Original Article

Quantitative Estimation of Immunomodulatory Flavonoid Quercetin by HPTLC in Different Leafy Vegetables Available in West Bengal

Tushar Adhikari, Prerona Saha*

ABSTRACT

Introduction: Leafy vegetables are commonly consumed medicinal plants used in various metabolic and infectious diseases. Additionally, their antiviral, antioxidant, anticancer, and antihypertensive activities have been reported in the literature. **Objectives:** To determine the quantity of immunomodulatory flavonoid, quercetin in different types of leafy vegetables available in West Bengal by using a simple validated HPTLC method. **Materials and Methods:** The chromatographic analysis was performed by using aluminium-backed silica gel 60 F_{254} plates with Toluene–Ethyl acetate–Formic acid 5:4:0.2 (%v/v) as the mobile phase. The method was validated according to the ICH guidelines. **Results:** The total flavonoid content (TFC) in the four leafy vegetables studied varied between 58.10 to 12.28 mg QE/g. Well-separated and compact spots (R*f*) of quercetin (0.55±0.03) were detected. The regression equation obtained was y = 0.0011x - 0.0569, with a correlation coefficient (*R^2*) of 0.9939. The linearity range (µg/spot) was 60-160. The LOD/LOQ (ng/spot) was 6.09/18.47. *Murraya koenigii* (0.1992±0.037 %w/w) contained the maximum amount of quercetin compared to *Ipomoea aquatic* (0.1501±0.039%w/w), *Coriandrum sativum* (0.1430±0.061%w/w) and *Trigonella foenum- graecum* (0.1201±0.055%w/w) in methanolic extract. **Conclusion:** This study revealed that the validated HPTLC method was simple, accurate and sensitive for separating and quantifying quercetin in different leaf vegetables. Quercetin content was highest in *Murraya koenigii* (0.1992±0.037 %w/w), and least in *Trigonella foenum-graecum* (0.1201±0.055 %w/w). The developed method might be used further in standardizing and quality control of secondary metabolites in herbal formulations.
Keywords: Flavonoid, HPTLC, Leafy vegetables, Quercetin.

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INTRODUCTION

Recently, the increasing prevalence of autoimmune disorders like cancer, and viral ones including, COVID-19 has been a serious issue in terms of prevention, diagnosis, prognosis, and therapy. Leafy vegetables are highly valued in both oriental and occidental therapies, have a long lineage of use as medical plants, and have been recognized as remedies for nearly every illness known to mankind.[1] They have been widely used in both Indian Ayurveda and With Unani medical systems, as well as traditional Chinese medicines since ancient times for the treatment of epilepsy, paralysis, gout, dropsy, chronic cough, diabetes, piles sinus, lung congestion, inflammation, infection, mitigation, hair treatment, breast enhancement, and aphrodisiac effects.[2] Many crop species have long been utilized as galactagogues to increase breastfeeding in weaning mothers, as well as for their medicinal properties[3] With the rising demand for safer pharmaceuticals, such traditional herbal remedies have been widely favored to prevent and cure human diseases due to their ease of availability

to local population and, most significantly, low toxicity.[4] As a result, usages of different types of leafy vegetables are being explored every day.[5] According to literatures, the pharmacological effects and availability of *Trigonella foenum-graecum*,[6] *Murraya koenigii*,[7] *Ipomoea aquatic*,[8] *Coriandrum sativum*[9] are due to the presence of unique bioactive substances such as steroidal diosgenin, alkaloid trigonelline, flavonoid quercetin, galactomannan, and the uncommon amino acid 4-hydroxyisoleucine. Quercetin has been touted as the most significant of these key bioactive substances and is well known for producing desired outcomes. Worldwide, the structures of more than 6000 distinct flavonoids have been determined. The common structure of these compounds is phenyl benzopyrone ring system (C6-C3-C6).[10] The leafy vegetables contain various different flavonoids with varying quantities.[11] Among those, 5 flavonoids – namely quercetin, apigenin, kaempferol, rutin, and luteolin – were present in most of the leafy vegetables in significant

amounts. Immunomodulators are substances which are synthetic or biological in nature, that help to modulate, suppress or stimulate both the adaptive and innate immune systems.[8] Immunomodulatory flavonoids from 40 leafy vegetables, belonging to 26 different plant families were reviewed.[12] Some of the flavonoids possessing immunomodulatory activity were identified; one among them being 'Quercetin'. Quercetin is a flavanonol glycoside found abundantly in leafy vegetables and possess immunomodulatory activity.[13] Quercetinits a bio flavonoids is used for anticancer,[14] anti-mutagenic,[15] anti-oxidative,[16] anti-inflammatory,[17] anticancer/ chemo-preventive, neuroprotective,[18] antihypertensive, and blood glucose-lowering activities.[19] Several analytical methods for the determination of quercetin have been reported in different literature. Several high-performance liquid chromatography (HPLC) [20] methods were developed for the quantification of quercetin either combination or in alone with other flavonoids in leafy vegetable juices or in pharmaceutical formulations. In order to simultaneously determine of kaempferol, naringenin, rutin, and quercetin, an LC-MS/MS method[21] was created. There hasn't been a single method published for the quantitative detection of quercetin by high performance thin layer chromatography (HPTLC) in several species of leafy vegetables.

Among the 40 different leafy vegetables reviewed, quantification of quercetin is already reported for 16 of them.[11] Others have reported qualitative analysis for the presence of quercetin. As a result, the objective of the current study was to create a quick, accurate, and reliable HPTLC densitometric technique for estimating the quantity of the the immunomodulatory flavonid quercetin (Figure 1) from the leafy vegetables of West Bengal to aid in the treatment of immune disorders that are frequently seen around the world.

MATERIALS AND METHODS

Plant Materials

Methi (*Trigonella foenum-graecum*), Curry (*Murraya koenigii*), Kolmishak (*Ipomoea aquatic*), and Dhonia (*Coriandrum sativum*) were purchased from a local market in Sodepur, West Bengal. The plants were collected and identified by the Central National Herbarium (CNH), Botanical Survey of India, Shibpur, West Bengal.

Chemicals

Standard quercetin was purchased from Loba Chemie Pvt. Ltd. All the solvents used were of HPLC grade and other chemicals used were of analytical reagent grade.

Sample preparation for analysis of quercetin in methanolic extract of Leafy vegetables

The air-dried leaves of four different leafy vegetables were coarsely powdered, separately, then exhaustively extracted by maceration with methanol for 7 days. The solvent was evaporated to dryness under reduced pressure by use of a rotary vacuum evaporator and each of the residues were separately dissolved in methanol in 50 ml volumetric flasks.[22]
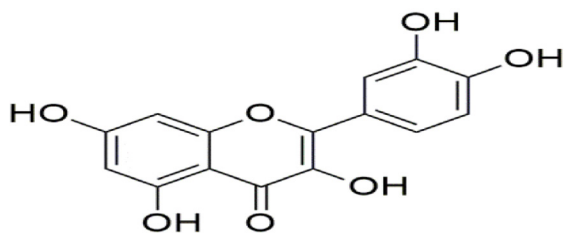

Figure 1: Chemical structure of quercetin.

Determination of Total Flavonoid Content
Preparation of Standard Quercetin for Calibration Curve

The extracts' total flavonoid concentration (TFC) was assessed using a colorimetric aluminum chloride test.[23] A stock solution (100 mg/ml) was made by dissolving 100 milligrams of quercetin in one ml of methanol. This standard solution was serially diluted to produce solutions with concentrations of 20, 40, 60, 80 and 100μg/ml. To this, add 4 ml of distilled water and 0.3 ml of 5 percent $NaNO_2$ in the test tube. Five minutes later, mix with 0.3ml of 10% $AlCl_3$. 1 M NaOH in 2 ml was added to the mixture after 6 min. The mixture was immediately diluted with distilled water to a level of 10 ml. A spectrophotometer was used to measure the absorbance at 510 nm.

Preparation of Samples for Total Flavonoid Content

The extracts were produced as 100 mg/ml stock solutions in methanol, which were then diluted to create various 0.5 mg/ml concentration solutions. The extracts were processed using a method similar to that described for quercetin, and a spectrophotometer set at 510 nm was used to detect absorbance. The total quantity of flavonoids present was calculated using the average absorbance value obtained from three measurements. Using a linear equation based on the standard calibration curve, the flavonoid concentration was represented as quercetin equivalent (mg QE/100g).

Chromatographic Conditions

On 10cm × 10cm aluminum-backed plates coated with 0.2mm layers of silica gel 60 F_{254}, densitometric HPTLC examination was carried out. The "CAMAG manual TLC Sampler 4" sample applicator, which is equipped with a CAMAG micro liter syringe, was used to apply samples to the TLC plates as 6mm bands. 150 L/s was applied at a continuous rate.[24] Toluene-Ethyl acetate-Formic acid 5:4:0.2 (% v/v/v) [25] was used as the mobile phase to carry out linear ascending development of the plates to a distance of 80 mm in a CAMAG manual Developing Chamber 2 that had been previously saturated with the mobile phase developed for 30 min at 22°C.

Method Validation

Precision: Instrumental precision, intra-assay precision, and intermediate precision of the method were determined.[26] Instrumental precision was measured by replicate ($n=3$) application of the same quercetin standard solution (concentration 100mg/ml). Intra-assay precision was evaluated by analysis of three replicate applications of freshly prepared standard solutions of same concentration, on the same day. Intermediate precision was evaluated by analysis of three replicate applications of standard solution of same concentration on three different days.

Limit of Detection and Limit of Quantification: The limit of detection (LOD) and limit of quantification (LOQ) values were determined as the amounts for which the signal-to-noise ratios were 3:1 and 10:1, respectively.

Recovery Studies: The accuracy of the method was established by performing recovery experiments at three different levels using the standard addition method. The values of percentage recovery and average value of percent recovery for quercetin were calculated. **Specificity:** The specificity of the method was ascertained by analyzing the standard quercetin and extract. The spot for quercetin in the sample was confirmed by comparing the R_f values and spectra of the spot with that of the standard. The peak purity of quercetin was assessed by comparing the spectra at three different levels, viz. peak start, peak apex, and peak end positions of the spot.

Quantification of quercetin in different varieties of methanolic extract of leafy vegetables

The test samples were applied on the plates and chromatograms were obtained under the same conditions as for the analysis of standard quercetin. The area of the peak corresponding to the R_f value of quercetin standard was recorded and the amount present was calculated from the graph.

RESULTS

Total Flavonoid Content Determination (TFC)

A colorimetric method based on aluminium chloride was used to measure the flavonoid content of a few leafy vegetable extracts. Equivalents of quercetin (QE) per gram of weight of dry extract were calculated using the calibration curve of quercetin (20-100 g/ml), and the results are shown in (Figure 2) as y = 0.0049x + 0.4664, R^2 = 0.9671. (Table 1). The content of flavonoid in methanol extracts ranged from 58.10 to 12.28 mg QE/g. The three plants with the greatest levels of flavonoids were *C. sativum L., M. koenigii,* and *T. foenum-graecum* (58.1±20.4, 49.1±5.77, and 42.07±16.34 mg QE/g, respectively), whereas *I. aquatica* had the lowest levels (12.28±1.5 mg QE/g).

The quantity and location of free hydroxyl (-OH) groups affect the efficacy of flavonoids, the secondary metabolites with immunomodulatory characteristics.[27] The methanol extract of *C. sativum L.* had a TFC of 60.87 mg QE/g dry weight, whereas the methanolic extract of *M. koenigii* had a TFC of 9.75 mg QE/g dry weight, according to D.T. Abeysinghe *et al.*[28] and Hasna Bouhenni *et al.*[29] The flavonoid concentration of leafy vegetables was shown to be considerably influenced by biological, environmental, genetic, seasonal, and yearly fluctuations, according to publications in the literature.

Method Validation

Calibration curve

The calibration plot shown in Figure 3 indicates the response is a linear function of concentration range 60-160 µg/ml quercetin. The correlation coefficient, intercept and the slope were 0.9939, -0.0569 and 0.0011 respectively. The linear regression data for the calibration curve of quercetin is shown in Table 2.
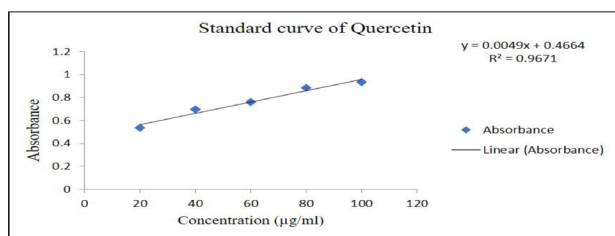

Figure 2: Calibration curve of standard quercetin.

Table 1: Total flavonoid content in the four selected leafy vegetables.

SL. NO.	Plant Name	Total Flavonoid Content (Mean ± SD; mg QE/g dry weight) *
1	*Murraya koenigii*	49.10 ± 5.77
2	*Trigonella foenum-graecum*	42.07 ± 16.34
3	*Ipomoea aquatica*	12.29 ± 1.51
4	*Coriandrum sativum*	58.10 ± 20.40

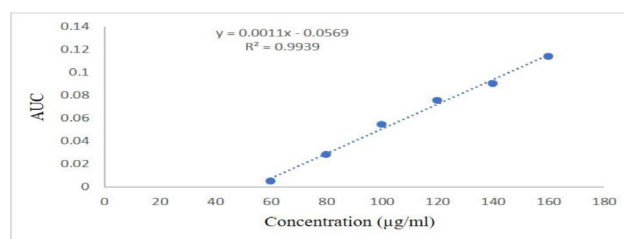*milligrams of quercetin equivalent (QE) per gram dry extract weight

Figure 3: Regression curve of standard quercetin.

Table 2: Linear regression data for the calibration curve of quercetin.

Linear Regression Parameter	Data
Linearity range (µg/spot)	60-160
Regression equation	y = 0.0011x - 0.0569
Correlation coefficient	0.9939
Slope	0.001075543
Intercept	-0.056913048
SE of intercept	0.004868565
SD of intercept	0.001987583

Table 3: Recovery study for proposed method (*n*=4).

Excess drug added to analyte (%)	Concentration found (µg±SD)	% recovery	% RSD
50	59.52±0.01	119.04	0.02
100	96.38±0.14	96.38	0.14
150	135.05±0.09	90.03	0.06

Recovery Studies

Results from recovery studies, listed in Table 3, were within acceptable limits (90.03 to 119.04 %), indicate the accuracy of the method was good.

Precision

Results from determination of repeatability and intermediate precision, expressed as SD (%) are show in Table 4. RSD was in the range 2.1 - 9.59 for repeatability and 1.44- 3.44 for intermediate precision. These low values indicated that the method is precise.

Limit of Detection and Limit of Quantification

LOD and LOQ of the proposed method was found to be 6.09 and 18.4 µg/spot, for quercetin, which indicated that the proposed method can be used in a wide range for detection and quantification of quercetin effectively.

Method Development

The mobile phase composition was optimized to establish a suitable and accurate densitometric HPTLC method for analysis of quercetin. The mobile phase Toluene–Ethyl acetate–Formic acid 5:4:0.2 (%v/v/v) resulted in a sharp, symmetrical, and well resolved peak at R*f* value of (0.55±0.03) (Figure 4).

Quantification of Quercetin in the Methanolic Extract of Different Varieties of Leafy Vegetables

Quercetin peaks from the methanolic extracts of different varieties of leafy vegetables were identified by comparing their single spot at R_f = 0.55±0.03 (Figures 5-8) with those obtained by chromatography

Table 4: Precision of the proposed method (*n*=4).

Conc. (µg/mL)	Repeatability (Intra-day precision)			Intermediate precision (Inter-day)		
	Area ± SD	Standard error	%RSD	Area ± SD	Standard error	%RSD
100	0.029493±0.002822	0.00162	9.598	0.033745±0.001161	0.00067	3.44
100	0.032183±0.000796	0.00045	2.473	0.038357±0.001315	0.000759	`3.42
100	0.051127±0.001074	0.00062	2.1	0.049447±0.000716	0.0004133	1.44
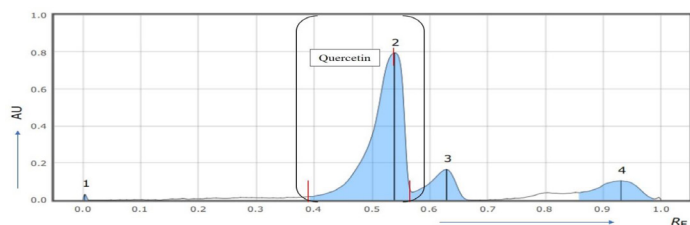
Figure 4: HPTLC chromatogram of standard quercetin.
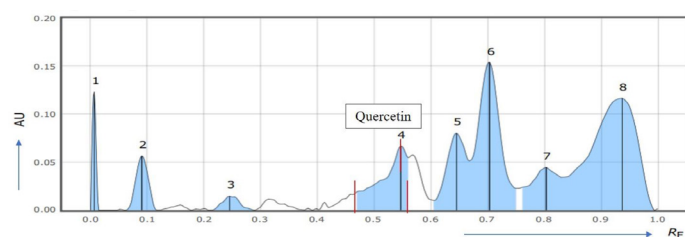
Figure 5: HPTLC chromatogram of methanolic extract of *Trigonella foenum-graecum* (Methi shak).
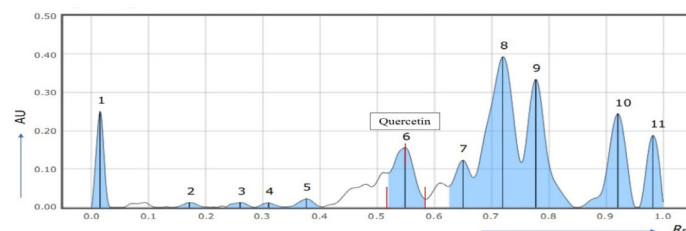
Figure 6: HPTLC chromatogram of methanolic extract of *Murraya koenigii* (Curry leaf).
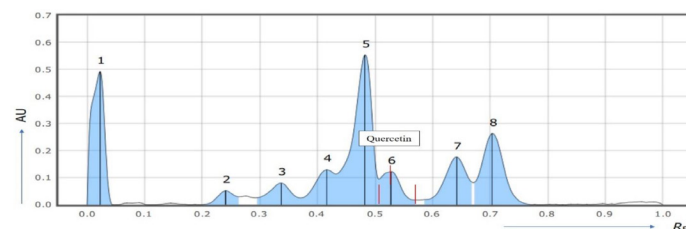
Figure 7: HPTLC chromatogram of methanolic extract of Coriandrum sativum (Dhania pata).
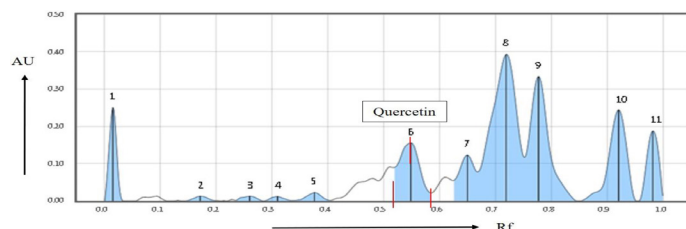
Figure 8: HPTLC chromatogram of methanolic extract of *Ipomoea aquatica* (Kalmi shak).

of the standard under the same conditions. The quercetin content in methanolic extracts of four different varieties of leafy vegetables was quantified by using the standard concentration and AUC as presented in Table 5.

DISCUSSION

For the identification and measurement of chemical compounds found in various green vegetables and plants, HPTLC is a useful method for quality evaluation.[30] The retention factor (R*f*) values produced from it can be used to identify chemicals as they are specific to each chemical in a given solvent system. To measure the herbal extract, the TLC process was improved. R*f* = 0.55±0.03 for quercetin was the value for the mobile phase, which was composed of toluene, ethyl acetate, and formic acid in the ratio (5:4:0.2% v/v/v) (Figure 1). The HPTLC technique created for quercetin quantification in the current work was shown to be user-friendly, precise, repeatable, and sensitive, and it can be used to analyze methanolic extracts of four distinct kinds of leafy vegetables. The maximum amount of quercetin was found to be present in *Murraya koenigii* (0.1992mg/100gm dry weight) and minimum amounts were present in *Trigonella foenum-graecum* (0.1221mg/100gm dry weight). Also, the reported HPTLC method would find wide applications in the standardization and quality control of herbal raw materials as well as formulations.

CONCLUSION

TLC is a crucial tool for standardising natural medicines and nutritious meals. It is not possible to identify herbal preparations using a single approach because of their chemical complexity brought on by the

Table 5: Quercetin content in methanolic extract of different leafy vegetables.

Sl. No.	SAMPLE	Quercetin Content (Mean ± SD, mg/100g dry weight)
1	*Murraya koenigii* (Curry)	0.1992 ± 0.037
2	*Ipomoea aquatic* (Kalmishak)	0.1501 ± 0.039
3	*Coriandrum sativum* (Dhoney)	0.1430 ± 0.061
4	*Trigonella foenum-graecum* (Methi)	0.1201 ± 0.055

presence of various bioactive ingredients. For the simultaneous quantitative detection of the immunomodulatory flavonoid quercetin in various green vegetable extracts, the presented HPTLC approach is an appealing easy, quick, and selective method. This technique might be used extensively for the direct, routine analysis and quality control of

associated extracts and medications. By using the approach described, bioactive components of additional plants with similar characteristics might be found and utilised to select genotypes appropriate for further development into cultivars tailored to the industry of producing natural health products; Additionally, statistical evidence shows that the technique is reliable and selective for the detection of quercetin, with the added benefits of quick turnaround, little sample preparation, and low cost. The comparative quercetin content analysis among the four commonly consumed leafy vegetables will be helpful for the nutritional choice in case of immunity boost up. However further study can be investigated to accomplish the quercetin content analysis for the remaining such common edible plants which will provide the nutritional indicator to enhance the immunity among common people of the society.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

QE: Quercetin; **HPTLC:** High performance thine layer chromatography; **HPLC:** High performance liquid chromatography; **TFC:** Total flavonoid content; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **AUC:** Area under curve; **RSD:** Relative standard deviation; **SD:** Standard deviation; **TLC:** Thin layer chromatography; **CNH:** Central National Herbarium; **R$_f$:** Retention factor; **SE:** Standard error; **LC-MS/MS:** Liquid chromatography- mass spectroscopy/ mass spectroscopy.

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GRAPHICAL ABSTRACT
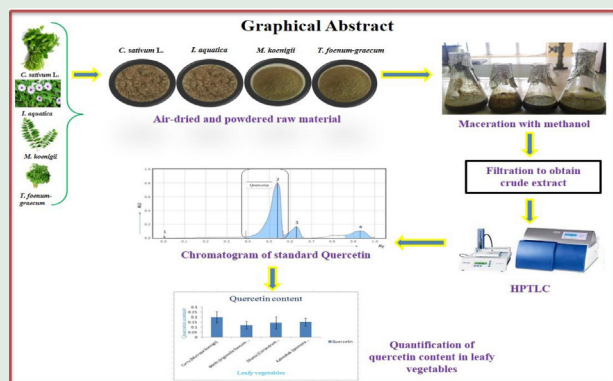
SUMMARY

Since ancient times, leafy vegetables are folk medicinal plants used in the treatment of verious metabolic and infectious diseases. This research work assessed the quentitative easimation of immunomoulatory flavonoid, quercetin from the leafy vegetables of West Bengal to support the treatment of prevalent immune related disorders worldwide. Based on the results of this study, it can be concluded that among the leafy vegetables studied, the highest amount of quercetin is present in *Murraya koenigii* (0.1992mg/100gm dry weight), followe by *Ipomoea aquatica* (0.1501±0.039 mg/100gm dry weight), *Coriandrum sativum* (0.1430±0.061mg/100 gm by weight) an fewer amounts were present in *Trigonella foenum-graecum* (0.1221mg/100 gm dry weight).