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SIMULTANEOUS QUANTIFICATION OF KAEMPFEROL AND QUERCETIN IN MEDICINAL PLANTS USING HPTLC

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Key words:

HPTLC, validation, kaempferol, quercetin, *Podophyllum hexandrum*, *Cassia angustifolia*

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ABSTRACT: Kaempferol and quercetin are the flavonoids which are widespread in fruits and vegetables exhibiting important biological activities. A simple and precise HPTLC method was developed and validated for simultaneous determination of kaempferol and quercetin in herbal drug extracts of *Podphyllum hexandrum* and *Cassia angustifolia*. The HPTLC of flavonoids was performed with toluene: acetone: formic acid (7:3:0.25) as mobile phases on RP-18 F254 TLC plates. Densitometric determination of flavonoids was performed at λ = 254 nm in reflectance/ absorbance mode. The results obtained indicated that the developed method was precise and accurate. Good resolution of kaempferol and quercetin was obtained from other constituents present in methanolic and hydrolysed extracts of *P. hexandrum* and *C. angustifolia* using proposed HPTLC method. Kaempferol was quantified in free (8.01and 2.42 µg/mg) as well as bound (17.54 and 8.13 µg/mg) forms and quercetin in free (4.5 and 0.93 µg/mg) as well as bound form (6.17and 4.8 µg/mg) in *P. Hexandrum* and *C. angustifolia.* Kaempferol was found to be present in high concentration and quercetin was in low concentration in these plants. Statistical data showed that method is sensitive and selective for simultaneous determination of flavonoids.

INTRODUCTION: Flavonoids are important bioactive polyphenolic compounds in kingdom Plantae. They are the largest groups of plant secondary metabolites which exist in the free aglycones and the glycoside forms with a diverse structure [1]. Amongst them kaempferol and quercetin are flavonols widely distributed in vegetables, fruits, beverages and reported to have important biological activities. *P. hexandrum* Royle syn. *P. Emodi* (berberidaceae) also known as Indian Podophyllum or Laghupatra. It is native to the foothills of Himalayas as well as the rain forests of Nepal and Kashmir.

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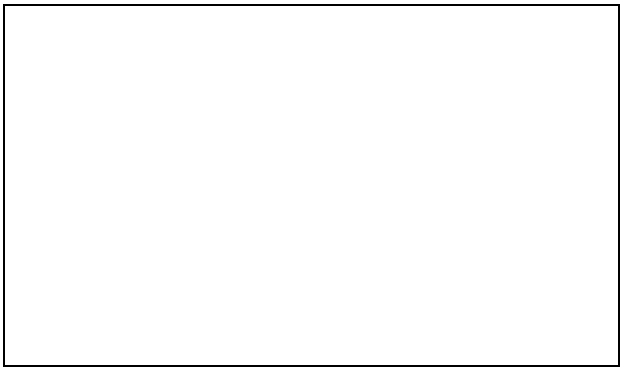
It is widely used for the treatment of cancer, biliary fever, septic wounds, constipation, cold, inflammation, burning sensation, mental disorder, and genital warts [2, 3]. The Podophyllum rhizome chemically contains lignans such as podophyllotoxin, flavonoids such as kaempferol and quercetin **(Fig.1)** both in free and glycoside form.

C. angustifolia Vahl. (Fabaceae) is an important medicinal shrub, commonly known as Senna cultivated in Somalia, Arabian peninsula, Punjab and in South India and recorded in many pharmacopoeias of the world [4, 5]. As it shows powerful cathartic properties, it is used for treatment of constipation. It is used as an anthelmintic and as a mild liver stimulant, febrifuge [6, 7]. The leaves and pods contain sennosides A, B, C, D, kaempferol and its glucosides, kaempferin,

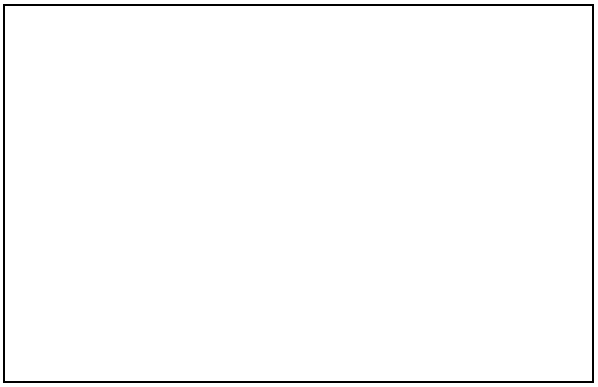
rutin, anthraquinone, essential oil, isorhamnetin, and emodin [8, 9].

Several chromatographic methods have been documented for identification and quantification of chemical constituents present in Podophyllum and *Senna* [2, 9, 10, 11, 12]. Quercetin and kaempferol are important constituents present in Indian Podophyllum and Senna as free and bound form and can be used as chemical markers. HPTLC is a simple, cost effective and an important tool for the qualitative, quantitative analysis of pharmacologically important compounds in medicinal plants and herbal formulations [2].

So far there is no method reported for simultaneous determination and validation of kaempferol and quercetin in Podophyllum and Senna using HPTLC in literature. The aim of present study was to develop a simple, precise, and accurate HPTLC method for the simultaneous determination of kaempferol and quercetin. The validation of proposed method was done according to the International Conference on Harmonization (ICH, 2002) guidelines [13].



KAEMPFEROL



QUERCETIN

FIG.1: CHEMICAL STRUCTURES OF *P. HEXANDRUM* AND *C. ANGUSTIFOLIA* CONSTITUENTS

Plant Material:

Rhizomes of Podophyllum and leaves of Senna were obtained from the local market, Mumbai, India. The plants were identified and their voucher specimens were deposited at the herbarium of Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Mumbai. The plant material was dried in hot air oven at 50ºC and powdered.

Chemicals:

Analytical grade methanol, toluene, acetone, formic acid were purchased from SD Fine Chemicals, Mumbai, India. Kaempferol (purity *97%) and Quercetin (purity *94%) were purchased from Total Herbs Solutions, Mumbai.

Preparation of Stock and Working Standard Solutions:

Stock solutions (1000.0 µg mL–1) of kaempferol and quercetin were prepared by dissolving 10 mg of each in a volumetric flask separately, sonicated and the volume of solution was made up to 10 ml with methanol. 100.0 µg mL–1 solutions were prepared by transferring 1ml of above stock solutions to 10 ml volumetric flask and adjusting volume up to 10 ml with methanol separately. These standard solutions were spotted to HPTLC plates to obtain kaempferol in the range of 0.1-2 µg per band and quercetin in the range of 0.2–0.6 µg per band.

Preparation of Sample Solutions:

1) Podophyllum dried rhizome powder (2g) and Senna dried leaf powder (5g) were extracted with methanol (50 ml and 125 ml respectively) under reflux on a water bath for 2 h at 70°C. The methanolic extract of Podophyllum was filtered through Whatman I filter paper and volume made up to 50 mL in a volumetric flask (sample solution A). The methanolic extract of Senna was concentrated to dryness and 0.1 gm of residue was taken and diluted to 10 ml with methanol in volumetric flask (Sample solution C). These extracts were used for TLC fingerprinting and co-chromatography with marker compounds i.e. kaempferol and quercetin.

2) Podophyllum dried rhizome powder (2g) was hydrolysed with 20% hydrochloric acid in 70%

methanol (50 ml) and Senna dried leaf powder (2g) was hydrolysed with 20% hydrochloric acid in methanol (50 mL) under reflux on a water bath for 2 h at 100ºC. The extract was filtered through Whatman I filter paper and the marc was washed with minimum amount of methanol (5 to 6 mL). The filtrate was transferred to a separating funnel. 100 mL of water was added and further extracted with ethyl acetate (50 mL× 4) and pooled. It was transferred to a 50 mL volumetric flask and the volume was made up to the mark with ethyl acetate (sample solution B and D respectively). This extract was used for co-chromatography with kaempferol and quercetin.

Chromatographic condition:

A DESAGA HPTLC system equipped with an automatic TLC sampler AS 30 (Biostep) equipped with 10-µL syringe under N2 gas flow. The densitometer consisted of a DESAGA CD 60 linked to PROQUANT software version 2.03 was used for the analysis. HPTLC was performed on pre-coated silica gel HPTLC 60F254 (20 cm × 10 cm) plate of 0.2 mm layer thickness. Samples and standards were applied on precoated plates, as 7 mm bands, with an automatic TLC applicator, 10 mm from the bottom, and 20 mm from the side, and the space between two bands was 7 mm of the plate.

Developing of the plate, about 80 mm, was performed at 25 ± 2°C with single run in toluene–acetone-formic acid (7:3:0.25) as mobile phase, in a Biostep glass twin-trough chamber (20×9×10 cm); previously saturated with mobile phase vapour for 30 min. After development, the plate was removed and dried. The spots were visualized under ultraviolet (UV) light at 254 nm with Pro Quant Software, using the deuterium light source, slit width 6 × 0.45 mm, scanning speed 20 mm s–1, and data resolution 50 µm step–1.

Procedure:

The sample solutions A, B, D (2 µl of each) and C (10 µl) along with kaempferol and quercetin standards were applied in triplicate on a TLC plate. As mentioned above, the plate was developed and scanned at 254 nm **(Fig. 2)**. The R_f and the colour of the resolved bands were noted. The peak areas were recorded and the amount of kaempferol and quercetin were calculated using their respective calibration curves.

Method Validation:

Limit of quantitation, limit of detection, linear dynamic range:

The linear range was initially found by spotting a wide range of kaempferol and quercetin standards (0.1µg-2µg). On the same TLC plate, Standard solutions of kaempferol of concentration 0.1, 0.5, 1, 1.5, 2 µg and quercetin of concentration 0.2, 0.3, 0.4, 0.5, 0.6 µg were applied as bands. The limits of detection (LOD) and (LOQ) were determined based on standard deviation of response and the slope of calibration curve.

Instrumental precision:

In order to determine the instrument precision, the same spot of kaempferol (0.1µg $spot^{-1}$) and quercetin (0.4µg $spot^{-1}$) were repeatedly scanned (n=5) and %RSD was calculated.

Inter-Day and Intra Day Variation:

Intra-Day precision was evaluated by analysis of triplicate applications of freshly prepared standard solutions containing 0.1, 1, 2 µg $spot^{-1}$ of kaempferol and 0.2, 0.4, 0.6 µg $spot^{-1}$ of quercetin on the same day and on three different days for inter day precision and % RSD was calculated.

Recovery:

The accuracy was assessed by performing recovery study of the compounds in the sample. The pre-analysed sample i.e. Podophyllum hydrolysed extract and Senna hydrolysed extract was spiked with three different concentration of kaempferol and quercetin. They were analysed by proposed HPTLC method. For each sample, the percent recoveries and average percent recoveries were calculated.

Specificity:

Specificity of the method was determined by analysing the standards and samples. The bands of kaempferol and quercetin from sample solutions were confirmed by comparing the R_f and spectra of the bands to those of standards. The peak purity of individual compound was determined by comparing spectra at three different levels, i.e. start, middle, end position of the bands.

Robustness:

To determine robustness of proposed TLC method, small deliberate changes in the chromatographic conditions were introduced such as change in mobile phase composition (± 0.1 mL of toluene and acetone), chamber saturation time (20,30and 40 min). The robustness of the method was determined with 0.5 µg spot^{-1} of each quercetin and kaempferol std. solution.

RESULTS AND DISCUSSION:

Optimization of chromatographic conditions: A suitable mobile phase for accurate analysis of the standards was selected by conducting trial experiments. Various mobile phases were tried to obtain best resolution of kaempferol and quercetin amongst which toluene: acetone: formic acid (7:3:0.25) gave the best resolution with R_f 0.46 and 0.39 respectively. These standards were also resolved from glycosides, flavonoids and lignans present in the sample extract **(Fig. 2)**. The identity of kaempferol and quercetin bands from the extracts of the plant powder of Podophyllum and Senna was confirmed by comparing their R_f values with those of the standards.

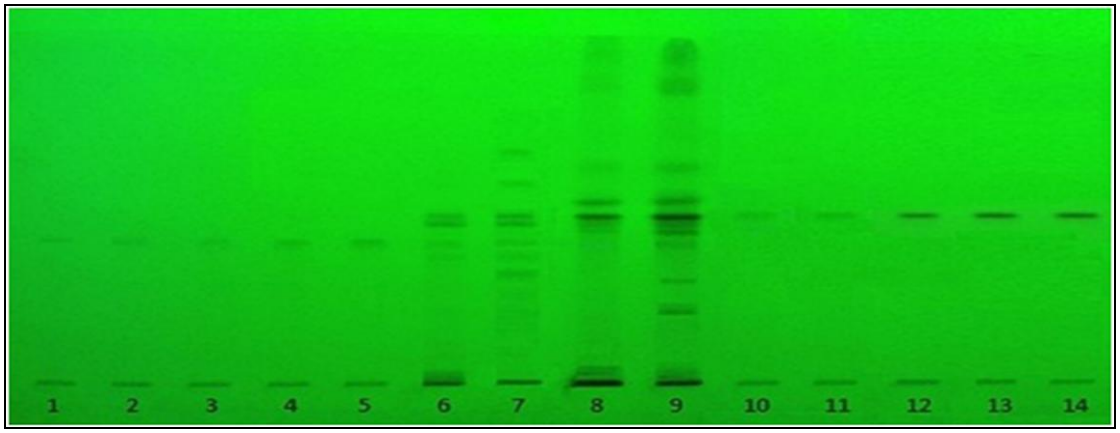


FIG. 2: CHROMATOGRAM (AT 254 nm)

Track 1-5 Quercetin (std); track 6, 7 *P. Hexandrum* methanolic and hydrolysed extract; track 8,9 *C. angustifola* methanolic and hydrolysed extract; track 10-14 Kaempferol (std)

Method validation:

The HPTLC method was validated for linearity, precision, accuracy and specificity. For kaempferol and quercetin linearity ranges were found to be 0.1 - 2 and 0.2 - 0.6µg spot^{-1}. The LOD values were 0.024 µg and 0.030 µg, LOQ values were 0.072 µg and 0.091µg for kaempferol and quercetin respectively. The average linear regression equation for kaempferol was y=770.93x+99.28 and for quercetin was y=1384.3x -271.2 **(Table 1)**.The results of %RSD intraday and inter day were in the range of 1.44 - 1.96 and 1.78- 1.99 for kaempferol respectively. And the results of % RSD for quercetin were in the range of 1.74-1.91 and 1.87-1.99 for intraday and inter day respectively for different concentrations as shown in **Table 2**. The low RSD values indicated that TLC densitometry method was precise and reproducible. The average recoveries at three different levels of kaempferol and quercetin were found to be 99.99% and 99.17% respectively as shown in **Table 3**. This indicated that the method was accurate.

TABLE 1: **METHOD VALIDATION PARAMETERS FOR THE QUANTIFICATION OF KAEMPFEROL AND QUERCETIN BY THE PROPOSED TLC DENSITOMETRY METHODS**

Sr. no	Parameter	Kaempferol	Quercetin
1	Instrumental precision (%RSD, n=5)	1.92	1.98
2	Accuracy (average% recover)	99.99	99.18
3	LOD (µg)	0.023	0.030
4	LOQ (µg)	0.072	0.091
5	specificity	specific	specific
6	Range (µg spot^{-1})	0.1-2	0.2-0.6
7	Robustness (% RSD)		
7a	Change in mobile phase composition	1.45	1.48
7b	Chamber saturation time	1.96	1.72

TABLE 2: INTRA-DAY AND INTER-DAY PRECISION OF KAEMPFEROL AND QUERCETIN

Marker	Concentration (µg/spot)	Intra-day precision	Inter-day precision
Kaempferol	0.1	1.78	1.93
	1	1.96	1.99
	2	1.44	1.78
Quercetin	0.2	1.88	1.97
	0.4	1.91	1.99
	0.6	1.74	1.87

TABLE 3: RECOVERY STUDY OF KAEMPFEROL AND QUERCETIN BY THE PROPOSED TLC DENSITOMETRIC METHOD

Marker	Amount of marker present (µg)	Amount of marker added	Amount of marker found	Recovery (%)	Average recovery (%)
Kaempferol	17.5	14	30.53±0.51	96.91±1.63	
	17.5	17.5	35.48±0.18	101.37±0.53	99.99
	17.5	21	39.16±0.23	101.71±0.61	
Quercetin	0.5	0.4	9.13±0.25	101.34±2.83	99.17
	0.5	0.5	10±0.26	100.12±2.58	
	0.5	0.6	10.5±0.4	96.06±2.78	

Simutaneous quantification of kaempferol and quercetin in plant extracts:

Though quantification of kaempferol and quercetin by TLC densitometric method was reported for other plants [14, 15, 16], this is the first report presenting that kaempferol and quercetin were simultaneously quantified in extracts of Podophyllum and Senna. Validated TLC method was used for quantifying and resolving kaempferol and quercetin in the extract. Mobile phases toluene–acetone–formic acid (7:3:0.25) showed highest selectivity for resolution of kaempferol and quercetin. Well-separated bands were observed in the densitogram **(Fig. 2)** for kaempferol (R_F =0.46), and quercetin (R_F =0.39), and extracts of Podophyllum and Senna. A typical 3D densitogram of standards and extracts is shown in **(Fig. 3)**. As kaempferol and quercetin were detected and resolved in all sample solution A, B, C and D; both compounds were present in free as well as bound form in Podophyllum and Senna **(Table 4)**. Quantification of kaempferol and quercetin was done from Podophyllum rhizome and Senna leaf using HPTLC by above validated method.

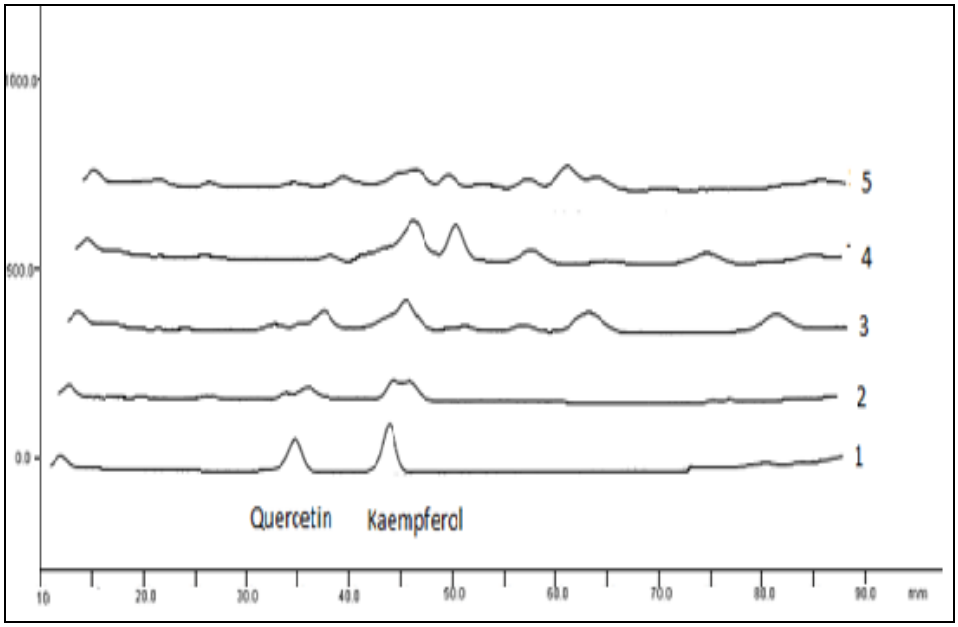


FIG.3: 3-D CHROMATOGRAM SHOWING PEAKS OF STANDARDS QUERCETIN AND KAEMPFEROL (1), *P. HEXANDRUM* METHANOLIC EXTRACT (2), *P. HEXANDRUM* HYDROLYSED EXTRACT (3), *C. ANGUSTIFOLIA* METHANOLIC EXTRACT (4), *C. ANGUSTIFOLIA* HYDROLYSED EXTRACT (5)

TABLE 4: KAEMPFEROL AND QUERCETIN CONTENT IN *P. HEXANDRUM* AND *C. ANGUSTIFOLIA* BY PROPOSED TLC DENSITOMETRIC METHOD

Sample	Kaempferol content (µg/mg)	Quercetin content (µg/mg)
A	8.01	4.5
B	17.54	6.17
C	2.42	0.93
D	8.13	4.8

CONCLUSION: HPTLC densitometric method has been developed for the simultaneous quantification of kaempferol and quercetin from herbal raw materials. The developed and validated HPTLC method is specific, selective, and reproducible and can be used for the quantitative analysis of these compounds with good separation and faster analysis speed. Under identical conditions, simultaneous chromatography of sample and standard led to satisfactory precision and accuracy.

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CONFLICT OF INTEREST: There is no conflict of interest as per my knowledge

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