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Rapid and sensitive High-Performance Thin-Layer Chromatographic (HPTLC) method for identification and quantification of luteolin by densitometry in Kasamarda (*Cassia occidentalis* L.)

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Kasamarda (*C. occidentalis* L.) is a traditional herb recently recognized as a potential nutraceutical in bone health. The current botanical nutraceutical regulations require consistent standardization for biological applications. The present study reported the standardization of bioactive flavonoid luteolin from *Cassia occidentalis* L. using validated high-performance thin-layer chromatographic (HPTLC) densitometric (DS) method. The mobile phase composition of toluene, ethyl acetate, and formic acid was optimized to separate and identify luteolin using silica gel $60F_{254}$ aluminum plates. The densitometric (DS) scanning was performed at 353 nm. This HPTLC-DS method was further validated as per ICH guidelines. The linearity was 200–700 ng/band with a correlation coefficient value of 0.994. The LOD and LOQ were found to be 54.06 ng/band and 163.84 ng/band, respectively. The recovery (88.38% and 100.72%) and precision (RSD,<5%) indicated method performance is robust and accurate for the routine analysis. Further, this bioactive flavonoid presence was confirmed and quantified by UV-spectrumin the sample matrix using this validated HPTLC-DS method. This HPTLC-DS method was robust, precise and accurate for quality control of active constituents present in *C. occidentalis* L.

Keywords: Densitometry, HPTLC, Luteolin, Quantification

IPC Code: Int Cl.[21]: A61K 9/00, A61K 31/352, A61K 31/7048, A61K 36/00, A61K 36/185, A61K 39/00, A61K 45/06

Cassia occidentalis L. (Family- Caesalpiniaceae) is an annual plant abundant in south Asia and the American region[1]. *C. occidentalis* L. is widely distributed in Asian countries like India, Pakistan, Bangladesh. In India, it is grown throughout the plains in the western and southern regions. *C. occidentalis* L., known as "Kasamarda," has been specified in various nighantus, viz., Dhanwantari, Rajnighantu, Rajballaba, Bhavaprakasa. *C. occidentalis* L. is known as a"famine food" or "edible weed of agriculture"[2,3]. The seeds are brewed into coffee-like refreshments for asthma and a blossoming mixture is utilized to treat bronchitis[3,4]. According to 'Bhavaprakasa,' Kasamarda' (*C. occidentalis* L.) is used in absorptionand stomach sickness[1]. Additionally, it is used in Jamaican society medication to cure dysentery, diarrhea, constipation, fever, cancer, dermatitis and reproductive diseases[5]. The roots are used as a diuretic, tonic and useful in treating tuberculosis and liver disease. The leaves are used in the treatment of urinary tract disorders[1]. In Ayurveda, the extract of *C. occidentalis* L. is used in the treatment of eye inflammation[5]. Bonnisan, an Ayurvedic medicine, contains this plant, used in newborns and infants for discomfort due to gastric wind[3,5]. Recently, Pal and co-workers found that *C. occidentalis* L. has osteogenic activity with anti-resorptive effect glucocorticoid-induced bone loss[7,8].

The *Cassia* species is well-known for the presence of anthraquinones like emodin, physcion and chrysophanol with flavonoids like luteolin, apigenin, vitexin, 7-heteroside of vitexin[9,10]. Flowers reported physcion, emodin and β-sitosterol[1] with new cycloartane triterpenoids and saponins by Li and co-workers[11]. These bioactive flavonoids luteolin (LT), apigenin, 4',7-dihydroxy flavone, 3',4',7-trihydroxy-flavone, isovitexin with emodin, has been isolated from *C. occidentalis* L. aerial parts[7,8].

As the nutraceutical market is emerging, the world is looking for new botanical ingredients. Indian herbs have always been in demand and have potential in therapeutics. Marker compound characterization and biochemical profiling are essential for these

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nutraceuticals. However, the chemical complexity of plant extracts is a challenge for their analysis. Recently, the Food Safety and Standards Authority of India (FSSAI) has issued a new list of botanical nutraceuticals. These contain indigenous, traditional and useful nutritional supplements from India. As per this regulation (schedule-IV), Kasamarda or *C. occidentalis* L. has been approved as a nutraceutical in India[12]. Thus, there is a requirement for the standardization and quantification of bioactive or analytical compounds for future application of this potential nutraceutical.

Many researchers have enlightened luteolin (LT) (3′, 4′, 5, 7-tetrahydroxyflavone) (Fig. 1) chemistry and its pharmacological role in antioxidant, anti-carcinogenic mechanisms. The scientific literature has reported luteolin as a potent anti-inflammatory activity by inhibiting nuclear factor kappa B (NF-kB) signaling in immune cells. In addition, available literature revealed that this bioactive flavonoid prevented bone loss in ovariectomized animal models and anti-inflammatory activity[13-16]. Nash and co-workers discussed that luteolin has potent antioxidant action, which may benefit bone health, suggesting its role in *C. occidentalis* potential bone health properties[7-8,17].

In *Cassia* species, during HPTLC studies and fingerprinting development, luteolin was found as a prominent compound. This chemical fingerprinting is a useful technique for evaluating medicinal plants using marker compounds for identification. However, a literature survey revealed that neither luteolin nor any other flavonoid had been quantitated from different morphological parts (flowers and aerial parts) of *C. occidentalis* L. using the HPTLC technique. Therefore, the HPTLC technique is more suitable for quantifying strong chromophores like luteolin due to eluting fluorescence at 254 and 366 nm for analysis[18].

Therefore, the present study aims for sensitive and robust method development for the standardization of *C. occidentalis* L. This research reports the identification and quantification of luteolin in *C. occidentalis* L. aerial parts and flowers by HPTLC-DS. Further, the method was validated for linearity, precision, the limit of detection and the limit of quantification, specificity, and accuracy as per ICH guidelines for its application in routine analysis of this traditional medicinal plant[19].

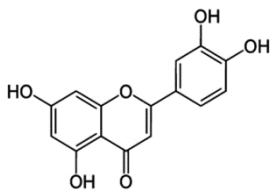

Fig. 1 — Structure of luteolin

Materials and Methods

Reagents and standards

Analytical grade and HPLC grade solvents were used, Silica gel $60F_{254}$ coated, aluminum sheet (Merck, India). Luteolin (LT) (≥98.0% purity) was isolated from methanolic extract of *C. occidentalis* L. in-house by column chromatography and confirmed by LC-MS/MS[1], H NMR, UV and FTIR techniques.

Plant materials

C. occidentalis L. plant was collected in September 2018 from Anand, Gujarat, India. It was identified and authenticated by the Botanical Survey of India, Pune, India, and voucher specimens were submitted (BSI/WRC/100-1/IDEN.CER./2019/26).

Preparation of plant extract

The *C. occidentalis* L. dried flowers and aerial parts were powdered and were soaked in methanol for 24 h using the maceration technique. After this, the material was filtered and the solvent was evaporated using a rotary evaporator. The yield of methanolic extract from *C. occidentalis* L. flowers (COF) and *C. occidentalis* L. aerial parts, leaves and stems (COAL) was 12.98% w/w and 12.30% w/w, respectively. This methanolic extract was subjected to TLC to develop a fingerprint. Different mobile phases compositions using different eluting solvents were used, but the combination of chloroform, ethyl acetate, toluene showed an excellent resolution of spots on the TLC plate.

Preparation of solution

Standard solution

A standard stock solution was prepared by weighing 1.0 mg luteolin reference standard and further, it was dissolved in 5 mL methanol using a sonicator. The final concentration (RS, 0.1 mg/mL) achieved by dilution with solvent and stored this RS at 4-8 °C until analysis.

Serial dilution

The serial dilution of samples from standard solution was prepared to obtain a concentration of 200-700 µg/mL.

Sample solution

The COAL and COF samples were accurately weighed separately and dissolved in 5 mL methanol using a sonicator in separate volumetric flasks. The final volume was made upto 10 mL using methanol (10 mg/mL).

HPTLC instrumentation

The method validation was performed using HPTLC-DS (CAMAG, Switzerland). ATS-4 TLC applicator (CAMAG, Switzerland) was used for a sample application using vision CATS software (version 2.5.18262.1) in the form of bands of width 8 mm. The 100 µL Hamilton Linomat syringe (CAMAG, 695.0014) were used for sample injection[20].

The pre-coated silica gel aluminum plate $60F_{254}$ of 20 X10 cm length with 0.2 mm thickness was used. The CAMAG twin trough glass chamber was used for mobile phase saturation at temperature 23±2°C and relative humidity of 33% and the plates were then dried after development[21].

The DS analysis performed using TLC Scanner 4 (CAMAG, Muttenz, Switzerland) at 353 nm with slit dimension kept at 6.0 X 0.45 mm, with the 20 mm/s scanning speed employed.

These parameters were kept constant throughout the sample analysis. The optimized mobile phase consisted of toluene, ethyl acetate and formic acid in a ratio of 5: 1: 0.5 *v*/*v*/*v*. The parameters are represented in Table 1.

Validation of HPTLC-DS method

The developed analytical method was validated for linearity, precision, accuracy, selectivity, sensitivity and robustness as per ICH guidelines.

Table 1 — Chromatographic conditions

Stationary Phase	Pre-coated activated silica gel plates $60F_{254}$ (Merck, India)
Mobile Phase (v/v)	Toluene: ethyl acetate: formic acid (30:15:1.5*v*/*v*/*v*)
Band Length and Injection volume	8 mm and 10 µL
Development chamber	Twin trough chamber: 20 cm x 10 cm
Pre-saturation	Mobile phase
Saturation time	20 min (20 cm x 10 cm)
TLC Scanner	Camag TLC scanner IV
Run distance	70 mm
Slit dimension for scanning	6.0 X 0.45 mm
Scanning wavelength	353 nm (D2 Lamp)
Measurement mode	Absorbance

Linearity

A series of dilutions of working standard solutions (n=6) were spotted on the TLC plates, covering the 200-700 ng/band range. The bands were spotted on TLC plates for luteolin to determine the linearity.

Specificity

The specificity of the HPTLC method was determined by analyzing reference standard and COAL, COF samples. The luteolin peaks were identified by comparing R_f and UV spectra with reference compounds in COAL and COF samples.

Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) based on signal-to-noise ratio were determined.

Precision

Intraday precision and inter day precision were determined of the same concentration (n=6) of luteolin. Intraday precision within a run is an assessment of precision on the same day during an analytical run. Inter day precision run assessment is on a different day with different analytes and the sample set in the laboratories.

Accuracy

Recovery studies were performed by spiking known amounts of luteolin by standard addition method (n=3) in sample solution at three different levels (80%, 100% and 120%) to determine the method's accuracy.

Selectivity

The selectivity of the analytical method was established by determining the regression coefficient. The luteolin was confirmed by comparing the R_f and spectra in the sample. The UV spectra of corresponding bands in standard and sample track were compared for the regression coefficient at three distinct area levels for luteolin.

Robustness

This developed HPTLC method was tested for itsrobustness using selected variables like (A) run distance, (B) saturation time and (C) detection wavelength.

Quantification of luteolin in *C. occidentalis* L. in flowers and aerial parts extracts

For the quantification purpose, three replicates of samples were subjected to analysis using optimized HPTLC conditions. First, the peak corresponding to

the luteolin in *C. occidentalis* L. was recorded and integrated to get the area under the curve. Then, the mean value of the peak area was inserted in the regression equation to find the luteolin concentration in the sample solution.

Results

HPTLC fingerprinting has been developed for methanol extract of *C. occidentalis* L. with standard luteolin. As per fingerprint, luteolin was present in aerial parts and flower extracts of *C. occidentalis* L.Further, luteolin was quantitated accurately using silica gel $60F_{254}$ HPTLC pre-coated plates using mobile phase toluene: ethyl acetate: formic acid (5:1:0.5, v/v/v). The chromatograms of luteolin and methanol extracts of aerial parts and flowers of *C. occidentalis* L. are shown in Figure 2. The R_f value of luteolin, as shown in the peak, was similar to the extract. The R_f value was optimized at 0.18. The UV spectra of luteolin reference standards were matched with the *C. occidentalis* L. sample at wavelength 353 nm. Quantification of luteolin in aerial parts and flowers of *C. occidentalis* L. by HPTLC technique was not to be found reported. So, this method was further validated for the quantification of the marker compound.

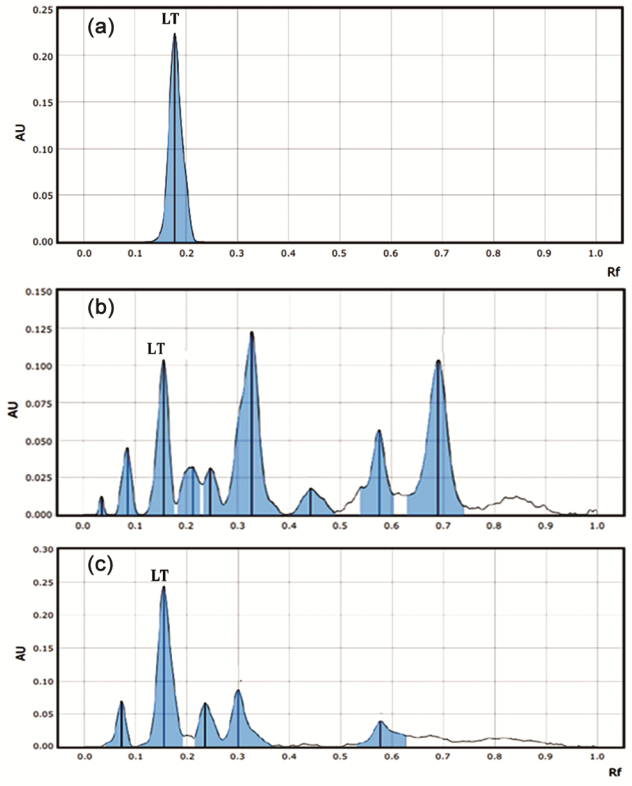

Fig. 2 — HPLTC chromatogram of (a) reference standard luteolin (b) *C. occidentalis* L. aerial parts (c) *C. occidentalis* L. flowers, (LT=luteolin)

An HPTLC chromatogram of luteolin standard and *C. occidentalis* L. extracts confirmed retention factor (R_f = 0.18) in the optimized method. In addition, the HPTLC-DS method was validated for precision, repeatability and accuracy, as represented in Table 2. The linearity was 200–700 ng/band with a regression coefficient value of 0.994 and the linear regression equation was y=1E-05x+0.001.

The methanolic extract of COAL and COF showed a peak with a similar R_f value as the luteolin (0.18) standard, shown in Figure 2a-c. COAL and COF's interday and intraday variations were expressed as a percent relative standard deviation (% RSD). It was found to be 4.12 and 4.37 (% RSD) of interday variations and it was found to be 3.52 and 3.15 (% RSD) of intraday. The regression coefficient (r^2) was found to be 0.994. The method was found to be linear with equation y = 1E-05x +0.001 (Fig. 3). The LOD was found to be 54.06 ng/band whereas LOQ was found to be 163.84 ng/band (n=6), represented in Table 2. The recovery was determined by spiking a known amount of luteolin in COAL and COF samples (n=3) at three concentration levels. The mean average recovery was found for COAL and COF as 88.38% and 100.72%, well within acceptable criteria for validation.

Table 2 — Validation parameters

Validation parameters	Results
Analyte	Luteolin
R_f	0.18
Linear range (ng/band)	200-700
Linear regression equation	y = 1E-05x +0.001
Regression coefficient (r^2)	0.994
LOD (n=6) (ng/band)	54.06
LOQ (n=6) (ng/band)	163.84
Average recovery (%)	
COAL	88.38
COF	100.72
Interday precision (n=6, % RSD)	
COAL	4.12
COF	4.37
Intraday precision (n=6, % RSD)	
COAL	3.52
COF	3.15
Robustness (% RSD)	
Change in run distance	4.14
Change in saturation time	4.94
Change in detection wavelength	3.97

The HPTLC profile of standard luteolin and methanol extract of *C. occidentalis* L. aerial extract and flower extract at wavelength 254 nm and 366 nm is represented in Figure 4a-b, with UV spectra comparison in Figure 5a-b. The identification fingerprinting showed well-separated compounds in both aerial parts and flowers. The luteolin showed resolved band at R_f 0.18 at 254 nm as dark grey colored and at 366 nm as a light grey colored band in reference compound and samples tracks. The quantification showed (n=3) luteolin content in *C. occidentalis* L. aerial (COAL) and flower (COF) parts as 127.0 µg/mg and 1.024 µg/mg, respectively.

Discussion

Kasamarda (*C. occidentalis* L.) is a traditional medicinal herb; it is also an approved botanical nutraceutical in India. It is a potential phytochemical ingredient in bone health application and is needed to establish chemical profiling[7-8]. The quantitative validation and standardization are important to formulate these medicinal plants to potential health benefit products[12,22]. In this research, the HPTLC-DS methodology was reported to quantify and identify flavone, 3',4',5,7-tetrahydroxyflavone [luteolin], from *C. occidentalis* L. The separation and optimization of these compounds were performed on HPTLC and further confirmed by densitometry while addressing R_f and UV spectra at 353 nm. The method performance showed linearity (200–700 ng/band) with a regression coefficient value of 0.994. The sensitive LOD and LOQ were (54.06 and 163.84 ng/band), respectively, as per ICH guidelines. Further, this bioactive flavonoid presence was confirmed and quantified by UV-spectrum in the sample matrix using this validated HPTLC-DS method. This HPTLC-DS method was robust, precise, and accurate for quality control of active constituents present in *C. occidentalis* L.

This study is the first report identifying and quantifying bioactive flavone from *C. occidentalis* L. by the HPTLC technique. Therefore, this HPTLC method could apply as a quality control tool for raw material, extracts and formulation of aerial parts and flowers of *C. occidentalis* L. These analytical parameters confirmed the presence and validated the content of this bioactive flavonoid in this traditional plant for its nutraceutical application[23].

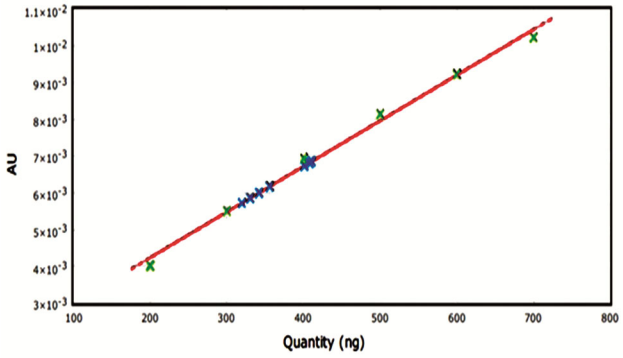

Fig. 3 — Linearity curve of luteolin

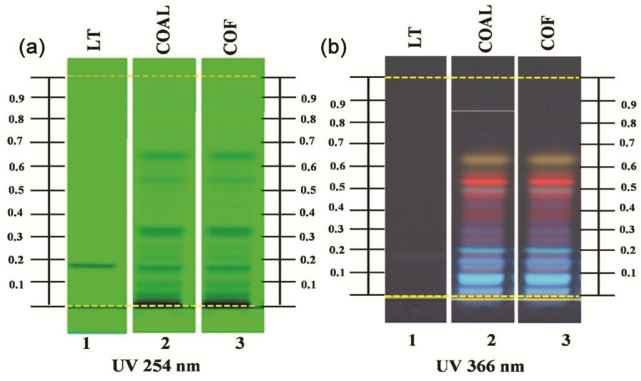

Fig. 4 — HPTLC profile of standard luteolin (LT) and methanol extracts of *C. occidentalis* aerial parts (COAL) and flowers (COF) at (a) UV 254 nm and (b) UV 366 nm

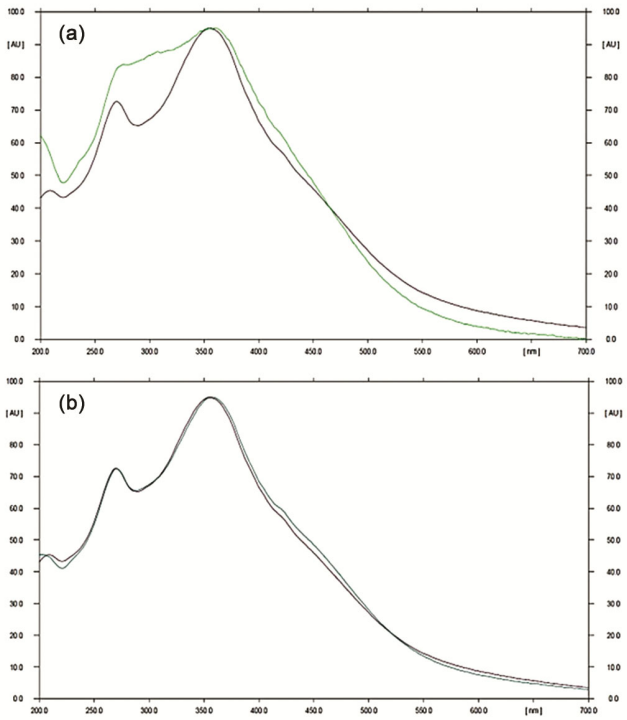

Fig. 5 — Spectral comparison of luteolin in *C. occidentalis* aerial parts (a) and *C. occidentalis* flowers (b)

Conclusions

Luteolin is reported to be one of the major constituents of *C. occidentalis* L. This study reported the confirmation and validation of luteolin from the *C. occidentalis* L. aerial part and flowers using a robust, precise, and accurate HPTLC-DS method with sensitive LOQ for this bioactive. These findings provide scientific evidence to support this traditional herb by establishing the identity and quantity of chemical and bioactive marker present in the plant *C. occidentalis* L. for potential nutraceutical application.

Acknowledgment

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Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

AG: Conceptualization; Methodology; Design; Writing - original draft; Visualization; Supervision; Resources; Project administration; Writing - review & editing. CG: Methodology; Validation; Software; Formal analysis; Writing - original draft. NG: Methodology; Formal analysis; Writing - review & editing. LH: Conceptualization; Resources; Writing - review & editing; Supervision; Project administration; Funding acquisition.

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