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Original Article

# EXTRACTION AND ISOLATION OF FLAVONOID QUERCETIN FROM THE LEAVES OF *TRIGONELLA FOENUM-GRAECUM* AND THEIR ANTI-OXIDANT ACTIVITY

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**ABSTRACT**

**Objective:** The present study was designed for isolation of bioactive flavonoid molecule quercetin from the leaves of *Trigonella foenum-graecum* and their subsequent characterization.

**Methods:** Crude extracts of fenugreek were prepared using various solvents such as hexane, ethyl acetate, and ethanol. The plant extracts were subjected for photochemical analysis and total flavonoid content. The extracts were then subjected to column chromatography followed by TLC. The isolated compound was subjected to FT-IR, $^{1}H$ NMR, $^{13}C$ NMR, mass spectroscopy and their free radical scavenging activity was studied.

**Results:** The ethanol extract showed the presence of higher flavonoid content when compared with other solvent extracts. The ethanol extract was subjected to fractionalization by column chromatography. The eluted fractions were run in TLC mobile phase with the different solvent ratio. The fractions showed $R_f$ value equal to standard quercetin in TLC were combined and crystallized. The characterization techniques confirmed that the isolated compound was found to be quercetin. The free radical scavenging activity suggests that the isolated compound quercetin could act as a potent source of antioxidants.

**Conclusion:** The flavonoid quercetin was isolated effectively from the leaves of *Trigonella foenum-graecum* and their antioxidant activity was studied.

**Keywords:** Antioxidant activity, DPPH, Flavonoids, NMR, Quercetin



## INTRODUCTION

Fenugreek (*Trigonella foenum-graecum*) is an annual herb that belongs to the family Leguminosae commonly grown in India, Pakistan, and some Middle Eastern countries, which has many beneficial medicinal effects [1]. Both the leaves and seeds of the fenugreek plants were widely consumed as food and medicine in Indo-Pak subcontinent and also in other countries. Fenugreek is rich in the source of vitamins, iron, β-carotene, etc [2]. It also has been reported to exhibit pharmacological properties such as antimicrobial, antiviral, antitumor, anti-inflammatory and antioxidant activity [3-5]. The fenugreek plant was included in normal diet generally because it has haematinic value [6].

Previous reports revealed that the fenugreek has medicinal properties such as hypoglycemic, anticancer, and gastro protective effects [7-9]. Despite the multiple bioactive properties that have been attributed to *Trigonella foenum-graecum*, the effects of this plant were widely studied. It was reported that the phytochemical analysis of fenugreek seeds contain a variety of alkaloids, flavonoids, saponins and carbohydrates [10]. There is an increased interest in the pharmaceutical properties of this plant. The bioactive compounds in the seeds of the fenugreek were extensively studied. There was only limited literature information was found about the bioactive compounds in the leaves of this plant. The phytochemical analysis of the leaves revealed the presence of two major flavonoids namely quercetin and kaempferol, either in the free form or as glucosides found in the aerial part of *Trigonella foenum-graecum* [11].

The search for naturally occurring quercetin has a great interest in industries as well as in scientific research. There was no qualitative and quantitative information on effective isolation on the flavonoid, particularly quercetin content in the leaves of the plant. As flavonoids are one of the major natural product subgroups in all the plants, it is useful to explore their content in the fenugreek leaves. Furthermore, there is a growing interest for naturally derived medications; effective methodology should be developed to extract the bioactive compounds from the leaves. In this study, we developed an analytical methodology for the qualitative and quantitative determination of a flavonoid quercetin from the leaves of fenugreek and their potential activity as a source of natural antioxidants.

## MATERIALS AND METHODS

### Plant collection and authentication

During the vegetative stage, the leaf samples of *Trigonella foenum-graecum* were collected from plants grown without pesticide in the field near to Madhuranthagam, Tamilnadu, India. The herbarium specimen was identified by Prof. P. Jayaraman, Institute of Herbal Botany, Plant Anatomy Research Centre, Chennai (Reg. No of the certificate: NO. PARC/2014/2254). The leaves cut down and separated from other parts of the plants, cleaned and dried for further experimental use.

### Preparation of plant extract

The plant materials (leaves of *Trigonella foenum-graecum*) were air-dried at room temperature (26 °C) for two weeks and later it was then ground to uniform powder. The extracts of leaf samples were prepared in a sequential procedure by soaking 100 g of dried powder in 900 ml of different solvents (hexane, ethyl acetate, and ethanol) for 48 h. At the end of respective extraction, the plant extracts were filtered using whatman filter paper. The filtrate was then concentrated under reduced pressure in vacuum at 40 °C for 25 min using a rotary evaporator (Superfit-ROTAVAP, India). The yields of extracts were calculated.

### Phytochemical screening

Phytochemical screening for major constituents was carried out using standard qualitative methods. Screening test was performed for carbohydrates, tannins, saponins flavonoids, cardiac glycosides, terpenoids, triterpenoids, phlobatannins, anthraquinones, alkaloids, quinones, phenols, coumarins, glycosides, proteins, steroids and phytosteroids by following the method of Harborne [12] and Edeoga [13].

**Estimation of total flavonoid content (TFC)**

Total flavonoid content (TFC) in the leaf extracts (hexane, ethyl acetate, and ethanol) were determined using the method described by Sakanaka *et al.* [14]. The flavonoid content was determined by aluminium chloride method using quercetin as standard. Extracts and quercetin were prepared in ethanol (1 mg/ml). 0.1 ml of extract was mixed with 0.9 ml of distilled water in test tubes, followed by addition of 75 µl of a 5% sodium nitrite solution. After 6 min, 150 µl of a 10% aluminium chloride solution was added and the mixture was allowed to stand for further 5 min after which 0.5 ml of 1M sodium hydroxide was added to the reaction mixture.

The reaction mixture was brought to 2.5 ml with distilled water and mixed well. The absorbance was measured immediately at 510 nm using a spectrophotometer. The determination was performed in three replicates. The absorbance was measured using various concentrations of quercetin (20-140 µg). Blank consist of all the reagents, except for the extractor quercetin standard solution is substituted with 0.1 ml of ethanol. Results were expressed in mg of quercetin equivalent/g of dry weight of the extracts.

**Column chromatography**

10 g of ethanol leaf extract were chromatographed over silica gel column (100-200 mesh) using solvents with increasing polarity. The admixture was packed on a silica gel column (Merck, India) and elution starts with 100% Hexane and then increased the polarity using Chloroform, Ethyl acetate and Ethanol and Methanol in the ratio of 90:10, 80:20, 70:30 and 50:50. All the collected fractions were run for TLC. Based on TLC profile fractions with similar $R_f$ values were pooled into some fractions.

**Thin layer chromatography (TLC) procedure**

The TLC developing was set as twin through chamber were examined in various solvent systems, such as toluene, ethyl acetate, chloroform and methanol in the ratio 9.5:0.5, 9:1, 7:3, 1:1. The fractions were run on silica gel 60 F254 pre-coated aluminum plate, of 0.2 mm thickness. The optimal solvent for the identification of compound was determined by varying the ratios of solvents for developing the solvent system. Visualization was carried out by dipping the plate in vanillin-sulphuric acid reagent and heating till the color of the spot appears. Retardation factor ($R_f$) was calculated using the formula,

$R_f$ = Distance moved by the solute/Distance moved by the solvent

**Infrared (IR) and nuclear magnetic resonance (NMR) analysis**

IR spectra were recorded on Bruker Alpha TKBR and ATR spectrophotometer. $^1H$ and $^{13}C$-NMR spectra were run on a Bruker AV NMR instrument equipped with 5 mm $^1H$ and $^{13}C$ operating at 500 MHZ, respectively with tetramethyl silane (TMS) as an internal standard.

**LC-MS-MS identification**

An Agilent 6400 series Triple Quad (QQQ) LC-MS (Agilent, Beijing, China) instrument equipped with an electro spray ionization source (ESI) was employed to analyze the composition of fractions. The optimized detection parameters were as follows: Chromatographic conditions were set up for the identification of an isolated compound. Full wavelength scanning was performed from 190 to 400 nm, the flow rate was 0.4 ml/min, sampling volume was 5 µl, and the column temperature was 30 °C. Mass spectrometry conditions: negative ion mode, atomization gas pressure-40 psi, dry gas velocity-9 l/min, drying temperature-350 °C, ionization voltage-3,000 V, electro spray ionization (ESI), detection of anion way-auto $MS^n$, scanning range-200~800 m/z.

**Determination of antioxidant activity of free quercetin and Q-PLAN using DPPH assay**

The free radical scavenging activity of isolated compound quercetin was measured *in vitro* by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay according to the method reported by Kumar *et al.* [15]. An aliquot of 0.5 ml of different concentration of sample solution in methanol was mixed with 2.5 ml of 0.5 mM methanolic solution of DPPH. The mixture was shaken vigorously and incubated for 37 min in the dark at room temperature. The absorbance was measured at 517 nm using UV-Vis spectrophotometer. Ascorbic acid was used as a positive control. DPPH free radical scavenging ability (%) was calculated by using the formula.

Percentage (%) of inhibition = [(absorbance of control–absorbance of sample)/(absorbance of control)] ×100.

**RESULTS AND DISCUSSION**

**Total yield extracts and photochemical analysis**

The plant materials were collected and dried. The plant extracts were collected with different solvents. The final yield of leaf extracts in different solvents was calculated and listed in table 1. Photochemical analyses were tested in various solvent leaf extracts. The presence and absence of photochemical parameters in all three leaf extracts were listed in table 2.

**Table 1: Final yields of the leaf extract in various solvents**

| Solvent | Yield (g) |
|---|---|
| Hexane | 2.869 |
| Ethyl acetate | 5.64 |
| Ethanol | 19.752 |

**Estimation of total flavonoid content (TFC)**

Isolation of flavonoid is the main target of our study; the TFC in all three leaf extracts were quantified. TFC was calculated according to the absorbance targeted for flavonoid quercetin. All three solvent extracts showed the presence of flavonoid quercetin (fig. 1). The hexane and ethyl acetate plant extracts showed about one fold of TFC and the ethanol extracts showed more than three folds of TFC with respect to absorbance with flavonoid quercetin.

**Column chromatography**

Ethanol extract (10 g) was subjected to column chromatography on silica gel (100-200 mesh) (Merck) eluted with mixtures of chloroform, ethyl acetate, ethanol and methanol of increasing polarity, to obtain fractions for yellow amorphous powder. About 121 fractions were eluted with different solvents with increasing polarity. Column fractions from 110 to 119 with ethyl acetate: ethanol (80:20) in the TLC mobile phase solvent ratio of chloroform: methanol (1:1) showed $R_f$ value of 0.46 equal to that of standard quercetin. The fractions were then combined and crystallized and the final yield approximately 100 mg. This process was repeated several times by using bulk quantity of samples until the desired amount of quercetin has been obtained.

**Fourier transforms infrared (FT-IR) spectrum analysis**

The FT-IR spectrum of isolated compound was shown in fig. 2 and their corresponding characteristic peak positions were listed in table 3. The broad absorption peak at around 3290 $cm^{-1}$ was assigned to the OH stretching vibration of phenol. C=O aryl ketonic stretching vibrations are observed at 1668 $cm^{-1}$. The absorption peaks positioned at 1612 $cm^{-1}$, 1516 $cm^{-1}$ and 1429 $cm^{-1}$ are assigned to the C---C, C=O and C=C aromatic stretching vibrations respectively. OH bending vibrations of phenols were observed at 1359 $cm^{-1}$. The absorption peak at 1315 $cm^{-1}$ and the peaks at the lower frequencies between 950 $cm^{-1}$ and 600 $cm^{-1}$ were assigned to the C-H bending vibrations of aromatic hydrocarbons. C-O stretching vibrations of aryl ether and phenols were observed at 1240 $cm^{-1}$ and 1210 $cm^{-1}$ respectively. C-CO-C stretching and bending vibrations of ketones were observed at 1163 $cm^{-1}$, which confirms that the isolated compound is flavonoid quercetin. This result is in good agreement with the previous literature for molecular structure of quercetin [16].

**Table 2: Phytochemical analysis of leaf extracts in various solvents**

| Test | Hexane | Ethyl acetate | Ethanol |
|---|---|---|---|
| Carbohydrates | + | + | + |
| Saponin test | – | + | – |
| Flavonoid test | + | + | + |
| Alkaloid test | + | + | + |
| Quinones | – | – | + |
| Glycosides test | – | – | – |
| Cardiac glycosides test | – | + | – |
| Terpenoids test | – | – | – |
| Triterpenoids | – | – | – |
| Phenols | + | + | + |
| Coumarins | – | + | – |
| Proteins | – | – | – |
| Steroids and Phytosteroids | – | – | – |
| Phlobatannins | – | – | – |

(+) = Presence, (–) = Absence

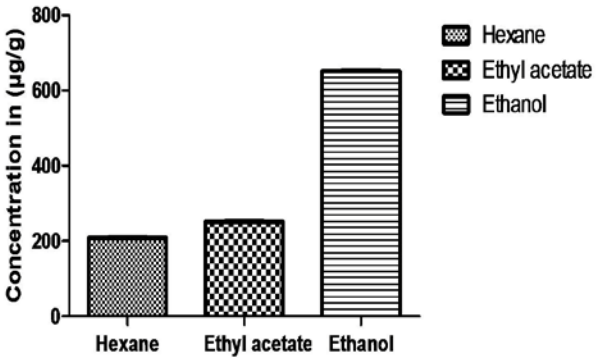


**Fig. 1: Estimation of TFC using various solvents**

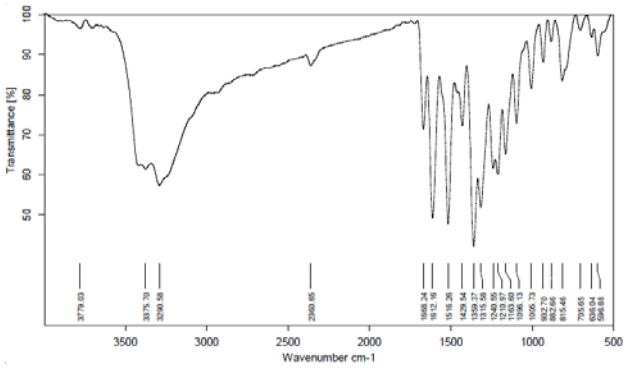


**Fig. 2: FT-IR spectrum of isolated compound**

**Table 3: Peak positions and probable inter-atomic bonds of isolated compound**

| Peak position | Inter-atomic bond |
|---|---|
| 3290.58 | O-H stretching vibration of phenol |
| 1668.24 | C=O Aryl ketonic stretch |
| 1612.16 | C---C Aromatic ring stretch |
| 1516.26 | C=O aromatic stretch |
| 1429.54 | C=C aromatic stretch |
| 1359.37 | O-H bending of phenols |
| 1315.58 | C-H bond in Aromatic hydrocarbon |
| 1240.55 | C-O stretch of Aryl ether |
| 1210.97 | C-O stretch of phenol |
| 1163.60 | C-CO-C stretch and bending in ketone |
| 932.70, 815.46, 705.65, 596.88 | C-H bending of aromatic hydrocarbons |

**Table 4: $^{1}$H NMR and $^{13}$C NMR data for isolated compound-quercetin**

| $^{1}$H NMR spectrum of quercetin | $^{13}$C NMR spectrum of quercetin |
|---|---|
| 6.19 (d, 1H, *J* = 7.2 Hz, *Ar-H*) | 93.8, 98.6, 103.4, 115.4,116.0 (*Ar-C*) |
| 6.41(d, 1H, *J* = 6.9 Hz, *Ar-H*) | 120.4, 122.4, 136.1, (*Ar-C*) |
| 6.88(d, 1H, *J* = 5.1 Hz, *Ar-H*) | 145.5, (*Ar-C*) |
| 7.54(q, 1H, *J* = 6.9 Hz, *Ar-H*) | 147.2, (*Ar-C*) |
| 7.66(d, 1H, *J* = 7.4 Hz, *Ar-H*) | 148.1, (*Ar-C*) |
| 9.36 (s, 2H, *Ar-OH*) | 156.6, (*Ar-C*) |
| 9.65(s, 1H, *Ar-OH*) | 161.1, (*Ar-C*) |
| 10.87(s, 1H, *Ar-OH*) | 164.3, (*Ar-C*) |
| 12.48(s, 1H, *Ar-OH*) | 176.2, (*Ar-C=O*) |

**NMR spectrum of isolated compound**

NMR studies were carried out to confirm the positions of proton and carbon binding sites. The isolated compound displayed a better resolved $^{1}$H-NMR spectrum. The $^{1}$H-NMR spectrum of the isolated compound showed aromatic hydrogen groups from 6.18-7.66 ppm and phenolic-OH groups from 9.36-12.48 ppm respectively (fig. 3). The $^{13}$C-NMR spectrum showed carbonyl group at 176.2 ppm and aromatic carbon group from 93.8-164.3 ppm was shown in fig. 4. The corresponding $^{1}$H NMR and $^{13}$C NMR peak positions for isolated compound were shown in table 4. The peaks present in the NMR spectrum showed resemblance with the pure quercetin which was

also confirmed by previous literatures [17, 18]. Thus, it can be confirmed that the isolated compound is found to be quercetin.

**Liquid chromatography/tandem mass spectrometry (LC/MS/MS identification)**

The isolated plant compound was analyzed by LC-MS-MS. It has been successfully applied for a quick separation and identification of the isolated compounds from fenugreek. The chromatogram of the isolated compound was shown in fig. 5. The fragment pattern m/z 302.95 was found in its first order mass spectrum, and it is speculated that they may correspond to the fragment patterns of quercetin. Comparison to the reference substance and a mass spectral library system confirmed that the isolated compound is found to be quercetin. The previous literature showed a similar peak, which confirms that the isolated compound is proved to be quercetin [19].

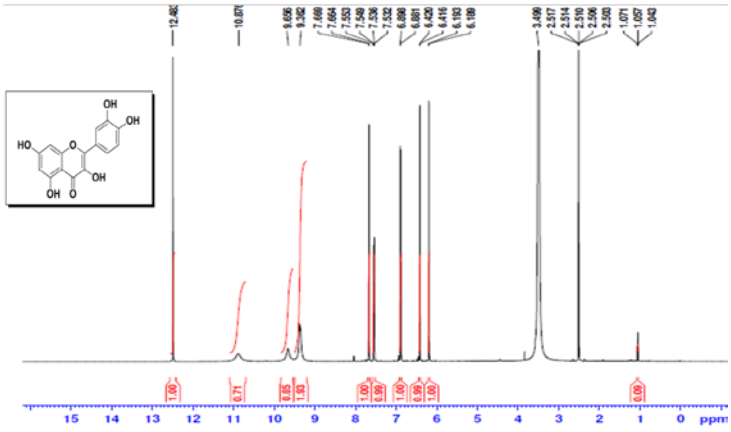


**Fig. 3: $^{1}$H NMR Spectrum of the isolated compound-quercetin**

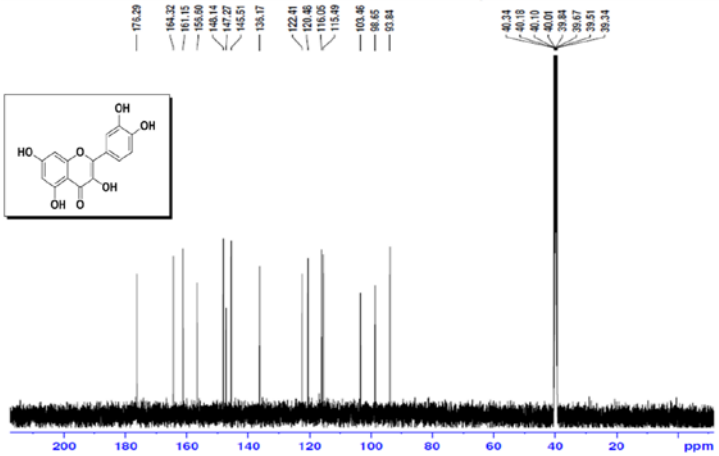


**Fig. 4: $^{13}$C NMR Spectrum of isolated compound-quercetin**

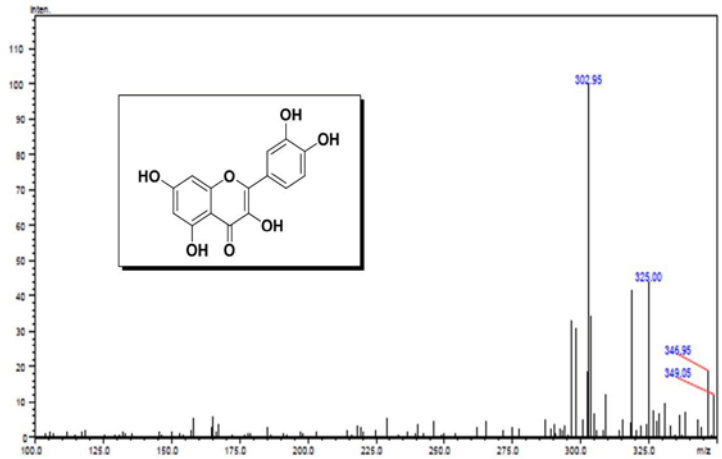


**Fig. 5: The total ion chromatogram of the isolated compound**

**Free radicals scavenging activity using DPPH assay**

Free radicals are generated by general metabolic process or from other external factors which are capable of initiating the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides [20]. The generated free radicals are scavenged by antioxidants. DPPH radical has been used to evaluate antioxidants for their free radical quenching activity. The antioxidants mechanism of isolated compound quercetin was evaluated for free radical scavenging activity against DPPH. The antioxidant activity of the isolated quercetin with respect to the standard molecule (ascorbic acid) was calculated (fig. 6). The decrease in the absorbance of the DPPH radical with respect to ascorbic acid is caused by antioxidant activity through the reaction between antioxidant quercetin and free radical results in the scavenging of radical by hydrogen donation [21]. Previous reports have also mentioned the efficient antioxidant activity of the compound quercetin [17]. Thus, the isolated compound quercetin showed potential antioxidant activity.

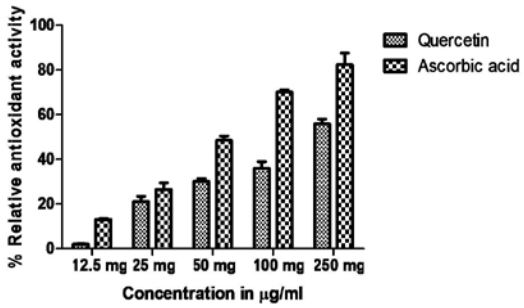


**Fig. 6: Free radical scavenging activity of isolated compound quercetin compared with ascorbic acid standard (Determined using DPPH assay)**

## CONCLUSION

From the present work, the compound plant quercetin has been extracted successfully from the leaves of *Trigonella foenum-graecum*. It is found that solvent plays the main role in the extraction of plant constituents. Ethanol is a highly polar solvent; it showed the presence of more flavonoid content when compared to the hexane and ethyl acetate. The identification of flavonoid quercetin was attempted by direct comparison with its retardation factor. The isolated constituent of quercetin was identified through FT-IR, NMR and mass spectroscopy. The isolated compound quercetin showed increased antioxidant activity with an increase in the treated concentrations. This method is simple, rapid and highly efficient extraction method for extracting the bioactive components from plants.

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## CONFLICTS OF INTERESTS

The authors declare no conflicts of interest

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