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Development and Validation of HPTLC Densitometric Method for Simultaneous Estimation of Quercetin and Kaempferol in Herbal Extracts and Polyherbal Formulation

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Abstract: Flavonoids are a class of phytochemicals, discovered in the 1930s, that generate pigment in plants and play various biological roles in other cellular processes. It is estimated that the average dietary intake of flavonoids in humans is several hundred mg per day. Quercetin and kaempferol, the most abundant dietary flavonoids, has been reported for its strong antioxidant capabilities, anti-obesity and anti-inflammatory activities. Therefore, a simple, reproducible, robust and precise high-performance thin layer chromatography (HPTLC) densitometric method was developed and validated for the simultaneous estimation of quercetin and kaempferol as per ICH guidelines. Quercetin and kaempferol were separated on aluminium-backed silica gel 60 F_{254} plates using toluene: ethyl acetate: methanol: formic acid (5:3:1:0.2, % *v/v*) as the mobile phase. This system was found to give compact bands of kaempferol and quercetin at R_f value 0.64 ± 0.05 and 0.69 ± 0.05, respectively. The proposed method was employed with a high degree of precision and accuracy for the simultaneous estimation of quercetin and kaempferol in methanolic extracts of petals of *Rosa damascena* Mill., whole plant of *Origanum vulgare* Linn., rhizomes of *Nardostachys jatamansi* DC., seeds of *Trachyspermum ammi* Linn. and *Apium graveolens* Linn., and in-house Unani formulation *Safoof-e-Muhazzil* containing these plants as ingredients.

Key words: ICH guidelines; Kaempferol; Natural bioactive compounds (NBCs); Quercetin; *Safoof-e-Muhazzil*; Secondary metabolites.

Introduction

Obesity is considered a major public health problem worldwide and its prevalence continues to rise uncontrollably [1]. Natural bioactive compounds are substances contained in foods that provide a verifiable benefit to human health [2]. Flavonoids comprise a large group of secondary metabolites widely distributed throughout the plant kingdom, including food plants. The major flavonoid is quercetin (QC), which belongs to the class called flavonols and is mainly found in apples, tea, onions, nuts, berries, cauliflower, cabbage and many other foods [3]. It is reported to exhibit a wide range of biological activities *e.g.* anti-obesity, modulate LDL-cholesterol levels and blood pressure [4,5], inhibit lipid peroxidation, platelet aggregation, capillary permeability and possess anti-lipase properties[6]. It has beneficial role in obesity, diabetes and their related disorders [3,7].

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Kaempferol (KF) is a flavonoid that has attracted great attention due to their ability to reduce the risks of chronic diseases through anti-inflammatory activity and to alter the immune function by inhibiting enzymes that are activated in inflammatory conditions [8,9]. It has been reported for its anti-obesity, anti-diabetic [4,10,11], antihyperlipidemic [10,12] and hepatoprotective effects [13]. *Safoof-e-Muhazzil*, an anti-obesity polyherbal Unani formulation contains, petals of *Rosa damascena* Mill., whole plant of *Origanum vulgare* Linn., rhizomes of *Nardostachys jatamansi* D.C., seeds of *Trachyspermum ammi* Linn., *Apium graveolens* Linn. and Lac resin from *Laccifer lacca* Kerr.[14,15]. All the herbal ingredients of the formulation are rich in QC and KF [16-23]. Since QC and KF were reported to play significant roles in the treatment of obesity, hyperlipidemia and diabetes and their related disorders, therefore, a HPTLC densitometric method was developed and validated for the simultaneous estimation of QC and KF in anti-obesity polyherbal Unani formulation (*Safoof-e-Muhazzil*) and its drug constituents.

Material and methods

Plant material

All the plants material were purchased from Samsi Dawakhana, Ballimaran, Delhi and each were authenticated by Dr. H. B. Singh, Scientist F and Head (RHMD), NISCAIR, New Delhi, India and voucher specimens (No. NISCAIR/RHMAD/Consult/-2010-11/1705/05) were deposited in the RHM Division, NISCAIR, New Delhi-110012.

Chemicals

QC and KF (purity ≥ 98.24 %) **(Fig. 1)** was purchased from Sigma Aldrich, India. Silica gel F_{254} HPTLC plates were purchased from Merck, Mumbai, India. Other analytical grade solvents and reagents were obtained from S.D. Fine Chemicals, Mumbai, India.

Preparation of sample solution

About 10 g of the crude drug powders and herbal formulation were weighed and extracted in Ultrasonicator (Toshniwal, India) separately in methanol. The extracts were filtered and fractionated with ethyl acetate in separating funnel. The ethyl acetate fractions were concentrated under reduced pressure using rotary evaporator (Hanshin, Korea). Samples for HPTLC were prepared (20 mg/ml) from the dried extracts in methanol and filtered through 0.45 ìm membrane filter and stored at 4°C before further analysis.

Preparation of standard solution

Accurately weighed 5 mg of QC and KF each reference standard and transferred to 10 ml volumetric flask. Methanol was added and sonicated in ultrasonic water bath to dissolve. Volume was made up with methanol to 10 ml. This gives concentration of 0.5 mg/ml. This solution was used as a reference solution (stock solution) for QC and KF.

Chromatographic conditions

HPTLC was performed on 10 cm × 10 cm aluminium backed pre-coated TLC plates with 0.2-mm layers of silica gel 60 F_{254} (Merck, Mumbai, India). Standard solutions of QC, KF as well as the sample solutions were applied to the plates as bands 5.0 mm wide, 10.0 mm apart, and 10.0 mm from the bottom edge of the same chromatographic plate by using a CAMAG (Muttenz, Switzerland) Linomat IV sample applicator equipped with a 100

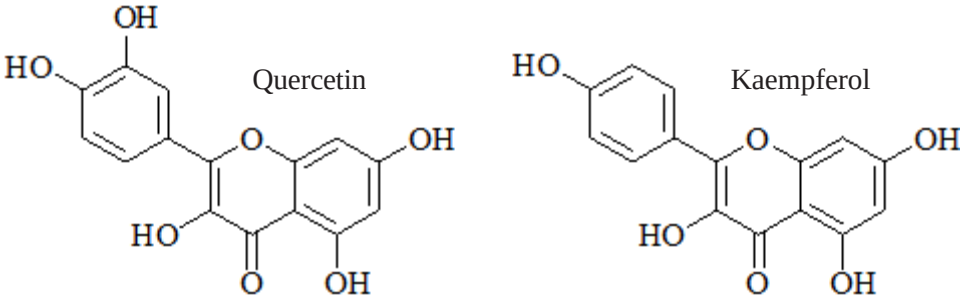

Fig. 1 Chemical structure of quercetin and kaempferol

µl Hamilton (USA) syringe. Ascending development to a distance of 80 mm was performed at room temperature (28 ± 2°C), with toluene-ethyl acetate- methanol-formic acid (5:3:1:0.2 % v/v) as mobile phase, in a CAMAG glass twin-trough chamber previously saturated with mobile phase vapour for 10 min. After development, the plates were dried in air and scanned at 366 nm with a CAMAG TLC scanner with WinCat software and using a deuterium lamp. The slit dimensions were 4 mm × 0.2 mm and scanning speed was 20 mm/S.

Method development

Various concentrations (100-1200 ng/spots) were made for the preparation of calibration curve from the prepared standard stock solution. The mobile phases tried for these purposes were toluene-ethyl acetate-methanol-formic acid (7:2:1.5:0.5, 5:3:1:0.2 and 6:3:1.5:0.5 % v/v) and ethyl acetate-methanol-water (6:3:1, 7:2:1 and 8:1.5:0.5 % v/v). The suitability of the solvent mixtures was decided by band separation, shape, slack of tailing, sensitivity of the assay, cost and the time required for analysis.

Method validation

Linearity

The linearity of QC and KF were checked between 100-1200 ng/spot concentration range. Graph was plotted between concentration and area for linearity.

Precision

Precision was determined at two levels according to ICH guidelines (repeatability and intermediate precision). Repeatability was determined as intraday precision whereas intermediate precision was determined by carrying out inter-day variation for the determination of QC and KF at three different concentration levels of 300, 600, and 900 ng/spot in triplicates.

Accuracy

Accuracy was determined as % recovery by the standard addition method. The pre-analysed sample of QC and KF (300 ng/spot, respectively) was spiked with the extra 0 %, 50 %, 100 % and 150 % of the standard QC and KF and the mixtures were reanalysed in triplicate by the proposed method. The % recovery and % RSD were calculated at each concentration level.

Robustness

Robustness of the method was determined by changing the mobile phase composition ethyl acetate- methanol-water. The change in R_f was recorded and % RSD was calculated.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined by standard deviation (SD) method. LOD and LOQ were determined by injecting blank sample to the chromatograph in triplicates, peak area of this blank was recorded. LOD and LOQ were determined using the slope of the calibration curve and SD of the blank sample using following formulae:

$$LOD = (3.3 \times SD / S); \quad LOQ = 10 \times SD / S$$

Where SD is the standard deviation of the blank response and S is the slope of the calibration curve.

Estimation of QC and KF in methanolic extract of plants and formulation

The test samples were applied and chromatograms were obtained in same conditions as that of standard QC and KF **(Fig. 2).** The peak area of the peak corresponding to the R_f of standard QC and KF was recorded and content of the same was calculated from the regression equation obtained from calibration curve.

Results and discussions

Calibration curve

The calibration curve area versus concentration was found linear in the range of 100-1200 ng/spot. Values of mean area with corresponding concentration, their standard deviation, % RSD and standard error are shown in Table 1. The linear regression data for the calibration curve showed a good linear relationship over the concentration ranges of 100-1200 ng/spot with respect to peak area. The R_f was found to be 0.64 and 0.69 for KF and QC, respectively.

Table 1. Linear regression data for the calibration curve

Parameters	Quercetin	Kaempferol
Linearity range (ng/spot)	100-1200	100-1200
Regression equation	y=257.4+11.62x	y=1654+12.08x
Correlation coefficient (R^2)	0.9965	0.9817
Slope ± SD	11.59±0.026	12.08±0.001
Intercept ± SD	285.42±24.54	1655.4±5.632
Slope without intercept ± SD	11.93±0.002	14.06±0.007
Standard error of slope	0.015	0.001
Standard error of intercept	14.168	3.252
95% Confidence interval of slope	11.524-11.942	12.078-12.084
95% Confidence interval of intercept	224.46-346.39	1641.4-1669.4

n=3, SD= Standard deviation

Validation of the method

Linearity

The linearity range of QC and KF were obtained as 100-1200 ng/spot as shown in Table 1. The regression equation were y=257.4+11.62x and y=1654+12.08x with correlation coefficient (R^2) of 0.9965 and 0.9818, respectively for QC and KF.

Precision

Precision was considered repeatability (intraday precision) and intermediate precision. Results of repeatability and intermediate precision were expressed in terms of % RSD and are shown in Table 2. The low values of % RSD indicated the repeatability and intermediate precision of the proposed method.

Accuracy

The accuracy of the proposed method was calculated by recovery analysis, which afforded the recovery of 98.41-101.81 % and 99.14-101.82 %, respectively for QC and KF after spiking the additional standard drug solution to the previously analysed standard solution. The values of % recovery are shown in Table 3, which indicates the accuracy of the proposed method.

Robustness

Robustness was determined to evaluate the influence of small but deliberate variation in the chromatographic conditions for the determination of QC and KF. There was no significant change in the R_f of QC and KF by changing the mobile phase. Low value of the % RSD indicated the robustness of the method as shown in Table 4.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ of the proposed method were determined by the standard deviation method and

Table 2. Precision of the proposed method

Conc. (ng/spot)	Quercetin		Kaempferol	
	Repeatability (% RSD)	Intermediate precision (% RSD)	Repeatability (% RSD)	Intermediate precision (% RSD)
300	1.628203	0.795963	0.498607	1.047508
600	0.56509	0.41744	1.053483	1.053483
900	1.968371	1.276677	0.087855	0.39281

n=3, RSD= relative standard deviation

Table 3. Accuracy of the proposed method

Excess drug added to analyte (%)	Theoretical content (µg)	Quercetin Recovery (%)	Kaempferol Recovery (%)
0	300	101.4412	99.16741
50	450	98.40333	99.14408
100	600	101.8183	100.8181
150	750	100.4357	100.0632

n=3, RSD= relative standard deviation

Table 4. Robustness of the proposed method

Conc. (ng/spot)	Mobile phase composition	Quercetin (% RSD)	Kaempferol (% RSD)
600	± 2	0.969206	0.475026
600	0 level	0.56509	1.053483
600	± 2	0.725727	1.387518

n=3, RSD= relative standard deviation

were found to be 23.49 and 76.38 ng/spot, and 34.28 and 84.95 ng/spot, respectively, for QC and KF, which indicated that the proposed method can be used in wide range for detection and quantification of QC and KF effectively.

Estimation of QC and KF in plant extracts and formulation

The peaks of QC and KF from the test samples were identified by comparing the R_f values obtained from the peaks with those of the standard. The R_f values of standard QC, KF and test samples were found identical (Table 5. Fig. 2, Fig. 3, Fig. 4). The details of result were given in Table 5, the contents QC and KF were based upon dry weight of extract. The formulation contains higher amount of KF in comparison to QC while *R. damascena* Mill. and *O. vulgare* Linn. contains highest amount of KF and *R. damascena* Mill. contains maximum QC content in comparison to other herbal drugs which supported the strong antioxidant behaviour of them[20, 24-26].

Table 5. Estimation of quercetin and kaempferol in plant samples and formulation

Test samples	Quercetin (% *w/w*)	Kaempferol (% *w/w*)
Rosa damascena Mill.	1.20 ± 0.13	1.13 ± 0.03
Origanum vulgare Linn.	0.96 ± 0.15	0.81 ± 0.13
Apium graveolens Linn.	0.78 ± 0.05	0.61 ± 0.07
Trachyspermum ammi Linn.	0.45 ± 0.09	0.77 ± 0.04
Nardostachys jatamansi DC.	0.51 ± 0.04	0.79 ± 0.15
Safoof-e-Muhazzil (Formulation)	0.62 ± 0.11	1.02 ± 0.06

n= 3, Mean ± SD

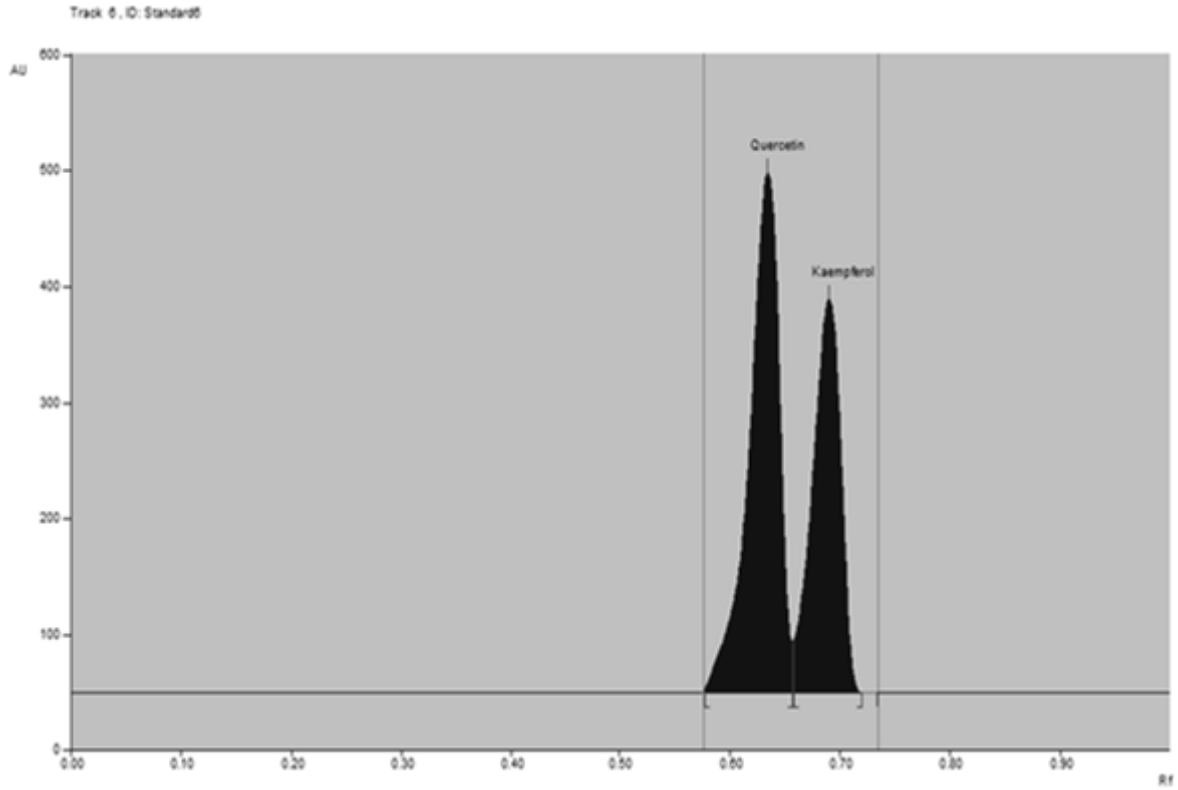

Fig. 2. HPTLC Chromatogram of standard quercetin and kaempferol

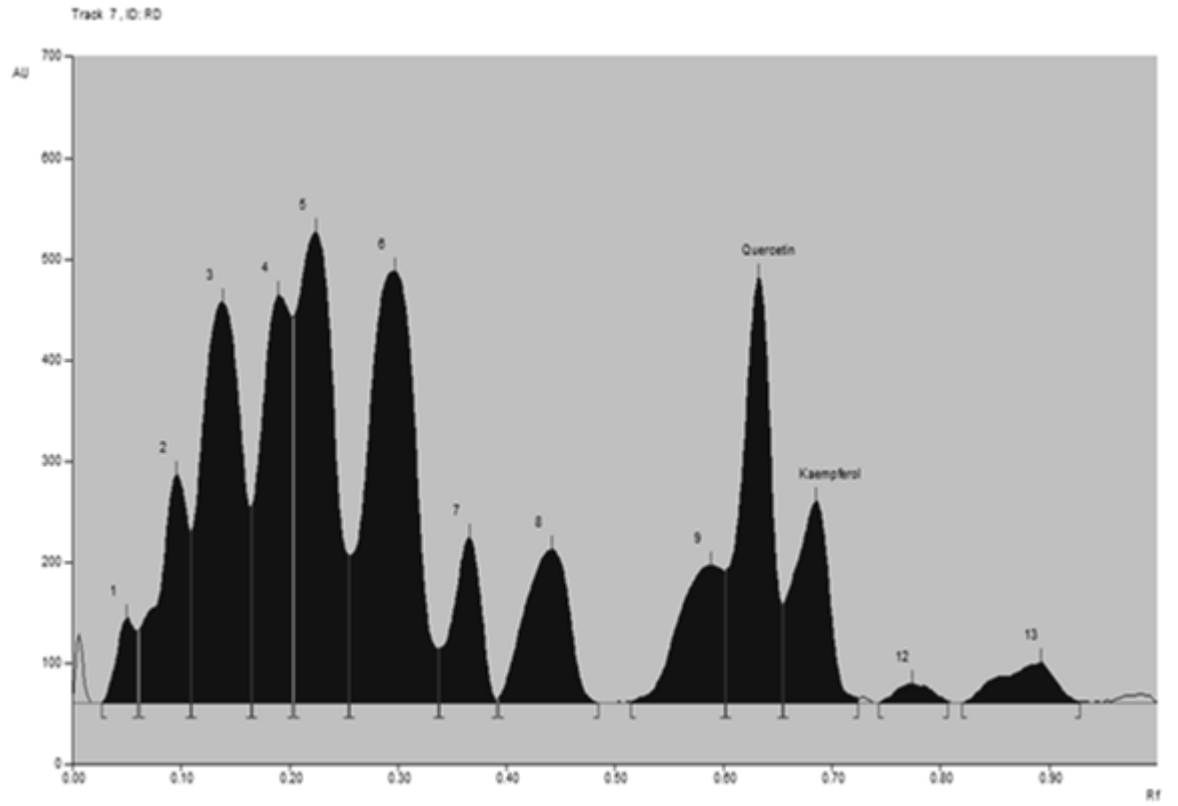

Fig. 3. HPTLC Chromatogram of methanolic extract of *R. damanscena* Mill.

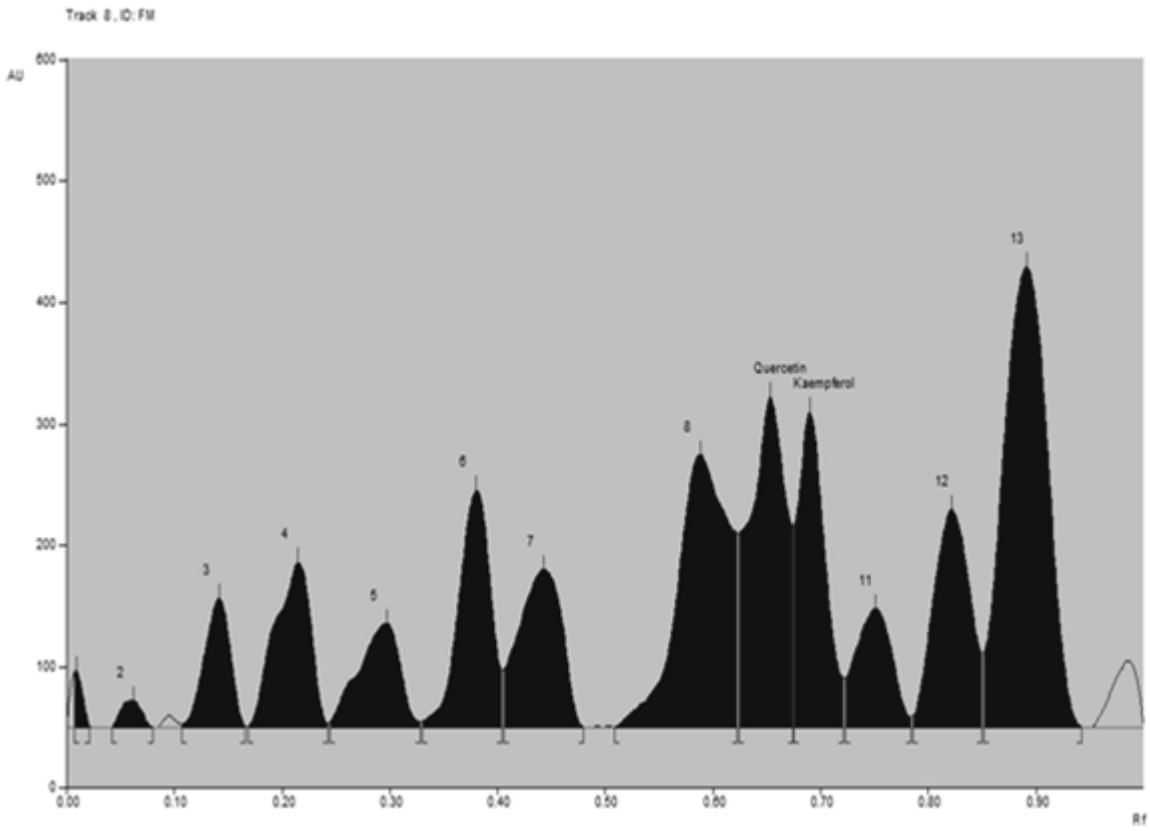

Fig. 4. HPTLC Chromatogram of methanolic extract of formulation, *Safoof-e-Muhazzil*

Conclusion

From the above studies, it can be concluded that HPTLC technique may successfully used for the estimation of quercetin and kaempferol in different plant extracts and various marketed and in-house polyherbal formulations. Statistical analysis proves that the method is simple, reproducible, robust and precise and selective for the analysis of quercetin and kaempferol. Therefore, this method can be successfully used for the routine analysis of quercetin and kaempferol in crude drugs, extracts and finished formulations without any interference that can be explored for standardisation and quality control of raw materials and marketed herbal products containing *R. damascena* Mill., *O. vulgare* L., *N. jatamansi* DC., *A. graveolens* L. and *T. ammi* L. as ingredients.

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