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VARIABILITY IN SOME ANTIDIABETIC FLAVONOIDS IN INDIGENOUS MEDICINAL *TRIGONELLA* SPECIES

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ABSTRACT

High performance liquid chromatography method was used to quantify rutin, quercetin and naringenin in seeds of six medicinal *Trigonella* species indigenous to India. For simultaneous analysis of rutin and quercetin the mobile phase was an isocratic binary system of methanol: distilled water (65:35, v/v), a flow rate of 1 ml/min, temperature of 40°C and detection wavelength of 230 nm and 298 nm respectively. For naringenin, an isocratic binary system of acetonitrile: water (65:35; v/v), a flow rate of 1 ml/min with 254 nm UV wavelength and a temperature of 40°C was used. The proposed methods were validated for analytical parameters such as accuracy, linearity, precision, robustness, limit of detection (LOD) and limit of quantification (LOQ) based on the International Conference on Harmonization (ICH) guidelines. In multiple accessions of *T. foenum-graecum* and *T. balansae*, rutin was the most abundant individual flavonoid as compared to quercetin and naringenin. In *T. glabra, T. gracilis, T. occulta* and *T. uncata* rutin could not be detected. While *T. glabra, T. gracilis* and *T. occulta* contained negligible levels of quercetin and naringenin, *T. uncata* displayed a flavonoid profile with the highest amount of naringenin among the tested species. The present work opens up new opportunities to explore the synergistic role of rutin with other phytoconstituents in *T. foenum-graecum* and *T. balansae* contributing to their antidiabetic potential.

Keywords: India, flavonoids, indigenous, *Trigonella*

INTRODUCTION

Flavonoids are dietary secondary plant metabolites with a wide range of biological effects, most importantly anti-oxidative, anti-inflammatory and antidiabetic **[1]**.

These structurally diverse polyphenols are divided into five subclasses: flavonols, flavones, flavanones, anthocyanins, and catechins **[2]**. The most common flavonol in diet quercetin (3,3′,4′,5,7-pentahydroxy flavone) and its glycosidic form quercetin-3-rutinoside also called rutin, rutoside and sophorin display prominent antidiabetic characteristics due to their ability to reduce inflammation and lower reactive oxygen species via several pathways **[3]**. The anti-diabetic effects of naringenin (4,5,7-trihydroxy-flavanone), a citrus flavanone, has been well documented by various *in vitro* and *in vivo* animal studies **[4]**. Numerous studies have focused on quercetin, rutin and naringenin as antidiabetic drugs to prevent and manage diabetes due to their ability to lower blood glucose and improve glucose tolerance by enhancing insulin sensitivity, stimulating insulin secretion, and reducing glucose production in the liver [5, 6]. The dietary intake of these antioxidant and anti-inflammatory flavonoids as the first line of intervention to reduce the pathogenesis of diabetes is well documented **[1]**.

The genus *Trigonella* (Leguminosae) contains medicinal and aromatic herbs with global distribution **[7]**. Many species are rich in nutraceuticals with antidiabetic potential **[8]**. In India, two species are particularly important as antidiabetic herbs: *T. foenum-graecum* L., known as fenugreek (methi), and *T. balansae* Boiss & Reut. (synonym *T. corniculata* L.) commonly called wild trefoil, kasuri methi, kasturi methi or sickle shaped fenugreek. *T. foenum-graecum* is highly valued for its diverse uses in medicine and nutrition. Traditionally, its seeds and leaves have been used for their health benefits, such as supporting heart health, lowering blood sugar, acting as a diuretic, and reducing blood pressure. Fenugreek is also known for its potential in managing diabetes and its complications, including issues like glycation, oxidative stress, high cholesterol, and insulin resistance **[8]**. Key compounds in fenugreek, such as diosgenin, trigonelline, 4-hydroxyisoleucine, and galactomannan, have been extensively studied for their benefits in managing diabetes **[9]**. The existence of flavonoids like quercetin, rutin, vetixin and isovetixin also contributes significantly to the antidiabetic potential of this plant **[10]**.

Besides *T. foenum-graecum*, seeds of *T. balansae*, commonly used as spice and flavouring agent in India, are rich in nutraceuticals (predominantly diosgenin and yuccagenin) with antidiabetic potential **[8]**. However, the presence and role of antidiabetic flavonoids has not been studied in this species. There is also a need to characterize the flavonoid profiles in *T. glabra* Thunb., *T. uncata* Boiss. & Noë. (hooked wild fenugreek), *T. occulta*

Delile ex Ser and *T. gracilis* Boiss. (slender fenugreek, Tinpaate Jhaar) used in traditional medicine in India as anti-inflammatory and antidiabetic agents **[11, 8]**. The present work aims to detect and comparatively quantify three important antidiabetic flavonoids in seeds of some indigenous medicinal *Trigonella* species using High Performance Liquid Chromatography (HPLC). The quantification of antidiabetic flavonoids would contribute towards the understanding of their combination with other phytochemicals responsible for the promising way by which some species can reduce blood sugar levels.

MATERIAL AND METHODS

Chemicals

The standards of rutin, quercetin and naringenin (purity >=99%) were purchased from Sigma-Aldrich (Aldrich Division, Steinbeim, Germany). Acetonitrile and methanol (HPLC grade) were purchased from Merck. Ultra-pure water was obtained using a Milli-Q purification system (Millipore, USA).

Plant material

Seeds of six *Trigonella* species, *T. foenum-graecum, T. balansae*, *T. glabra, T. gracilis*, *T. uncata* and *T. occulta* (two accessions) were collected from India [7, 8]. The herbarium of these species has been submitted to Botanical Survey of India, Western Regional Center and/or Agharkar Research Institute Pune, India for archival and reference purpose. Multiple accessions of *T. foenum-graecum* and *T. balansae* were analysed to represent chemical diversity present across different geographic locations. For this, seeds of four accessions of *T. foenum-graecum* and three accessions of *T. balansae* sampled from different geographic locations were procured from South Australian Research and Development Institute (SARDI), South Australia through National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India **(Table 1)**.

Table 1: *Trigonella* species used for quantification of flavonoids

Sr. No.	Species	Identity number	Country of collection
1	*T. balansae* Boiss. &Reut.	BSI 141702	India (Nagaur Rajasthan)
		EC 583500	Jordan
		EC 583507	Pakistan
		EC 583509	Turkey
2	*T. foenum-graecum* L.	BSI 141589	India (Jodhpur Rajasthan)
		EC 583588	Iran
		EC 583590	Egypt
		EC 583591	Syria
		EC 583592	Saudi Arabia
3	*T. glabra* Thunb.	BSI 141536	India (Ratnagiri Maharashtra)
4	*T. gracilis* Benth.	BSI 141605	India (Ganderbal Jammu &Kashmir)
6	*T. occulta* Delile.	BSI 141554	India (Pune Maharashtra)
		BSI 141598	India (Amravati Maharashtra)
7	*T. uncata* Boiss & Noe.	BSI 207560	India (Bharatpur, Rajasthan)

EC: Accession identity number- National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India
BSI - Herbarium submitted at Botanical Survey of India, Western Regional Center. Pune, India

Preparation of rutin, quercetin and naringenin standard solution

Ten mg of rutin, quercetin and naringenin was dissolved in 1ml of HPLC grade methanol and syringe filtered (0.22 µm). The solution was diluted with methanol to prepare a 1 mg/ml stock solution which was later appropriately diluted with methanol to prepare calibration solution concentrations: (5 µg – 100 µg)/ml

Preparation of sample seed extract

Seeds (1 g) were powdered to a fine powder using liquid nitrogen. 0.1g of finely ground seed powder was added to 2 ml of methanol. The solution was sonicated with an ultrasonic probe sonicator (LABMAN Pro-650) at 20 ± 5°C, power rate 20% for a process time of 30 minutes per sample. After extraction, the extract was centrifuged at 10,000 rpm for 15 minutes; the supernatant was concentrated in a hot water bath at 72°C and the resultant crude extract was suspended in 1 ml HPLC grade methanol and syringe filtered through a 0.22 µm filter membrane before HPLC analysis.

HPLC chromatographic conditions

Quantification of flavonoids was performed using HPLC (Shimadzu LC-20 system with LC-20 AD pump and photodiode array detector SPD-M40 having a wavelength range of 190 nm-800 nm, Shimadzu Corporation, Kyoto, Japan. Chromatographic estimations were performed using an equilibrated reverse phase Shimadzu Shim-Pack Solar C-18 column (partical size 5 µm, 4.6. I.D. × 250 mm). For simultaneous quantification of rutin and quercetin, the mobile phase was an isocratic binary system of methanol: distilled water (65:35, v/v), a flow rate of 1 ml/min, temperature of 40°C and detection wavelength of 230 nm and 298 nm respectively. For naringenin, an isocratic binary system of acetonitrile: water (65:35; v/v), a flow rate of 1 ml/min with 254 nm UV wavelength and a temperature of 40°C was used. Standard solutions of flavonoids (10 µl, ranging from 5 µg – 25 µg) and seed extract (10 µl) were injected into HPLC. The peak areas obtained were plotted against the concentration of flavonoids injected. Area vs. concentration calibration curves were treated by the least square regression analysis. The regression equation with slope, intercept and coefficient of correlation was calculated. In the sample, peak identification was done by comparing with the retention time (RT) and UV absorption spectrum of standard. Post run data acquisition was done on the LC Solutions Chromatographic software. All the samples were analysed in triplicate to determine the average content. The content of flavonoids in sample extract was expressed as mg/ g of seeds.

Method Validation

The method was validated for linearity, limit of detection and quantification (LOD and

LOQ), precision (intraday and interday variation and repeatability), accuracy (recovery) and robustness following ICH Q2 (R1) **[12]** and some reports in literature **[13]**.

RESULTS

Optimization of chromatographic conditions (Mobile phase and detection wavelength)

Various proportions of methanol–water or acetonitrile–water system chosen did not yield satisfactory simultaneous separation of rutin, quercetin and naringenin. Mixtures of methanol–water or acetonitrile–water in different ratios gave a better separation of rutin and quercetin but could not separate quercetin from naringenin. Thus, rutin and quercetin were quantified simultaneously using a mobile phase of methanol: water in the ratio 65:35 v/v with minimum peak trailing. Naringenin was quantified separately using acetonitrile: water (65:35; v/v) as mobile phase. Using DAD, the spectro-chromatograms of all flavonoids at several wavelengths were detected simultaneously. In the spectrogram of rutin, only one peak appeared at 230 nm Hence, 230 nm was confirmed as detection wavelength for rutin. In the spectrogram of quercetin two clear peaks appeared at 280 and 298 nm separately. The peak at 298 nm was higher than the peak at 280 nm, that is quercetin's UV-absorbing value at 298 nm was higher than the value at 280 nm. Hence, 298 nm was confirmed as determination wavelength for quercetin. Likewise, in naringenin the presence of a single peak with maximum absorption was obtained at 254 nm.

System suitability (Stability, linearity and detection limit)

Standard solution (100 µg/ml) of rutin, quercetin and naringenin were injected and analysed under the specified chromatographic conditions for average peak area and retention time at an interval of 0, 12, 24 and 48 h. The standard solution was stable and did not show degradation till 48 h. The area vs. concentration calibration curve exhibited a linear relationship for all flavonoids within the concentration range of 5 µg – 25 µg/ml with an acceptable regression coefficient. Detail information regarding the calibration curve, linear range, LOD and LOQ is listed in **Table 2**.

Precision

Precision was determined on two levels, which included repeatability (intra-assay precision) and intermediate precision (ruggedness). Inter and intraday precision was carried out by injecting 10 µl of a standard solution of flavonoids (concentration of 100, 200 and 400 µg/ml) into the column, in six replicates (inter-day reproducibility n=3, intra-day reproducibility n=3), under specified chromatographic conditions. The percent relative standard deviation (%RSD) of peak area of standard calculated was < 2% which

is ideal as per ICH Q2 (R1) guidelines **(Table 3)**.

Accuracy

To measure recovery, the samples were spiked with three different amounts of standard stock solution (100, 200, and 400µg/ml) and injected three times with optimum chromatographic conditions. The percent recovery of spiked samples ranged from 97.4155% to 117.29 which is ideal as per ICH Q2 (R1) guidelines (80-120% for trace levels analytes **Table 4**).

Robustness

Parameters of robustness studied included change in organic phase concentration (methanol or acetonitrile) and change in column temperature (± 5.0°C). The change in retention time and drug concentration obtained from the variation parameters were compared to those obtained for the normal method conditions. Throughout robustness testing, 10 µl of standard flavonoid solution at a concentration of 100 µg/ ml was used. **Table 5** summarises the results obtained during robustness testing.

Quantification of Rutin, quercetin and naringenin from *Trigonella* species

The validated analytical method was used to determine total rutin and quercetin content simultaneously in different samples **(Figure 1)**. Naringenin was quantified separately in all samples. All the samples were tested in triplicate to determine the average content. In five accessions of *T. foenum-graecum* sampled, the average rutin content varied from 4.375 ± 0.36 - 9.027 ± 0.57 mg/ g of seeds. The content of quercetin varied from 0- 0.0152 ± 0.02 mg/ g of seeds while that of naringenin ranged from 0 to 0.2286 ± 0.10 mg/ g of seeds. The content of rutin, quercetin and naringenin in *T. balansae* was comparable to *T. foenum-graecum*. In the four accessions of *T. balansae*, the average content of rutin varied from 7.115 ± 0.64 to 8.158 ± 0.34 mg/g of seeds. The content of quercetin and naringenin were 0.0173±0.011 - 0.0252± 0.3 mg/ g and 0-0.0202 mg/ g of seeds respectively **(Table 6)**. In *T. glabra, T. gracilis, T. occulta* and *T. uncata* rutin could not be detected. While *T. glabra, T. gracilis* and *T. occulta* contained negligible levels of quercetin and naringenin, *T. uncata* displayed a flavonoid profile with the highest amount of naringenin (3.195 ± 0.15 mg/ g of seeds) among the tested species **(Figure 2)**.

Table 2: Regression data, LODs, and LOQs for rutin, quercetin and naringenin analysed by HPLC.

Compound	Concentration (µg/ml)	Slope equation (n=3)	Regression Coefficient (r^2)	Limit of detection (LOD, µg/ml)	Limit of Quantification (LOQ µg/ml)
Rutin	5–25	Y= 3016.8x – 8646.5	0.9994	1.74438	5.286
Quercetin	5–25	Y= 15040x – 23588	0.9994	1.716938	5.202844
Naringenin	5–25	Y= 5983.5x – 7477	0.9987	2.58759	7.84118

Table 3: Intra and Inter day variability

Compound	Concentration (µg/ml)	Intra-day precision		Inter-day precision	
		Mean area ± SD	RSD (%)	Mean area ± SD	RSD (%)
Rutin	100	232563.67 ± 2855.73	1.228	239367.34 ± 4107.24	1.716
	200	550030.67 ± 1294.78	0.235	553615 ± 2971.26	0.537
	400	1462781.34 ± 10353.26	0.708	1494754.34 ± 26495.37	1.773
Quercetin	100	1750871.34 ± 20544.04	1.174	1762912 ± 18257.56	1.036
	200	3481026.67 ± 8924.11	0.256	3448387.67 ± 23881.12	0.693
	400	6873602.34 ± 22607.79	0.329	6814903.67 ± 37846.72	0.555
Naringenin	100	394999 ± 2066.17	0.523	396705.67 ± 2411.01	0.608
	200	747675.67 ± 2689.30	0.360	757014.67 ± 10099.14	1.334
	400	2854282.34 ± 3164.32	0.111	2870479.34 ± 19221.24	0.670

Table 4: Recovery data of spiked standard to the *T. foenum-graecum* seed extracts

Compound	Original (µg/ml)	Spiked (µg/ml)	Measured ± SD (µg/ml)	Recovery (%)	%RSD
Rutin	1516.785	100	1632.66 ± 4.863	112.441	0.298
	1565.773	200	1768.49 ± 11.146	97.4155	0.630
	1547.462	400	1958.79 ± 9.044	101.234	0.462
Quercetin	0	100	98.20 ± 0.839	98.790	0.839
	0	200	203.22 ± 0.673	101.370	0.331
	0	400	423.36 ± 3.481	105.219	0.822
Naringenin	39.289	100	147.670 ± 3.35	106.01	2.272
	39.289	200	277.97 ± 5.789	117.29	2.083
	34.858	400	497.329 ± 9.007	114.02	1.811

Table 5: Method Validation for Robustness

Compound	Parameter	Method	Condition	Active Drug (%)	Retention Time
Rutin	Mobile phase (methanol: water)	Original Method	65:35	100	4.087
		Changed Method	60:40	94	5.903
			70:30	153	4.033
	Temperature	Original Method	40 ° C	100	4.087
		Changed Method	35 ° C	121	4.28
			25 ° C	107	4.34
Quercetin	Mobile phase (methanol: water)	Original Method	65:35	100	6.464
		Changed Method	60:40	98	7.012
			70:30	103	7.039
	Temperature	Original Method	40 ° C	100	6.464
		Changed Method	35 ° C	94	7.29
			25 ° C	86	7.039
Naringenin	Mobile phase (acetonitrile: water)	Original Method	65:35	100	3.779
		Changed Method	60:40	98	7.4
			70:30	104	5.05
	Temperature	Original Method	40 ° C	100	3.779
		Changed Method	35 ° C	105	4.75
			25 ° C	122	5.9

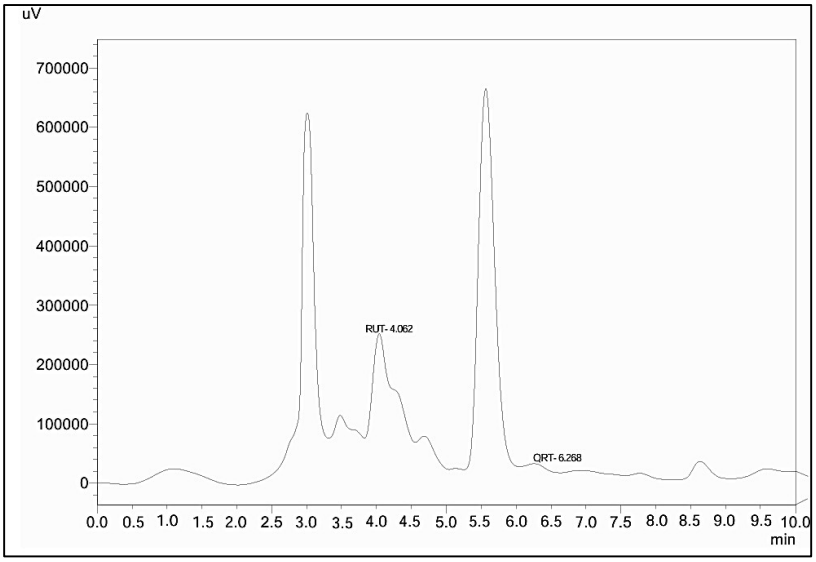

Figure 1: Representative chromatogram showing the total runtime and separation of rutin and quercetin in *T. balansae*

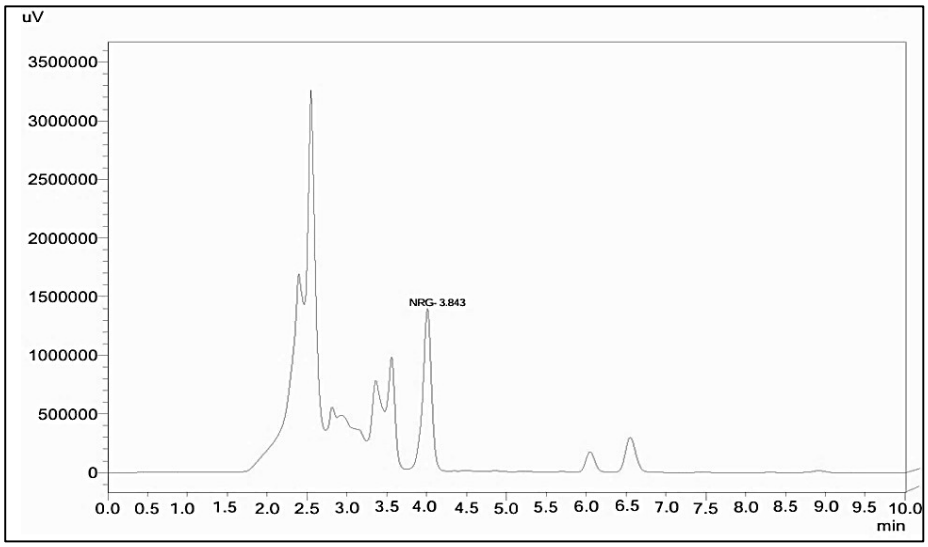

Figure 2: Representative chromatogram showing the total runtime and naringenin in *T. uncata*

Table 6: Individual flavonoid content in tested *Trigonella* species

Sr. No.	Species	Country of collection	Rutin (mg/ g seeds)	Quercetin (mg/ g seeds)	Naringenin (mg/ g seeds)
1	*T. balansae*	India	8.158 ± 0.34	0.0173±0.011^{BQL}	nd
		Jordan	7.115 ± 0.64	0.0185± 0.1^{BQL}	0.0202 ± 0.00^{BQL}
		Pakistan	8.049 ± 0.26	0.0252± 0.3^{BQL}	nd
		Turkey	8.103 ± 0.77	0.0175 ± 0.0^{BQL}	0.0191 ± 0.00^{BQL}
2	*T. foenum-graecum*	India	8.258 ± 0.34	0.0334 ± 0.017^{BQL}	0.2286 ± 0.10
		Iran	9.027±0.57	nd	0.0194 ± 0.021^{BQL}
		Egypt	7.422 ± 0.08	0.0152 ± 0.02^{BQL}	nd
		Syria	4.375 ± 0.36	nd	0.0215 ± 0.00^{BQL}
		Saudi Arabia	8.696 ± 0.51	0.0138 ± 0.0^{BQL}	nd
3	*T. glabra*	India	nd	0.0212 ± 0.011 ^{BQL}	0.0350 ± 0.018^{BQL}
4	*T. gracilis*	India	nd	0.0507 ± 0.0 ^{BQL}	0.5379 ± 0.015
5	*T. occulta*	India	nd	0.0105 ± 0.01^{BQL}	0.0296 ± 0.01^{BQL}
6		India	nd	nd	0.0208 ± 0.001^{BQL}
7	*T. uncata*	India	nd	nd	3.195 ± 0.150

BQL: values below LOQ; nd: not detected

DISCUSSION

The present study is the first report on quantification of three important antidiabetic flavonoids in Indian *Trigonella* species using HPLC method validated as per ICH guidelines for its sensitivity (LOD and LOQ), linearity, precision, stability, accuracy and robustness. All the results were obtained within the specified limits. Sonication is considered as a simple and effective method for extraction of flavonoids as compared to reflux, hot water extraction or solvent extraction using soxhlet **[14]**. Ultrasonic wave can quickly induce acoustic cavitation of plant cell wall so as to enhance the extraction of phytoconstituents from plant matrices. In fenugreek, the solvent with highest yields of flavonoids by sonication was methanol **[15]**. To avoid the degradation of flavonoid compounds and decrease energy consumption, extraction temperature below

30°C and ultrasonic time of not more than 30 mints is recommended **[16]**. Hence, in the present work, flavonoids were extracted in methanol by sonication of finely powdered seeds at 20 ± 5°C for 30 mints.

In the species tested, the content of quercetin and naringenin was well within the range reported in seeds of some legumes like *Glycin max, Phaseolus mungo, Psoralea corylifolia,* and *Raphanus sativus* **[17]**. However, in *T. foenum-graecum* and *T. balansae* the amount of rutin was appreciably higher than these legumes. In *T. foenum-graecum* and *T. balansae* accessions sampled, the average rutin content is within the range reported in seeds of medicinal plants like *Fagopyrum esculentum* **[18]**, and *Sophora japonica* **[19]**. The variation in rutin content in the accessions of *T. foenum-graecum* and *T. balansae* can be attributed to differences in geographical location, altitude and environmental conditions as reported by Alaghemand *et al* 2012 **[20]**. Previous studies have report that *T. foenum-graecum* seed extracts contained high amount of rutin **[21, 22]**, while quercetin was poorly detected **[23]**. Eaknai *et al* (2022) [20] confirmed that rutin was the main active ingredient in fenugreek seed extracts using LC-MS. In agreement to present results, Eaknai *et al* (2022) **[20]** reported as high as 7.73 ± 0.40 mg/g of rutin in seed extracts of fenugreek. Ahmed *et al* (2023) **[15]** compared the content of rutin, quercetin, luteolin, and kaempferol in *T. foenum-graecum* accessions from Egypt, India, Saudi Arabia, Yemen and Iran. In agreement to Ahmed *et al* (2023) **[15]**, in the present study also the content of flavonoids showed variation in different accessions of *T. foenum-graecum* and rutin was the most abundant individual flavonoid in seeds followed by quercetin. However, we recovered a higher content of rutin in *T. foenum-graecum* as compared to Ahmed *et al* (2023) **[15]**. This may be due to difference in the bioactive extraction method employed. In the present study liquid nitrogen was used to grind the seeds to a very fine powder which may have led to a higher yield of flavonoids by sonication.

Rutin, a dietary flavonoid glycoside of quer cetin is found in many vegetables, fruits, and medicinal herbs. It has a wide range of activities, including antioxidant, anti-inflammatory, antiallergic,anti-aggregative, cholesterol-lowering, anti-diabetic and neuroprotective properties **[5, 6]**.

Increasing research has confirmed the role of diosgenin, trigonelline, 4-hydroxyisoleucine, and galactomannan in the antidiabetic, antiobesity, anti-inflammatory, antioxidant and anticancer activity of fenugreek seeds **[8]**. The presence of appreciable amount of rutin in fenugreek seeds releveled by the present study strongly supports the fact that a substantial portion of fenugreek's health benefits may also rely on

the presence on rutin. Although needed to be tested with more accessions, variation observed in rutin content among the samples from different geographic origins also opens up the possibility of using rutin, in addition to trigonelline and diosgenin as a biomarker for quality control evaluation of fenugreek seed powder and marketed formulations. Rutin can also be used as an additional chemical marker to assess the effects of agrotechnological factors on the chemical composition of fenugreek seeds.

T. balansae (synonym *T. corniculata* Kasuri/champa/Nagpuri methi) is cultivated in India and Pakistan and is morphologically and genetically distinct from *T. foenum-graecum*. Due to its strong aroma, it is a more popular spice and flavouring agent in India than *T. foenum-graecum*. Its young tops are used as a green vegetable and its seeds are used for the treatment of swellings and bruises **[24]**. *T. balansae* also has significant nutraceutical potential, being abundant in phytochemicals with hypoglycaemic and antidiabetic potential **[25]**. With comparable level of total phenolic (TPC) and total flavonoid content (TFC), *T. balansae* seed extract had a better DPPH radical scavenging and total antioxidant activity as compared to *T. foenum-graecum* **[8]**. Despite its importance, research on the phytochemical profiling of *T. balansae* remains scanty. The present report is the first to reveals that *T. balansae* like *T. foenum-graecum* contain comparable and higher amounts of rutin than quercetin and naringenin. In addition to the presence of diosgenin, yamogenin, choline, betain **[26, 27]**, the antioxidant and antidiabetic potential of *T. balansae* may be attributed to the presence of these flavonoids. This work opens up new opportunities to explore the synergistic role of rutin with other phytoconstituents in *T. balansae* and *T. foenum-graecum* to understand the promising way by which these species can reduce blood sugar levels associated with diabetes. *T. glabra, T. gracilis, T. occulta* and *T. uncata* were distinct in their flavonoid profile when compared to *T. foenum-graecum*. Our results showed for the first time that naringenin, rather than rutin or quercetin is the more abundant individual flavonoid in *T. uncata*. This species is included in the Indian herbals along with *T. foenum-graecum* **[24]**.

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