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HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC (HPTLC) METHOD FOR SIMULTANEOUS QUANTITATION OF LUTEOLIN AND QUERCITIN FROM DRIED WHOLE PLANT POWDERS OF *GMELINA ARBOREA* LINN. AND *KALANCHOE PINNATA* (LAM.) PERS..

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Abstract:
Objectives: To develop and validate High Performance Thin Layer Chromatographic (HPTLC) method for simultaneous quantitation of luteolin and quercitin from dried whole plant powders of Gmelina arborea Linn. and Kalanchoe pinnata (Lam.) Pers..
Methods: Chromatography was performed using methanolic extracts of dried whole plant powder of Gmelina arborea Linn. and Kalanchoe pinnata (Lam.) Pers.. Separation of luteolin and quercitin from methanolic extracts of both the plant materials was achieved on Silica gel 60F_{254} TLC plates using chloroform : methanol in the volume ratio of 96.0 : 4: 0 as the mobile phase. Detection and quantitation of luteolin and quercitin was done by densitometric scanning at λ=254 nm. The developed HPTLC method has been validated using International Conference on Harmonization (ICH) guidelines.
Results: The validated HPTLC method was used for simultaneous quantitation of luteolin and quercitin from methanolic extracts of dried whole plant powders of Gmelina arborea Linn. and Kalanchoe pinnata (Lam.) Pers. using their respective calibration curves. Amounts of luteolin and quercitin present in dried plant powder of Gmelina arborea Linn. are 0.9236mg/g and 0.0836 mg/g respectively. Amounts of luteolin and quercitin present in dried plant powder of Kalanchoe pinnata (Lam.) Pers are 0.0529mg/g and 0.2331mg/g.
Conclusion: The developed method is simple, precise and accurate and can also be used for routine quality control analysis and for the quantitation of luteolin and quercitin in herbal raw materials as well as in their formulations.
Keywords: *Luteolin, quercitin, Gmelina arborea Linn. and Kalanchoe pinnata (Lam.) Pers., High Performance Thin Layer Chromatography, Method validation*

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INTRODUCTION:

Gmelina arborea belonging to the family Verbenaceae is a fast growing deciduous tree found throughout Indiaand also in Pakistan, Bangladesh, China, Japan, Myanmar, Nepal, Pakistan, Sri Lanka, Thailand. It is a one of theherbs mentioned in all ancient scriptures of Ayurveda. It is known to have been used in traditional Indian medicine.It is an important timber-yielding tree that grows naturally in the tropical and subtropical regions of Southeast Asia and has also been introduced as a plantation species outside these regions (1,2).

The whole plant is medicinally very important. Folklore states that it promotes digestive power, improves memoryand is useful alteration of fever, heart disease, nervous disorders and piles. The drug has been known to be used forsnake-bites and scorpion-stings. The root of *Gmelina arborea* Linn. is one of the ingredients of "*dashmuladikwath*"and "*bhrahatpanchamool*" of ayurveda, which constitutes a number of ayurvedic preparations, used as tonics (3).

Plant contain phyto-chemicals like apigenin, luteolin, quercetin, hentriacontanol and *β*-sitosterol(4).

Kalanchoe pinnata (Lam.) Pers. belonging to family, Crassulaceae,is a glabrous, ornamental, crassulenscent herb, cultivated in houses and gardens(5, 6, 7). The leaves of parnabija have great medicinal value and are used for medicinal purpose both, internally as well externally. The leaves possess various properties like haemostatic, refrigerant, emollient, mucilaginous, vulnerary, depurative, anti-inflammatory, disinfectant and tonic. They are useful in vitiated conditions of vata and pitta, cuts and wounds, hemorrhoids, menorrhagia, discoloration of the skin, boils, sloughing ulcers, ophthalmic, burns, scalds, corn, diarrhea, dysentery, vomiting and acute inflammations(8). Plant contain phyto-chemicals likeIsocitric acid & citric acid, Bufadienolides, Phenols, Phenylpropanoids and Flavanoids like Syringic acid, caffeic acid , 4-hydroxy-3-methoxy-cinnamic acid, 4-hydroxybenzoic acid, p-hydroxycinnamic acid, paracoumaric acid, ferulic acid, protocatechuic acid, phosphoenolpyruvate, protocatechuic acid .Triterpenoids and Steroid like: α-amyrin, α-amyrinacetate, β-amyrin, β-amyrinacetate.(9)

Both the plants possess high medicinal value because they are reported to contain array of therapeutically important classes of phytochemicals such as triterpenoids, flavones, aglycones, glycosides and a range of fatty acids and esters. Flavone aglycones, luteolin and quercitin have been reported to be present in considerable amounts in leaves of *Gmelina arborea Linn.*(10) and Kalanchoe pinnata (Lam.) Pers.(11).

Therefore, a High Performance Thin Layer Chromatographic method has been developed for simultaneous quantitation of these two flavonoids from dried whole plant powder of *Gmelina arborea* Linn. and *Kalanchoe pinnata* (Lam.) Pers which may be used as an important quality control method for standardization of these two plant materials.

Literature survey revealed that HPTLC methods have been reported for the qualitative and quantitative analysis of quercitin and luteolin from different sources such as Boerhaavia Diffusa L1, selected medicinal plants of Margalla hills and surroundings2, *Bauhinia variegata, Bacopa monnieri, Centella asiatica, Ginkgo biloba, Lonicera japonica, Rosa bourboniana, Rosa brunonii,* and *Rosa damascena3*. However, in the present research work, the separation and quantitation of luteolin and quercitin was achievedusing a simple mobile phase and in single run. Thus, making the developed HPTLC method, cost effective and less time consuming compared to all of the above methods reported.

MATERIALS AND METHODS:

Experimental reagents

Analytical grade methanol (99.9%) and chloroform (99.4%) were procured from Qualigens Fine Chemicals (Mumbai, India).
TLC plates, precoated with Silica gel 60F254,with aluminium sheet support were obtained from E. Merck (India).

Reference standards

The reference standards,luteolin (purity ≥ 98%) and quercitin (purity ≥ 95%), were purchased fromSigma-Aldrich Chemie GmbH (Aldrich Division,Steinbeim, Germany).

Plant material

Both the plants *Gmelina arborea* Roxb. and *Kalanchoe Pinnata* (Lam.) Pers. were collected from Keshav Shrishti, Maharastra. The plant material of *Gmelina arborea* Roxb. was authenticated from Agharkar Research Institute, Pune, India (Auth.15-193). Herbarium of *Kalanchoe Pinnata* (Lam.) Pers. was authenticated from Botanical Survey of India, Pune, India (Certificate No. BSI/WRC/Cert./2014) and collection no. HSQ 01.

Both plant materials were washed with water to remove soil particles, dried in shade, finely powdered

and then sieved through BSS mesh size 85 and stored in an airtight container at room temperature (25 ± 2° C).

Preparation of solutions

Preparation of stock solution and working solution of standard luteolin (1000 µg/mL)

Stock solution of 1000 µg/mL of luteolin was prepared by dissolving 10.0 mg of accurately weighed luteolin in 5.0 mL of methanol in a 10.0 mL standard volumetric flask. The standard volumetric flask was then sonicated in an ultrasonic bath (Model: TRANS-O-SONIC, Frequency: 50 Hz) for 5.0 minutes for complete dissolution of luteolin. The contents were then diluted up to the mark with methanol to obtain a solution of 1000.0 µg/mL of luteolin. 0.50 mL of the above stock solution of luteolin was then transferred to 50.0 mL volumetric flask and the contents were diluted to 50.0 mL using methanol to obtain working solution of standard luteolin with concentration 10.0 µg/mL.

A similar procedure was followed for preparation of standard stock and working solution of quercitin.

Preparation of sample solutions

Sample Preparation

About 1.000 g of dried whole plant powder of *Gmelina arborea* Roxb. was accurately weighed and transferred to a stoppered conical flask and 10.0 mL of methanol was then added to it. The flask was then shaken at 50 rpm, on a conical flask shaker overnight at room temperature (25ºC + 2ºC). The contents of the flask were filtered through Whatman No.41 filter paper (E. Merck, Mumbai, India). The filtrate was further used as sample solution for the assay experiment. The sample solution was filtered through 0.45µm filter paper before analysis.

The same procedure was followed for preparation of extract of whole plant of *Kalanchoe Pinnata* (Lam.) Pers.

Preparation of mobile phase

The mobile phase used in the present research work was prepared by mixing chloroform : methanol , 96.0 : 4.0 (v/v). It was then sonicated for 5 minutes.

Chromatographic conditions

Chromatographic separation was carried out on aluminium plates (20cm x 20cm), precoated with Silica Gel 60 F_{254}, with thickness of 200µm. Sample solution and standard solutions of different concentrations were applied to plates, as 6 or 8 mm bands at 10 mm from the bottom edge of the plate by means of CAMAG Linomat V Automatic sample applicator fitted with a 100µL syringe (Hamilton, Bonaduz, Switzerland). The plates were developed in a CAMAG (Muttenz, Switzerland) glass twin-trough chamber saturated with mobile phase comprising of chloroform and methanol in volume ratio of 96.0 : 4.0.The optimized chamber saturation time for the mobile phase was 20 minutes at room temperature ($28^{\circ}C \pm 2^{\circ}C$). The plates were developed to a distance of 90 mm from the bottom edge of the plate.The plates were air dried for 10 min after development and scanned at λ=254 nm in absorbance/reflectance mode, using a CAMAG TLC Scanner III and winCATS software version 1.4.2

METHOD VALIDATION

ICH guidelines were followed for the validation of the analytical methods developed (precision, accuracy, Ruggedness, Robustness, linearity, LOD, LOQ, specificity)[12].

Linearity

Linear working range for luteolin

The linear working range of each standard was determined by applying 10.0µg/mL of each standard solution in the volume range of 2µ- 22µL as bands on TLC plate to obtain concentrations in the range of 0.02µg/band to 0.22µg/band.Calibration curves of luteolin and quercitin were obtained by plotting mean peak areas against corresponding concentrations. Good correlation was obtained between mean peak areas and corresponding concentrations for both the standards. The results are listed in Table 2.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) were determined at signal to noise ratios of 3:1 and 10:1, respectively. The LOD and LOQ values obtained for both the components are listed in Table 2.

System suitability

The system suitability test was carried out to confirm that the system used for the analysis gives precise, accurate and reproducible results. System suitability test was carried out by applying standard solutions of luteolin with concentration of 0.06µg/band and quercitin with concentration of 0.07µg/band on same TLC plate in six replicates under specified chromatographic conditions. The chromatograms for both standards were recorded.

The values of percent relative standard deviation (%R.S.D) for peak area and retention factor (R_f) of luteolin and quercitin were found to be less than 2, indicating that the method was suitable for analysis.

Precision

The precision of the method was studied by determining repeatability and intermediate precision.

Repeatability was carried out by analysing three different sample solutions of each plant material on same day. Each sample solution was applied in triplicate on same TLC plate and analysed using optimised chromatographic conditions.

Intermediate precision was carried out on three successive days. Each sample solution was applied in triplicate on a TLC plate each day and analyzed using the optimized chromatographic conditions.

The precision results were expressed as percentage relative standard deviations of peak areas of luteolin and quercitin and are listed in Table 1. The results indicate that the proposed method is precise and reproducible.

Table 1: The precision results were expressed as percentage relative standard deviations of peak areas of luteolin and quercitin

Results of Precision Precision	Luteolin	Quercetin
Repeatability (% R.S.D.)		
whole plant powder of *Gmelina arborea* Roxb. (n=3) (on the same day)	1.06	0.92
whole plant powder of *Kalanchoe Pinnata* (Lam.) Pers. (n=3) (on the same day)	1.04	1.06
Intermediate Precision (% R.S.D.)		
whole plant powder of *Gmelina arborea* Roxb. (n=9) (For three successive days)	1.02	0.92
whole plant powder of *Kalanchoe Pinnata* (Lam.) Pers. (n=9) (For three successive days)	1.03	1.03

Specificity:

The specificity of the proposed HPTLC method was ascertained by comparing UV absorption spectra of luteolin and quercitin standards with those in the sample. The spectra were compared at three different positions namely at the peak start, peak centre and peak end. Good correlation was obtained between UV spectra obtained from luteolin and quercitin standards and luteolin and quercitin from the sample at all the positions.The peaks of luteolin and quercitin were not masked by any peaks arising due to other components or impurities present in the sample solution.

Ruggedness

Ruggedness of the method was studied by determining the effects of small variations of mobile phase composition (±2%), and flow rate (1.00 ± 0.05 mL/min). Effect of these deliberate changes on the response (area) and retention time of standard solution of luteolin and quercitin was observed during the analysis. The results were expressed in terms of % mean difference. Values within a difference range of ±5% were accepted.

The amount of luteolin and quercitin from dried whole plant powders of *Gmelina arborea* Roxb. and *Kalanchoe pinnata* (Lam.) Pers. obtained by altered method to that obtained by normal method did not show any significant differences. Thus, it can be concluded that the method is robust.

Stability of the standard luteolin and quercitin solution

The stabilities of standard luteolin and quercitin solution were determined by comparing the peak areas of standard solution of luteolin and quercitin at different time intervals, for a period of minimum 48 hours, at room temperature. Standard solutions of luteolin with concentration of 0.06µg/band and quercitin with concentration of 0.07µg/band were applied as bands on same TLC plate at different time intervals and analyzed under the optimized chromatographic conditions. Low values of percent relative standard deviation (less than 2) for peak areas of luteolin and quercitin indicated that the peak area values remained unchanged over a period of 48 hours and no significant degradation was observed within the given period, indicating that standard solutions of luteolin and quercitin were stable for a period of minimum 48 hours.

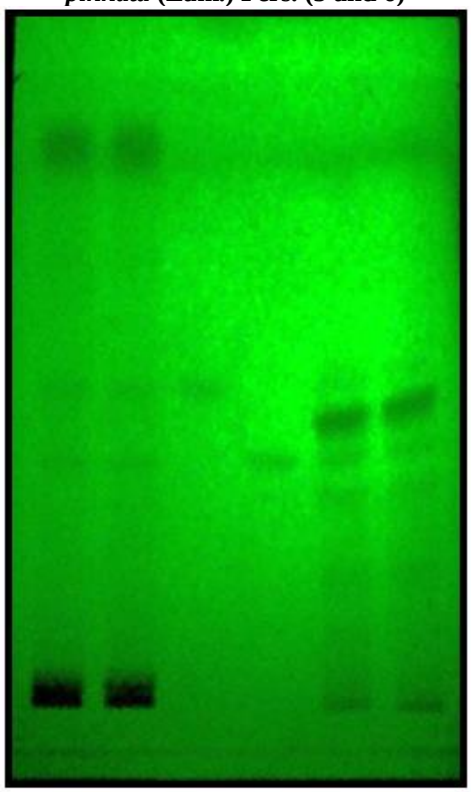
Assay

The validated HPTLC method was used for simultaneous quantitation of luteolin and quercitin from the sample solutions of dried whole plant powders of *Gmelina arborea* Roxb. and *Kalanchoe pinnata* (Lam.) Pers.

10µL of methanolic extracts of dried whole plant powders of *Gmelina arborea* Roxb. and *Kalanchoe pinnata* (Lam.) Pers. were applied as bands on the TLC plate. The plate was developed using optimized chromatographic conditions. The chromatograms

were obtained. To ascertain the repeatability of the method, the assay experiment was repeated seven times. The values of amounts of luteolin and quercitin present in dried whole plant powders of *Gmelina arborea* Roxb. and *Kalanchoe pinnata* (Lam.) Pers., values of standard deviation (S.D.) and the percent relative standard deviation (%R.S.D.) were calculated. Amounts of luteolin and quercitin present in the sample solutions were determined from their respective calibration curves, by using the peak areas of luteolin and quercitin recorded for both the sample solutions. The results of assay experiment are listed in Table 2.

TLC plate showing separation of standard quercitin (3), standard luteolin (4) and methanolic extract of dried plant powderof *Gmelina arborea* Linn. (1 and 2) and methanolic extract of dried plant powder of *Kalanchoe pinnata* (Lam.) Pers. (5 and 6)



Accuracy

Recovery tests were carried out to further investigate the accuracy of the method by adding three different concentration levels of the mixed standard solutions to known amounts of *Gmelina arborea* Roxb. and *Kalanchoe pinnata* (Lam.) Pers.. The resultant samples were then extracted and analyzed with the described method. The mean percentage recoveries were calculated using the formula: Recovery (%) = [(amount found – original amount) / amount added] x 100. Values within the range of 85 – 115% were accepted. Results obtained are tabulated in Table 2.

RESULTS:

Different mobile phases were triedin order to resolve flavones, quercitin and luteolin from methanolic extracts dried whole plant powder of *Gmelina arborea* Linn. and *Kalanchoe pinnata* (Lam.) Pers.. However, the mobile phase comprising of chloroform: methanol, 96.0: 4.0 (v/v), resulted in a good separation of luteolin (R_f = 0.31) and quercitin (R_f = 0.18) from one another and also from other phytochemicals present in the methanolic extracts of selected plant materials. Detection was carried out densitometricallyatλ = 254 nm as both luteolin and quercitin showed maximum response at this wavelength. The identity of the bands of luteolin and quercitin in the sample solution was confirmed by comparing their R_f value in sample with that of reference standards.

Table 2: LOD and LOQ values obtained for both the components

Parameters	Observations	
	Luteolin	**Quercitin**
Linear Dynamic Range (µg/band)	0.01 - 0.30	0.01 - 0.15
Linear Working Range (µg/band)	0.02 - 0.22	0.03 - 0.11
Correlation coefficient (r)	0.999	0.999
Limit of Detection (LOD) (µg/band)	0.01	0.01
Limit of Quantitation (LOQ) (µg/band)	0.02	0.03
Assay (mg/g)		
Plant powder of *Gmelina arborea* Linn.	0.9236	0.0836
Plant powder of*Kalanchoe pinnata* (Lam.) Pers.	0.0529	0.2331
Percent Recovery		
Plant powder of *Gmelina arborea* Linn.	98.56	99.17
Plant powder of*Kalanchoe pinnata* (Lam.) Pers.	99.08	99.23

CONCLUSION:

A High Performance Thin Layer Chromatographic method has been developed and validated for simultaneous quantitation of flavone aglycones, luteolin and quercitin from methanolic extracts of dried whole plant powders of *Gmelina arborea* Linn. and *Kalanchoe pinnata* (Lam.) Pers.

The developed method is simple, precise and accurate and can also be used for routine quality control analysis and for the quantitation of luteolin and quercitin in herbal raw materials as well as in their formulations.

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