer's suggestion to avoid redundancy. The sentence has been revised to remove "and other countries" for conciseness. It now reads: "Fenugreek is cultivated in several countries worldwide, including the Mediterranean region, North Africa, and India."

**Reviewer Comment 3:** "The USE protocol was selected due to the range of advantages USE presents, including high extraction efficiency and the use of the least possible sample amount, solvent volume, and extraction time." The phrase "the range of advantages USE presents" could be simplified to "the numerous advantages of USE."
Response: We agree with the reviewer's suggestion to simplify this sentence. The revised sentence now reads: "The USE protocol was selected due to the numerous advantages of USE."

**Reviewer Comment 4:** "The method avoids the need for an extra step of seed crushing and sieving via the use of an ultrasonic dismembrator with small amounts of sample (10 mg) and low solvent volumes." Consider breaking this into two sentences for clarity, as the crushing and sieving highlight the simplicity of the method, while the use of small sample amounts and solvent volumes emphasizes cost-efficiency and eco-friendliness.
**Response:** We appreciate this suggestion to enhance clarity. The sentence has been split into two, as follows: "The method avoids the need for additional steps, such as seed crushing and sieving, by using an ultrasonic dismembrator. Furthermore, the method employs small sample amounts (10 mg) and low solvent volumes, ensuring cost-efficiency and eco-friendliness."

***Competing Interests:*** No competing interest

Reviewer Report 22 November 2024

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**Abdulrahman Koshak**
[1] King Abdulaziz University, Jeddah, Saudi Arabia
[2] King Abdulaziz University, Jeddah, Saudi Arabia
[3] King Abdulaziz University, Jeddah, Saudi Arabia

The article presents a novel analytical technique for quantifying the marker compound TG in fenugreek using UPLC-MS/MS. However, it would benefit from a more comprehensive discussion of the disadvantages associated with traditional methods in comparison to the developed approach. For example, elaborating on the advantages of UPLC-MS/MS, the types of solvents commonly used in traditional methods, their potential drawbacks, and how the new method overcomes these challenges would help to better clarify the research gap.

**Title**: Include the keywords "green" and "UPLC-MS/MS" to emphasize the innovative and advantageous aspects of the study.

**Abstract**:
- Clearly articulate the research gap and the drawbacks of traditional methods for quality analysis of fenugreek.
- Avoid mentioning (TG) in brackets, as it has already been identified earlier in the text.
- In the results section, include the units for extract yield and TG concentration for clarity.

**Formatting**:
- Ensure the correct format (UPLC-MS/MS) is consistently used throughout the article.
- Avoid using the symbol "±" for reporting Standard Deviation; opt for a more descriptive format instead.

**Results**:
- Provide the percentages of Trigonelline found in all samples to facilitate comparisons with values reported in the literature.

**Discussion**:
- The first paragraph contains repetitive information regarding the introduction and methodology. Revise this section to focus directly on the findings and the specific research gap addressed by the study.
- Compare your findings for the amount of TG with those in the literature, expressed in percentages, to provide a clearer context and strengthen the relevance of your results.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Not applicable

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Pharmacognosy and natural products

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 27 Nov 2024

**Salma Hago**

Title Reviewer Comment: Include the keywords "green" and "UPLC-MS/MS" to emphasize the innovative and advantageous aspects of the study. Response: While we appreciate this suggestion, we have chosen to retain the current title because the study has already been published. Changing the title could cause confusion and inconsistency in citations and references to the work. However, we have ensured that the terms "green" and "UPLC-MS/MS" are included in the "Keywords" section to emphasize the innovative and advantageous aspects of the study.

Abstract Reviewer Comment 1: Clearly articulate the research gap and the drawbacks of traditional methods for quality analysis of fenugreek.
Response: The abstract has been revised to better articulate the research gap and the limitations of traditional methods, including their inefficiency, reliance on high solvent volumes, and lack of sensitivity.

Reviewer Comment 2: Avoid mentioning (TG) in brackets, as it has already been identified earlier in the text.
Response: The brackets around "(TG)" have been removed.

Reviewer Comment 3: Include the units for extract yield and TG concentration in the results section for clarity.
Response: Units for extract yield (mg/100mg) and TG concentration (ppb) have been added to the results section of the abstract.

Formatting Reviewer Comment 4: Ensure the correct format (UPLC-MS/MS) is consistently used throughout the article.
Response: The manuscript has been reviewed, and we have ensured that "UPLC-MS/MS" is consistently formatted throughout.

Reviewer Comment 5: Avoid using the symbol "±" for reporting Standard Deviation; opt for a more descriptive format instead.
Response: The symbol "±" has been replaced with descriptive phrasing (standard deviation, SD).

Results Reviewer Comment 1: Provide the percentages of Trigonelline found in all samples to facilitate comparisons with values reported in the literature.
Response: Percentages of Trigonelline in all samples have been added to Table 4. However, direct comparisons with literature values are not feasible because this study focuses on developing a green-sensitive method for trace analysis rather than optimizing an extraction method for high yields. Our approach utilized intact, uncrushed fenugreek seeds, whereas previous studies employed powdered seeds, which likely resulted in significantly lower TG %. Despite this difference, our method provides a rapid and reliable method to detect trace amounts of Trigonelline in fenugreek samples.

Discussion Reviewer Comment 1: The first paragraph contains repetitive information regarding the introduction and methodology. Revise this section to focus directly on the

findings and the specific research gap addressed by the study.
Response: The first paragraph of the discussion has been revised to eliminate repetitive content. It now focuses on the study's findings and how they address the research gap.

Reviewer Comment 2: Compare your findings for the amount of TG with those in the literature, expressed in percentages, to provide a clearer context and strengthen the relevance of your results.
Response: The findings for TG content, expressed in percentages, have been compared with values from the literature to provide a clearer context and emphasize the significance of our results.

***Competing Interests:*** No competing interest

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