ORIGINAL ARTICLE

Validation of HPTLC method for the analysis of luteolin in *Cardiospermum halicacabum* Linn.

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Background: Luteolin is one of the constituent of *Cardiospermum halicacabum*, which is traditionally used as an important component of plant-based medicine. **Aim and Objective:** This study presents the first report of thin layer chromatography (TLC) densitometric method, which has been developed and validated for quantification of luteolin from aerial parts of *C. halicacabum.* **Materials and Methods:** Chromatographic separation was achieved on silica gel 60 F_{254} plates with using the solvent system of Toluene: Ethyl acetate: Formic acid (10:9:1). Detection of luteolin was carried out in the absorption-reflection mode at 254 nm. TLC plates were dried under stream of hot air and then subjected to densitometric scanning using a Camag TLC scanner III (Camag, Switzerland) with win CATS software in the absorbance-reflectance scan mode. The accuracy of the method was checked by conducting various validation parameters according to ICH (International Conference on Harmonizaation) guidelines. **Results:** The system was found to give compact spots for luteolin (retention factor, R_f = 0.55). The calibration plot was linear in the range of 500-3000 ng of luteolin. The correlation coefficient of 0.997 was indicative of good linear dependence of peak area on concentration. The concentration of luteolin was found to be 0.50% w/w in aerial parts of *C. halicacabum.* The limit of detection (LOD) and limit of quantification value for luteolin were found to be 22.45 ng and 68.03 ng, respectively. Recovery values from 99.37 and 99.80 to 100.58 showed excellent reliability and reproducibility of the method. **Conclusion:** The proposed HPTLC method for quantitative monitoring of luteolin in *C. halicacabum* can be used for routine quality testing of *C. halicacabum* extract used in formulations.

Key words: *Cardiospermum halicacabum*, high performance thin layer chromatography, luteolin

INTRODUCTION

Cardiospermum halicacabum Linn., commonly known as Ballon vine, is an important medicinal herb belonging to family Sapindaceae. It is an annual or sometimes perennial climber, found as a weed throughout India, ascending up to 1200 m in the hills.[1]

The herb is used as diuretic, stomachic, rubefacient, in rheumatism, lumbago and nervous disorders and in preparing hair oil for curing dandruff and alopecia. The juice of the herb is used to cure earache and asthma. The decoction is administered in piles. The pungent vapours from the crushed leaves are inhaled to relieve headache. The root is mucilaginous and considered emetic, laxative and anti-rheumatic. It is effective in didymitis and early stages of hydrocela and is used in asthma and colic. It is useful in amenorrhoea, gonorrhoea, nervous diseases, haemorrhoids and erysipelas.[2-4]

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The plant yielded stigmasterol, stigmasterol-β-D-glucoside, β-sterol-β-D- glucoside, apigenin and luteolin. The ethanolic extract of seeds showed presence of arachidic acid and β-sitostrol-beta-D-galactoside.[5,6]

Luteolin, 3′,4′,5,7-tetrahydroxyflavone, belongs to a group of naturally occurring compounds called flavonoids that are found widely in the plant kingdom. Flavonoids are polyphenols that play an important role in defending plant cells against microorganisms, insects and UV irradiation.[7] Evidence from cell culture and animal and human population studies suggested that flavonoids are also beneficial to human and animal health. Because of their abundance in foods, e.g., vegetables, fruits and medicinal herbs, flavonoids are common nutrients that are antioxidants, estrogenic regulators and antimicrobial agents.[8] It has been noticed that flavonoids may be a cancer preventive.[9,10] Plants rich in luteolin have been used in Chinese traditional medicine for treating various diseases such as hypertension, inflammatory disorders and cancer. Having multiple biological effects such as anti-inflammation, anti-allergy and anticancer, luteolin functions as either an antioxidant or a pro-oxidant biochemically.[11-13] Luteolin's anti-cancer property is associated with the induction of apoptosis and inhibition of cell proliferation, metastasis and angiogenesis [Figure 1].[14,15]

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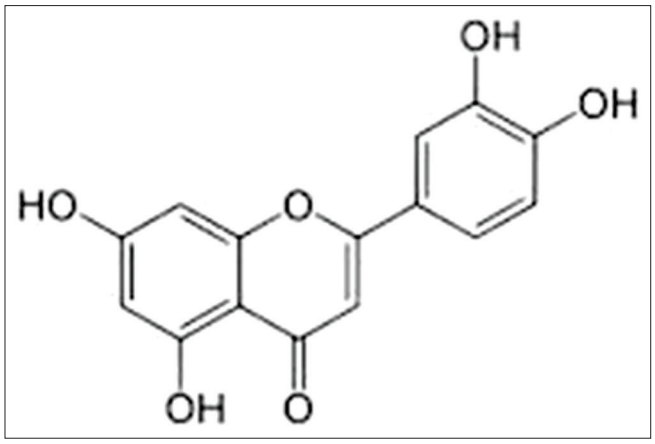


Figure 1: Luteolin

In the recent years, advancement in chromatographic and spectral fingerprints played an important role in the quality control of complex herbal medicines.[16] Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines, since they may appropriately represent the chemical integrities of the herbal medicines and their products, and therefore, be used for authentication and identification of herbal plants.[17] High performance thin layer chromatography (HPTLC) is a more efficient faster method and the results are more reliable and reproducible. Combined with digital canning profiling, HPTLC also provides accurate and precise retention factor (R_f) values and quantitative analysis of sample by *in situ* scanning densitometry aided by formation of easily detected derivatives by post-chromatographic chemical reactions as required along with a record of the separation in the form of a chromatogram, with fractions represented as peaks with defined parameters including absorbance (intensity), R_f, height and area.[18]

Literature survey revealed that no method has been reported for quantification of luteolin from aerial part extract of *C. halicacabum*. Hence, a densitometric HPTLC method has been developed in the present work for quantification of luteolin from methanolic extract of aerial parts of *C. halicacabum* that may be useful to pharmaceutical industry for the authentication, standardisation and quantification of luteolin component in herbal formulations.

MATERIALS AND METHODS

Plant Material

C. halicacabum Linn. Family Sapindaceae was selected for present research work encompassing the phytochemical and pharmacological studies. The aerial parts of plant were collected in July from New Vallabh Vidyanagar Campus and authenticated by a Taxonomist, Dept. of Biosciences, Sardar Patel University, Vallabh Vidyanagar, India. It's voucher specimen no. (MRK/CH-1/5/ARGH-12) has been preserved in Department of Pharmacognosy, A. R. College of Pharmacy, Vallabh Vidyanagar.

Reagents and Chemicals

All chemicals, including solvents, were of analytical grade from E. Merck, India. HPTLC plates Si 60 F_{254} and standard luteolin were purchased from E. Merck.

Preparation of Crude Extract

Accurately weighed 5 g of the coarse powder of aerial parts of *C. halicacabum* was extracted with methanol (50 ml) under reflux on a water bath. The extract was filtered, concentrated and transferred into an amber coloured bottle. Thereafter, 10 mg of extract was transferred in a 10-ml volumetric flask and the volume was made up with methanol.

Preparation of Standard Solution

Stock solution of luteolin was prepared by dissolving 5 mg of accurately weighed luteolin in methanol and making up the volume of the solution to 10 ml with methanol.

Analytical Procedure

Chromatography Conditions

A Camag TLC system equipped with Camag Linomat V an automatic TLC sample spotter, Camag glass twin trough chamber (20 cm × 10 cm), Camag scanner 3 and integrated win CATS 4 Software were used for the analysis. TLC was performed on a pre-coated TLC plate as 8-mm wide bands with an automatic TLC sampler (Linomat V) under a flow of N_2 gas, 10 mm from the bottom and 10 mm from the side, and the space between two spots was 15 mm of the plate. The linear ascending development was carried out in a Camag twin trough chamber (20 cm × 10 cm) which was pre-saturated with 20 ml mobile phase Toluene: Ethyl acetate: Formic acid (10:9:1) for 20 min at room temperature (25 ± 2°C and 40% relative humidity). The length of the chromatogram run was 8 cm. Subsequent to the development, TLC plates were dried under a stream of hot air and then subjected to densitometric scanning using a Camag TLC scanner III (Camag, Switzerland) with win CATS software in the absorbance-reflectance scan mode. Quantitative evaluation of the plate was performed in the absorption-reflection mode at 254 nm. Quantification of luteolin in the methanolic extract of aerial parts of *C. halicacabum* was performed by external standard method, using pure luteolin as standard.

Stock solution of luteolin (500 µg/ml) was prepared in methanol and different amount (500-3000 ng spot) were applied on a TLC plate, using Linomat V for preparing six point calibration graphs of peak area versus concentration in nanogram. The regression equations and correlation coefficients (r^2) were reported in results.

Detection and Quantification

Thirty microlitres of sample solution was applied on TLC plates and developed, scanned as above. Peak area was recorded and the amount of luteolin was calculated using the calibration curve.

Specificity

The specificity of the method was ascertained by co-analyzing standard and sample. The band for luteolin in sample was confirmed by comparing the R_f (0.55) and absorption spectra of the spot to that of reference compound. The peak purity of luteolin peak in sample track was assessed by comparing the spectra at peak start, peak apex and peak end positions of the band. Good correlation was also obtained between standard and sample overlay spectra ($r^2 > 0.99$).

Method Validation

The method was validated for precision, accuracy (ICH guideline, 1996-2005) and repeatability. Instrumental precision was checked by repeated scanning of the same spot 500 and 3000 ng six times and was expressed as coefficient of variance (%RSD). Method precision was studied by analyzing the standards 500 and 3000 ng per spot under the same analytical procedure and lab conditions on the same day and on different days (inter-day precision) and the results were expressed as % RSD. Accuracy of the method was tested by performing the recovery studies of the pre-analysed sample with standard at three levels; percentage recovery and average percentage recovery were calculated.

Linearity and Detection Limit

Linearity was checked by applying standard solution of luteolin at six different concentration levels. The calibration curve was drawn in the concentration range of 500-3000 ng per spot. The equations for the calibration curve of luteolin and the correlation coefficient of the calibration plot was indicating good linearity. Results of regression analysis on the calibration curve and detection limits are presented in Table 1.

Precision Studies

To determine the precision of the developed assay method 500-3000 ng per spot of the luteolin standard was analysed five times within the same day to determine the intra-day variability. The RSD values for intra-day and inter-day precision were tested on the same concentration levels on 2 days, and the RSD values are reported in Table 2.

Sample Analysis and Recovery Studies

This developed TLC method was subsequently applied for the analysis of luteolin in the methanolic extract of aerial parts of *C. halicacabum*. The luteolin content by this proposed method was found to be 0.50%. For the examination of recovery rates, 80%, 100% and 120% of pure luteolin was added to pre-analysed sample and quantitative analysis was performed. The average recovery is listed in Table 3.

RESULTS AND DISUSSION

Chromatographic fingerprint analysis has shown to be a rational and feasible approach for the quality assessment and species authentication of traditional medicine. It utilises chromatographic techniques to construct specific patterns of recognition for herbal drugs. The developed fingerprint pattern of components can then be used to determine not only the absence or presence of markers of interest but the ratio of all detectable analytes as well.

Although HPTLC has a few limitations, such as the limited developing distance and lower plate efficiency compared with high performance liquid chromatography (HPLC) and gas chromatography (GC), it is still an effective tool for quality evaluation of herbal drugs due to its simplicity, low cost and requirement; it has been successfully utilised to develop the chromatographic fingerprint for herbal

Table 1: Linearity regression data for quantification of luteolin by proposed TLC densitometric method

Parameters	Results
R_f	0.55
Dynamic range (ng/spot)	500-3000
Equation	Y=1.962×+523.3
Slope	1.962
Intercept	523.3
Limit of detection	48.94 ng/spot
Limit of quantification	148.31 ng/spot
Correlation coefficient	0.997
Specificity	Specific

TLC – Thin layer chromatography

Table 2: Precision data of luteolin by proposed TLC densitometric method

Concentration (ng/spot)	Inter-day precision (*n*=3)		Intra-day precision (*n*=3)	
	Peak area (mean±S.D.)	% RSD	Peak area (mean±S.D.)	% RSD
500	1431.33±2.08	0.1454	1432.8±11.45	0.7997
1000	2451±27.51	1.1225	2398.6±60.41	2.5187
1500	3544±46.03	1.2988	3511±30.61	0.8718

TLC – Thin layer chromatography; RSD – Relative standard deviation; S.D. – Standard deviation

Table 3: Recovery studies of luteolin at 80%, 100% and 120% addition by the proposed TLC densitometric method

Amount of luteolin added (ng/spot)	Amount of luteolin found (ng/spot)	Recovery (%)
800	803	100.37
1000	994	99.4
1200	1190	99.16

TLC – Thin layer chromatography

drugs. Moreover, the above-mentioned shortcomings can be overcome by separately developing fractions of different polarity on two or several thin layer plates. Thus, the unique feature of the picture-like image of HPTLC coupled with digital scanning profile is gradually attractive to herbal analysis to construct the herbal chromatographic fingerprint. HPTLC could provide adequate information and parameters for comprehensive identification and differentiation of two closely related herbal medicines.

TLC Densitometric Quantification of Luteolin using HPTLC

There is no report of quantification of luteolin in aerial parts of *C. halicacabum* by HPTLC. Hence, we developed a simple and precise method for quantification of the marker compound. The TLC procedure was optimised with a view to quantify the herbal extracts. The mobile phase Toluene: Ethyl acetate: Formic acid (10:9:1) consisting of gave better, sharp and well-defined peak resolution. The spot at R_f = 0.55 was identified as luteolin with the help of chromatogram of the standard compound. The well-defined spots were obtained when the chamber was saturated with mobile phase for 20 minutes at room temperature. The TLC plate was visualised at 254 nm after derivatization. A photograph of a TLC plate after chromatography of luteolin standard and extract showed the identity of luteolin bands in the sample chromatogram; this was confirmed by the chromatogram obtained from the sample with that obtained from the reference standard solution [Figure 2] and by comparing retention factor of luteolin from sample and standard solution. The peak corresponding to luteolin from the sample solution had same retention factor as that from luteolin standard (R_f = 0.55) [Figure 3].

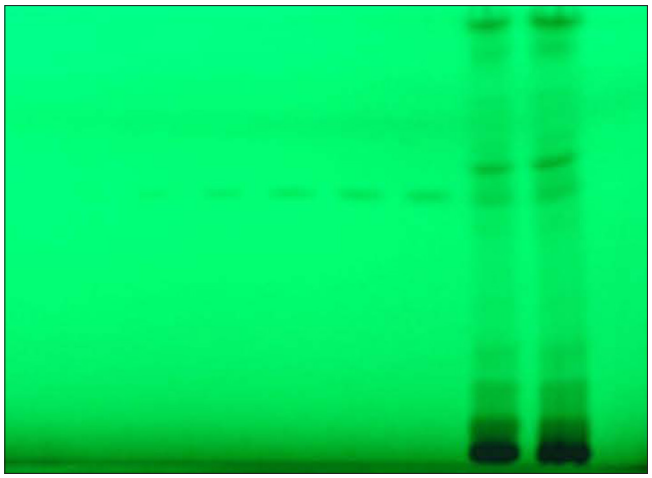


Figure 2: TLC fingerprinting for detection of luteolin in methanolic extract of *Cardiospermum halicacabum*

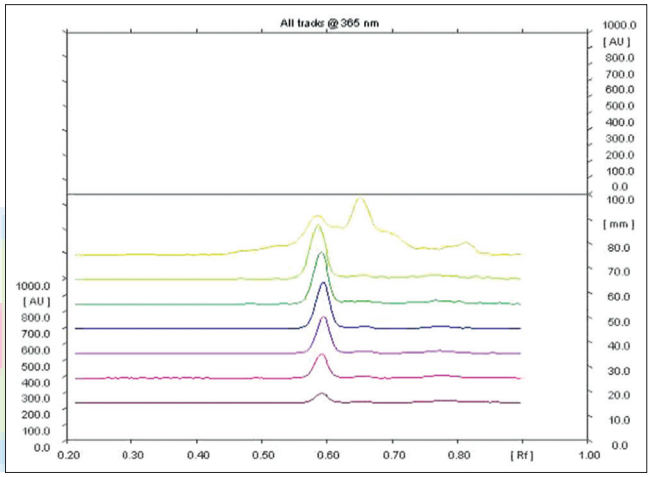


Figure 3: 2D overlaid chromatogram of methanolic extract of *Cardiospermum halicacabum* and standard luteolin AQ1

The TLC densitometric method was validated in terms of precision, repeatability and accuracy [Tables 1-3 and Figures 2-5]. The linearity range for luteolin were 500-3000 ng/spot with correlation coefficient, intercept and the slope 0.997, 523.3 and 1.962, respectively (Y = 1.962X + 523.3). The measurement of the peak area at six different concentration levels showed low values of %CV (<2%) for inter-day (0.14-1.28) and intra-day (0.79-2.51) variation for different concentrations of luteolin which suggested an excellent precision and reproducibility of the method [Table 2]. The limits of detection (LOD) and quantification (LOQ) were 48.94 ng and 148.31 ng, respectively, which indicate the adequate sensitivity of the method [Table 1].

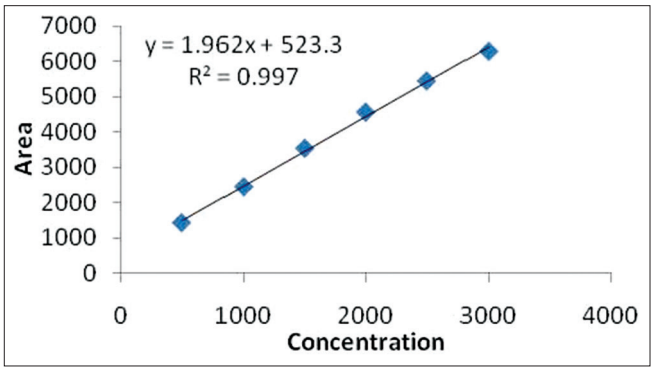
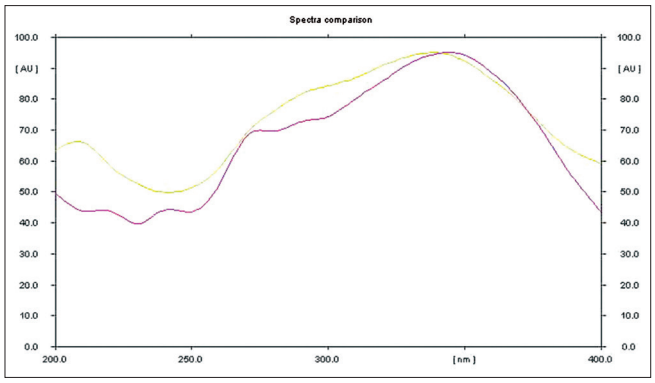


Figure 4: Calibration curve plot for luteolin

Method Validation Parameters for Quantification of Luteolin by using Proposed HPTLC Densitometric Method

Results from recovery studies, listed in Table 2, were within acceptable limits (99.16-100.37%), indicating the accuracy of the method was good. Low % RSD value (between the peak area values) proved the ruggedness of the method, indicating that luteolin is stable during the extraction procedure as well as during analysis. The contents of luteolin quantified using TLC densitometric methods were found to be 0.50% w/w in aerial parts of *C. halicacabum*.



AQ1 **Figure 5:** Overlaid UV/VIS spectrum of standard track and sample track for luteolin

CONCLUSION

A rapid, simple, precise, accurate and specific HPTLC method for quantitative estimation of luteolin present in the aerial parts of *C. halicacabum* has been developed and validated. This was the first time that quantification of luteolin in *C. halicacabum* using mobile phase Toluene: Ethyl acetate: Formic acid (10:9:1) was carried out. The data could be used as a quality control standard. The method used in this work resulted in good peak shape and enabled good resolution of luteolin from other constituents of the plant materials. Because recovery (99.16-100.37%) was close to 100%, there was no interference with the luteolin peak from other constituents present in the plant. The HPTLC images shown in Figure 2 indicate that all sample constituents were clearly separated without any tailing and diffuseness. The activity of a plant extract is always influenced by the quantity of active principle present in the extract. Since luteolin is used in various diseases, it is essential to develop a standardisation method from which one can optimise its quantity in the herbal formulations.

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