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ORIGINAL ARTICLE

Development of Validated High-performance Thin-layer Chromatography Method for Simultaneous Determination of Quercetin and Kaempferol in *Thespesia populnea*

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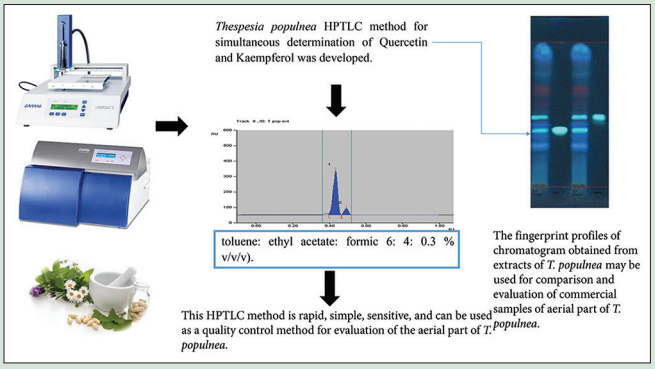
ABSTRACT

Introduction: *Thespesia populnea* L. (Family: Malvaceae) is a well-known medicinal plant distributed in tropical regions of the world and cultivated in South Gujarat and indicated to be useful in cutaneous affections, psoriasis, ringworm, and eczema. Bark and fruits are indicated in the diseases of skin, urethritis, and gonorrhea. The juice of fruits is employed in treating certain hepatic diseases. The plant is reported to contain flavonoids, quercetin, kaempferol, gossypetin, Kaempferol-3-monoglucoside, β-sitosterol, kaempferol-7-glucoside, and gossypol. T. populnea is a common component of many herbal and Ayurvedic formulation such as Kamilari and Liv-52. **Objective:** The present study aimed at developing validated and reliable high-performance thin layer chromatography (HPTLC) method for the analysis of quercetin and kaempferol simultaneously in *T. populnea*. **Method:** The method employed thin-layer chromatography aluminum sheets precoated with silica gel as the stationary phase and toluene: ethyl acetate: formic acid (6:4:0.3 v/v/v) as the mobile phase, which gave compact bands of quercetin and kaempferol. **Result:** Linear regression data for the calibration curves of standard quercetin and kaempferol showed a good linear relationship over a concentration range of 100–600 ng/spot and 500–3000 ng/spot with respect to the area and correlation coefficient (R2) was 0.9955 and 0.9967. The method was evaluated regarding accuracy, precision, selectivity, and robustness. Limits of detection and quantitation were recorded as 32.06 and 85.33 ng/spot and 74.055 and 243.72 ng/spot for quercetin and kaempferol, respectively. **Conclusion:** We concluded that this method employing HPTLC in the quantitative determination of quercetin and kaempferol is efficient, simple, accurate, and validated.

Key words: High-performance thin-layer chromatography, kaempferol, quercetin, *Thespesia populnea*



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INTRODUCTION

The global resurgence of interest in herbal medicines has led to an increase in their demand leading to a decline in their quality, primarily due to lack of adequate regulations pertaining to drugs.[1] Identification and quality evaluation of crude drugs are fundamental requirements. Directive on analytical control of crude drugs must take account of the fact that the material to be examined has complex and inconsistent composition. Therefore, the analytical limits are not as precise as those for single chemical entity. Adequate standard using chemical, instrumental, and physicochemical methods is necessary. The WHO has emphasized the need to ensure quality using modern analytical techniques and setting up physicochemical standards.[2]

Thespesia populnea is a tree growing well along warm coastal areas from the east coast of Africa and South and Southeast Asia to Melanesia, Micronesia, and Polynesia. It is currently naturalized in tropical climates throughout the world from the Caribbean to Africa.[3,4] *T. populnea* is commonly known as paraspipalo. The plant is claimed to be useful in cutaneous affections, such as scabies, psoriasis, ringworm, guinea worm, and eczema. A decoction of the bark is given internally in the diseases of skin and that of the fruits as an antidote for poisoning. The juice of fruits is used in the treatment of certain hepatic diseases. Various parts of the plants have high tannin content, and plant extracts have been shown to have antibacterial and antiviral activity.[5,6] The seed possesses purgative properties. The plant has been shown to be effective in malaria.[7]

The plant is reported to have antifertility, antibacterial, anti-inflammatory, antioxidant, peroxisome proliferator-activated receptors-γ agonist activity, purgative, hepatoprotective, antisteroidogenic activity, anti-implantation activity, cytotoxicity and superoxide anion generation, antinociceptive and antipsoriatic activities.[8-22] It has been demonstrated that the biological effects of *T. populnea* are mainly due to the presence of flavonoid compounds. It is highly valued for its action on the liver and has been incorporated as one of the major ingredients in many

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herbal formulations indicated in the treatment of liver disorders.[23-26] High-performance thin-layer chromatography (HPTLC) is a more effective technique for the simultaneous determination in single samples in routine analysis.[27] Literature survey showed the nonexistence of any analytical method for the estimation of quercetin and kaempferol in *T. populnea*.[28-32]

The aim of the present investigation is to develop a validated HPTLC method for the simultaneous determination of quercetin and kaempferol. We have developed a method using toluene: ethyl acetate: formic acid (6:4:0.3 v/v/v) as mobile phase on Silica Gel 60 F_{254} HPTLC Plates (0.2 mm; MercK, India). Quantitative estimation was accomplished by densitometric scanning with ultraviolet detector at 366 nm wavelength.

MATERIALS AND METHODS

Instrumentation

A Camag Linomat V sample applicator (Muttenz, Switzerland) was used to dispense the aliquots of the standard stock solution and the prepared samples. The plates were saturated in a twin trough chamber; slit dimension settings of length 4 and width 0.3 mm, monochromator bandwidth 20 nm and scanning rate of 20 mm/s spraying rate, 10 s/μL; data resolution, 100 mm/step. Zones were quantified using Camag TLC Scanner III densitometer controlled by Win CATS version 1.4.3.6336 software in the absorption mode using a deuterium source and a filter with a wavelength of 366 nm. The chromatographic plates used were aluminum plates precoated with Silica Gel 60 F_{254} (20 cm × 20 cm, 0.25 mm) (E. Merck, Darmstadt, Germany).

Pure standards

Reference standards quercetin (100%), KAEMPFEROL (99.68%), were procured from Natural Remedies Pvt. Ltd., Bengaluru, Karnataka, India.

Plant material

Plants of *T. populnea* were collected in full bloom (September 2012) from the local nursery farm, Anand, Gujarat. Botanical identification of *T. populnea* was done by the taxonomist of Sardar Patel University. A voucher specimen (APC/2012-2013/01) was deposited in the Department of Pharmacognosy, Anand Pharmacy College, Anand, Gujarat, India. The plant material was dried under shade and stored in closed boxes at room temperature until the isolation procedure started.

Chemicals and reagents

All chemicals used throughout this work were of analytical grade and the solvents were of spectroscopic grade. These included hydrochloric acid, toluene, ethyl acetate, acetic acid, sodium carbonate, formic acid, methanol, and chloroform (S. D. Fine Chemicals, Ahmedabad, Gujarat, India).

Standard solution

The standard stock solution was prepared by weighing 10 mg of the authentic sample of quercetin and kaempferol, then quantitatively transferred to 10 mL volumetric flask and volume was adjusted with methanol, kept in the refrigerator, and tightly closed. One milliliter of the stock solution was withdrawn and accurately transferred to 10 mL volumetric flask and volume was made up with methanol (stock solution A). Calibration curve was constructed according to the requirements of the International Conference on Harmonization (ICH) guidelines.

Sample preparation

Ten grams of accurately weighed dry powder of aerial parts of *T. populnea* was extracted using methanol and water. The hydroalcoholic of *T. populnea* was hydrolysed by refluxing with 2N HCl: toluene (1:1 v/v) for 3 h. The hydrolysate after neutralizing with 5–10% Na_2CO_3 was allowed to separate. The aqueous phase was further extracted using ethyl acetate (3 × 25 ml). Collect ethyl acetate extract and evaporate it. The stock solution was prepared by weighing 100 mg of the sample, then quantitatively transferred to 100 mL volumetric flask and volume was adjusted with methanol, kept in the refrigerator, and tightly closed.

Calibration

Calibration curve was constructed according to requirement of the ICH guidelines. Each concentration was applied to a plate (20 cm × 10 cm) in triplicates of 6 mm band length with a distance of 11.7 mm between each two bands. The distance from the plate side edge was 12 mm and from the bottom of the plate was also 8 mm. The application rate was 15 μL/s, the bands were developed using toluene: ethyl acetate: formic acid (6:4:0.3 v/v/v) after saturation for 20 min. The development time was 15 min, the plates were air-dried for 10 min showing R_f = 0.39 ± 0.01 and 0.50 ± 0.04, respectively, for quercetin and kaempferol. Standard zones were quantified by linear densitometric scanning using Camag TLC scanner in the absorbance mode at λ 366 nm, the wavelength corresponding to the maximum sensitivity. Deuterium lamp was utilized as a source of radiation. Evaluation was done using linear regression analysis through peak areas.

Sample assay

After preparation as previously described, sample and standard solution were spotted in triplicates on a plate, developed under the same conditions as described for the standard. After development and drying of the plates, the analyte was found to be completely separated from other components; hence, the linear and compact zones were scanned at λ 366 nm and peak areas for quercetin and kaempferol were recorded.

RESULTS AND DISCUSSION

Different proportions of toluene, ethyl acetate, and formic acid were tried as the mobile phase on silica gel HPTLC plates and a ratio of (6:4:0.3 v/v/v) gave good resolution. Well-resolved symmetric band for quercetin and kaempferol in extract was obtained under the optimized conditions using precoated HPTLC plates [Figure 1]. Quercetin and kaempferol on derivatization with

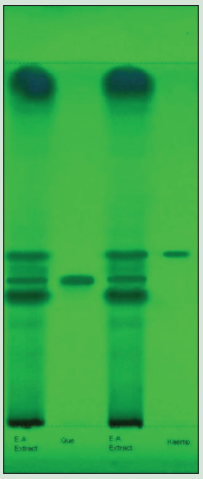

Figure 1: Ultraviolet mode at 254 nm Que-standard Quercetin, E.A. extract-ethyl acetate extract, kaemp-standard kaempferol

boric acid (10%), oxalic acid (3%) reagent gave yellow fluorescence [Figure 2]. Standard quercetin (R_f = 0.39) and kaempferol (R_f = 0.50) [Table 1] showed single peak in HPTLC chromatogram [Figures 3 and 4] and HPTLC chromatogram of *T. populnea* [Figures 5 and 6].

Linearity

The linearity of the HPTLC method was evaluated by analyzing a series of different concentrations of the standard quercetin and kaempferol where each concentration was applied triplicate. Linear regression data for the calibration curves of standard quercetin showed a good linear relationship over the concentration range of 100–600 ng/spot with respect to the area [Table 2]. The correlation coefficient (R^2) was 0.9955 and linear regression equation was found to be: $y = 16.2168x - 1816.2$, where y is the spot area and x is the concentration of the analyte [Figure 7], and kaempferol showed a good linear relationship over the concentration range of 500–3000 ng/spot with respect to the area [Table 3]. The correlation coefficient (R^2) was 0.9967 and linear regression equation was found to be: $y = 4.7753x - 1788.8$ [Figure 8].

Method precision (repeatability)

The precision of the instruments was checked by repeatedly injecting (n = 6) standard solutions of quercetin and kaempferol. Repeatability of HPTLC instrument was assessed by applying the same sample solution 6 times on a plate with the automatic spotter using the same syringe and taking repeated scans of the sample spot 6 times for both quercetin and kaempferol without changing the position of the plate [Table 4].

Intermediate precision (reproducibility)

The intraday and interday precisions of the proposed methods were determined by estimating mixed standard solutions of quercetin and

Table 1: Optimized chromatographic conditions for quercetin and kaempferol

Parameter	Conditions
Mobile phase	Toluene: ethyl acetate: formic acid (6:4:0.3)
Stationary phase	Precoated silica gel G60-F254 (100 mm × 100 mm, thickness layer 0.2 mm)
Temperature	27°C
Distance run (mm)	80
Chamber saturation time (min)	20
Scanning speed (mm/s)	20
Detection wavelength (nm)	366
Retention factor (R_f)	
QUE	0.39
Kaempferol	0.50
Diluent	Methanol

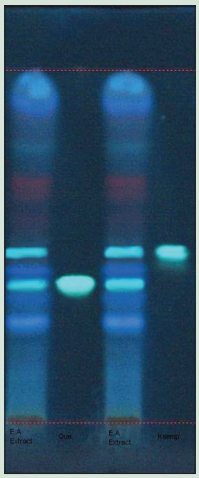

Figure 2: Fluorescence mode after derivatization Que-standard Quercetin, E.A. extract-ethyl acetate extract, kaemp-standard kaempferol

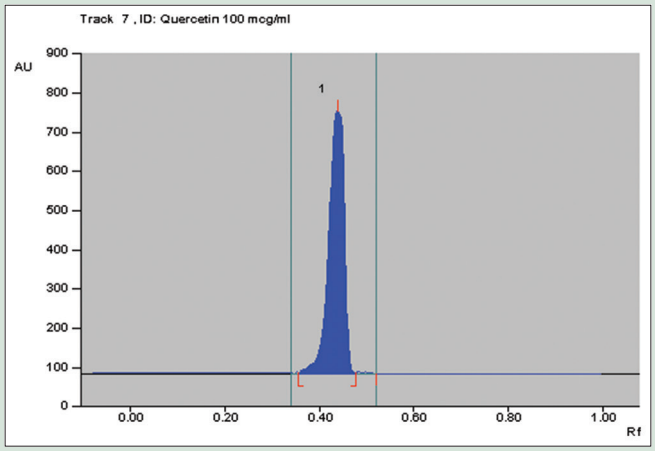

Figure 3: Chromatogram of standard quercetin

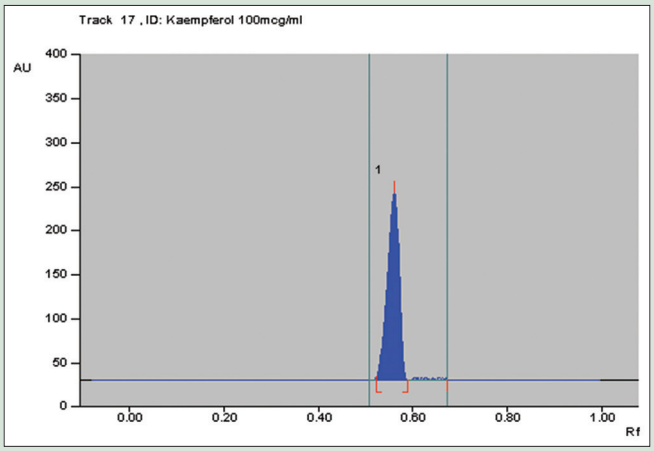

Figure 4: Chromatogram of standard kaempferol

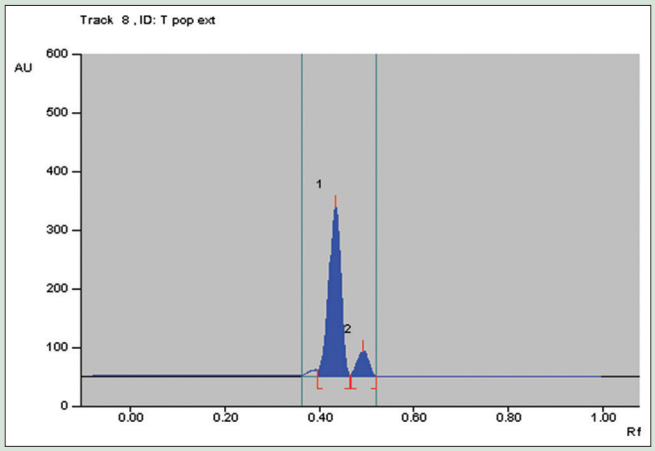

Figure 5: Chromatogram of *Thespesia populnea* extract

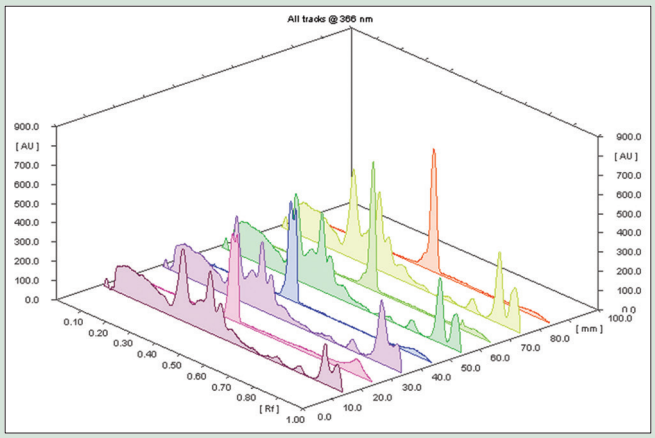

Figure 6: Densitogram scanning (overlay spectra) of extract of *Thespesia populnea*

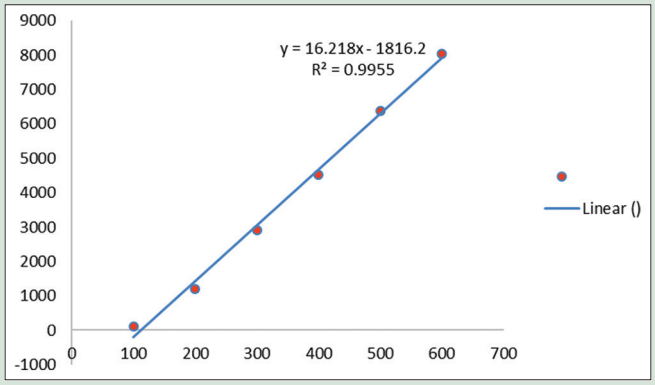

Figure 7: Calibration plot for quercetin standard

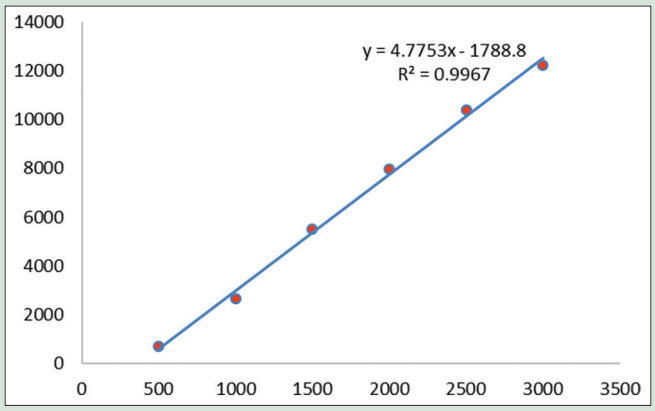

Figure 8: Calibration plot for kaempferol standard

kaempferol for three different concentrations (100, 200, 300 ng/spot for quercetin and 500, 1000, 1500 ng/spot for kaempferol) 3 times on the same day and on three different days. The results are reported in terms of relative standard deviation (RSD) [Table 4].

Accuracy (percentage recovery)

The accuracy of the methods was determined by calculating recoveries of quercetin and kaempferol by the standard addition method. Known amounts of standard solution of quercetin (100, 200, 300 ng/mL) and kaempferol (500, 1000, and 1500 ng/mL) were added to prequantified sample solutions. The amounts of quercetin and kaempferol were estimated by applying values of peak area to the regression equations of the calibration curve [Table 4].

Table 2: Linear regression data of quercetin

Concentrations (ng/spot)	Area, mean±SD (*n*=6)	Coefficient of variation
100	110.04±12.565	0.15
200	1198.7±45.451	0.68
300	2920.74±45.206	0.74
400	4518.52±12.354	0.24
500	6375.98±23.458	0.19
600	8036.65±18.145	0.24

SD: Standard deviation

Table 3: Linear regression data of kaempferol

Concentrations (ng/spot)	Area, mean±SD (*n*=6)	Coefficient of variation
500	669.9±12.44	0.12
1000	2639.1±25.12	0.34
1500	5496.4±08.25	0.09
2000	7981.2±12.58	0.13
2500	10,785.6±36.89	0.41
3000	12,138.45±28.73	0.29

SD: Standard deviation

Table 4: Summary of validation parameters of high-performance thin layer chromatography

Parameters	Quercetin	Kaempferol
Recovery, %	98.23-99.64	99.14-99.78
Precision (coefficient of variation)		
Intraday (*n*=3)	0.10-0.49	0.26-0.73
Interday (*n*=3)	0.13-0.48	0.12-0.39
Specificity, %	99.86	99.12
Solvent suitability	Solvent suitable for 24 h	Solvent suitable for 24 h

Limit of detection and limit of quantification

The limit of detection (LOD) with S/N of 3:1 and the limit of quantification (LOQ) with S/N of 10:1 were calculated for both drugs using the following equations according to the ICH guidelines:

$LOD = 3.3\times \sigma/S$

$LOQ = 10 \times \sigma/S$

where σ is the standard deviation of the response and *S* is the standard deviation of the *y*-intercept of the regression line.

CONCLUSION

This HPTLC method was developed for quantitative analysis of quercetin and kaempferol in the aerial part of *T. populnea*. RSD values were <2% which indicated that the precision of the method is reasonably acceptable. Recovery of quercetin was 98.23%–99.64% and kaempferol was 99.14%–99.78% which showed the reliability and suitability of the method. The fingerprint profiles of chromatogram obtained from extracts of *T. populnea* may be used for comparison and evaluation of commercial samples of aerial part of *T. populnea*. Shorter processing time, small sample volumes, single optimized extraction using inexpensive chemicals, and small mobile phase volumes are the inherent advantages of this method compared with HPLC. This HPTLC method

is rapid, simple, sensitive, and can be used as a quality control method for evaluation of the aerial part of *T. populnea*.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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