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Method development, optimization and validation of RP-UFLC method for bioactive flavonoids from *Cassia auriculata*

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Abstract
Cassia auriculata (CA) Linn (vernacular name: Avartaki,) is a traditional ayurvedic medicine used in the treatment of Diabetes mellitus. The present study was aimed for extraction, isolation, method development, optimization and validation of RP-UFLC method for bioactive flavonoids such as quercetin-3-*O*-rutinoside & quercetin from *Cassia auriculata*. Reverse phase Ultra Fast Liquid Chromatography method was developed with help of UV detector for isolated compounds. Method validation was performed as per international conferenece of harmonization guidelines for system suitability, linearity, accurcay, precision, specificity, robustness, limit of detection and limit of quantification of quercetin and quercetin-3-*O*-rutinoside. Intra-day and inter-day precisions for quercetin 3-O-rutinoside and quercetin was found precise as ≤ 2.0% &< 4.0% in intraday exercise & ≤2.0% &< 3.0% in inter day exercise. The concentration found in *Cassia auriculata* are 0.05 µg/mL of quercetin and 0.16 µg/mL of quercetin-3-*O*-rutinoside. The proposed method for simultaneous estimation of quercetin 3-O-rutinoside and quercetin seems to be accurate, precise, reproducible and repeatable.

Keywords: *Cassia auriculata*, flavonoids, quercetin, quercetin-3-*O*-rutinoside

Introduction
Botanical drugs systematic standardisation is important to identify its bioactive compounds. The traditional and modern scientific data strongly recommends, need to establish the chemical identity of bioactive compounds in natural products. Isolation, identification and analytical quantification techniques must optimise to finalise identity of these compounds in different fractions and extracts. Currently many natural products have been subjected to bioprospecting and quality control for use in different therapeutic area [1]. Botanical drugs have been used in patients with diabetes mellitus (DM) and other complications like diabetic retinopathy, diabetic peripheral neuropathy, dyslipidemia, hypertriglyceridemia etc. Compounds with different structure with different chemical moieties are responsible for the therapeutic activity in the treatment of DM [2]. Further Isolation and identification of compounds can support their traditional claim [3]. Several phytoconstituents such as alkaloids, glycosides, flavonoids, saponins, dietary fibres, polysaccharides, glycolipids, peptidoglycans, amino acids and others obtained from various plant sources that have been reported as potent hypoglycaemic agents [4, 5].

The genus Cassia (Fam: Leguminosae) comprises of 580 species of herbs, shrubs and trees, which are widely distributed throughout the world, of which only twenty species are indigenous to India [6]. According to ayurveda the leaves and seeds are acrid, laxative, antiperiodic, anthelmintic, ophthalmic, liver tonic, cardiotonic and expectorant. The leaves and seeds are useful in leprosy, ringworm, flatulence, colic, dyspepsia, constipation, cough, bronchitis, cardiac disorders [7]. In India, Cassia species is used as a natural pesticide in organic farms. The extracts of Cassia species have been used as a therapy for various skin ailments, rheumatic disease and as laxatives. The plant as a whole has been used as antidiabetic, antidysentric, antimicrobial and for various skin diseases from ancient times. In Ayurveda, a traditional Indian system of medicine, prescribes several herbal plants for health and therapeutic benefits [8, 9]. Among them, *Cassia auriculata* seeds are used to treat various gastrointestinal disorders [10]. The extracts of the flowers and seeds of *Cassia auriculata* have been found to possess antidiabetic activity [11, 12]. In our earlier publication chemical studies have revealed the presence of flavonoids in *Cassia auriculata* [13]. Flavonols, primarily in the form of glycosides, exist in part that is edible within numerous plants including leafy vegetables, many fruits, bulbs and tubers, herbs, spices, tea, and wine [14]. Among these, quercetin has the highest amount of flavonol molecules.

Quercetin is a flavonol and quercetin-3-*O*-rutinoside is a flavonoid glycoside, both belongs to a class of plant secondary metabolites, flavonoids. The reports suggested that flavonoids are molecules capable to interact with more than one target, which allow to define them as privileged structures in accordance with Patchett's definition [15]. Moreover, recent investigations have demonstrated that flavonoids are very promising antidiabetic agents [16]. Currently, the National Institutes of Health Clinical Centre is investigating the use of quercetin on glucose absorption in obesity, and obesity with type 2 diabetes patients on oral glucose tolerance test [17].

Here isolation, identification and quantitation of quercetin-3-*O*-rutinoside & quercetin from Cassia auriculata are done using reversed phase ultra-fast liquid chromatography (RP-UFLC) and validation parameters such as system suitability, specificity, accuracy, precision, linearity, limit of quantification, limit of detection and robustness are performed as per ICH [18].

Materials and methods

Collection and identification of plant material

Seeds were collected from District-Ahmednagar, Maharashtra. The plant material was authenticated at BSI-Pune by comparing morphological features (twigs arrangement, flower etc.) The herbarium of the plant specimen has been deposited at BSI-Pune (BSI/WRC/IDEN.CER/2016/53).

Extraction Procedure

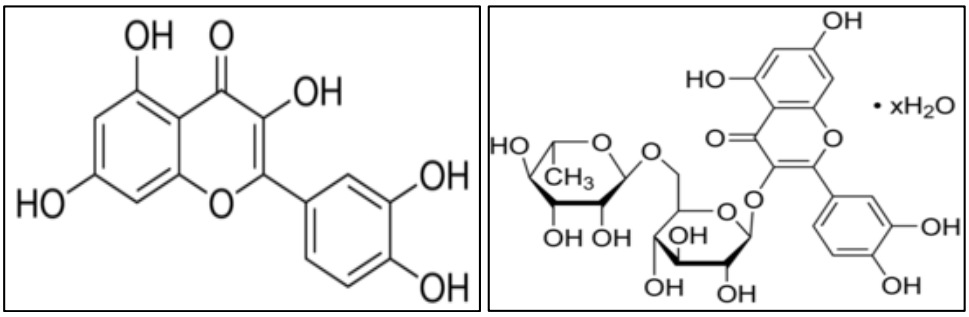
Seeds cleaned off adhering dust and unwanted plant material, shade dried, cut and pulverized (powdered). Pulverized (250 g) were extracted with Ethanol (6 litre × 3 × 14 hours) at room temperature. The ethanol soluble were filtered and concentrated under reduced pressure on rotary evaporator to yield a brownish extract (50 g, 5% based on dry powder weight). The dried extract was successively fractionated in petroleum ether (40.5 gm) [CA-PE], n-butanol, (5.8g) [CA-NB] acetone: methanol 1:1 (26.8g) [CA-AM] and methanol: water 1:1 (21.23g) [CA-MW]. The solvents were chosen for larger delivery of bioactive compounds which are polar and mid-polar.

The CA-NB extract, 5.8g was separated by CC using 25% methanol in ethyl acetate as a mobile phase to collect 20 fractions. Fraction 15 (0.260g) was further separated by CC using 5% methanol in chloroform as a mobile phase to obtain 7 fractions. Fraction 7 (0.360g) was further separated by CC using 5% methanol in chloroform as a mobile phase to obtain 6 fractions. Fraction 2-4 from the former CC were combined with fractions 1-2 from the later (0.0254mg) and subjected to successive preparative TLC in 5% methanol in chloroform as a mobile phase to isolated fraction 6 as CA quercetin (13.3 mg).

Further CA-MW extract, 26.8g was separated by CC using 1% to 10 % methanol in ethyl acetate as a mobile phase to collect 15 fractions. Fraction 5 (0.532g) was further separated by CC using 5% methanol in ethyl acetate as a mobile phase to obtain 6 fractions. Fraction 3 (11.0 g) was obtain CA quercetin-3-*O*-rutinoside (7.8 mg) [19].

Quercetin : Yellow amorphous powder; 1H (DMSO-d4, 500 MHz) δH 7.67 (1H, d, J = 2 Hz, H-2'), 7.53 (1H, dd, J = 8, 2 Hz H-6'), 6.88 (1H, d, J = 8 Hz, H-5'),6.40 (d, J = 2 Hz, H-8), 6.18 (d, J = 2 Hz, H-6). ESI+MS [M + H] + m/z 303.

Quercetin-3-O-rutinoside :Whitish amorphous powder; 1H (DMSO-d4, 500 MHz) δH 7.55 (1H, dd, J = 8, 2 Hz, H-6'),7.54 (1H, d, J = 2 Hz, H-2'), 6.87 (1H, d, J = 8 Hz, H-5'),6.39 (d, J = 2 Hz, H-6), 6.20 (d, J = 2 Hz, H-8), 5.35-3.00 (sugar protons), 1.00 (3H, d, J = 6 Hz). ESI+MS [M + H] + m/z 611.



Quercetin Quercetin-3-*O*-rutinoside

Fig 1: Structures of isolated quercetin-3-O-rutinoside and quercetin.

Ultra fast liquid chromatography (UFLC) analysis

Chemicals & Reagents- Methanol (Rankem, India), Water (double distilled), Formic acid (Loba chemie India). Quercetin was procured from United states pharmacopeia (Maryland, United States, Lot number-7035PO) and quercetin-3-O-rutinoside procured from Sigma-aldrich (Missouri, United States, Lot number-BCBL7548V)

Reverse phase-ultra fast liquid chromatograph instrumentation-Chromatographic analysis was performed using a Shimadzu Nexera X2series UFLC system (Shimadzu, Kyoto, Japan) consisting of a solvent delivery unit (LC-30AD), degasser (DGU-20A_{5R}), Ultra violet (UV) detector (SPD-20A), autosampler (SIL-30 AC) and column oven (CTO-20AC) set at ambient temperature. Chromatographic data was monitored and processed using Shimadzu Lab solution software. Separation was achieved on acquity C18, 100 × 2.1 mm, 1.8µm (Acquity, CA, USA).Stepwise gradient elution was carried out using 0.1% Formic acid in water (solvent A) and Methanol (solvent B) delivered at a flow rate of 0.350 mL/min according to the following program: 0–0.10 min 40% B; 0.10–6.0 min 90% B; 6.0–8.0 min 100% B; 8.0–12.0 min 40% B. 12.0–14.0 min 40% B. The injection volume was 1µL.The UV detector was set to monitor the wavelength of maximum absorption of Quercetin 3-O-rutinoside and Quercetin at 350 nm.

Preparation of standard and sample solution-Quercetin-3-O-rutinoside and quercetin dissolved in methanol to prepare 200 µg/ml solution and 80 µg/ml solution respectively in methanol. From this stock final concentration injected as 40 µg/ml in RP-UFLC each standard. *Cassia auriculata* sample

concentration optimised to 3 mg/ml as a stock solution in methanol. Before injection standards and sample pass through a membrane filter of 0.45µm.

Method validation

The validation parameters such as accuracy, precision, linearity, limit of detection and limit of quantification, Robustness, specificity and system suitability has been evaluated as per ICH guideline [18, 20].

Linearity

Linearity for Quercetin-3-O-rutinoside & Quercetin was plotted from the standard solution for different concentration range of 50- 150 % µg/ml. Linear regression analysis was followed and correlation coefficients (r^2) was used as measure of linearity.

Accuracy

Accuracy is expressed as the closeness of agreement of trueness. Accuracy was evaluated to determine recovery of the standard solution form the sample solutions. Mean percentage (%) recovery of analytes was used as a measure of accuracy. Each sample concentration was injected in triplicate and the percentage recovery was determined. Accuracy was evaluated to determine recovery of the standard solution form the sample solutions. Mean percentage (%) recovery of analytes was used as a measure of accuracy [21].

Precision

Precision of the analytical method is demonstrated by showing System Precision, Method Precision and Intermediate Precision. The level of agreement in between individual test results carried out with differentiation of number of analysis (System precision), different series of analysis (Method precision) and same method and analyte analysis during different time or by different analyst (Intermediate precision). Intraday and Inter day precision was carried out to measure the reproducibility and repeatability of intermediate precision. RSD is calculated utilizing the equation RSD %= (SD/ Mean) × 100, where SD is the standard deviation, and Mean is the mean of UFLC responses [22].

Robustness

Robustness of the method was investigated under a variety of conditions including changes of composition of buffer in the mobile phase, flow rate, extraction time, wavelength and temperature. This deliberate change in the method has no effect on the peak tailing, peak area, and theoretical plates and finally, the method was found to be robust.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD can be defined as the lowest amount of analyte that can be detected but not quantitated. Quantification limit (LOQ) of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy LOD and LOQ can be calculated as per following equation: LOD =3.3 σ/S and LOQ =10 σ/S, where σ is standard deviation of regression line and S is slope of calibration curve.

Specificity

The specificity of the method confirms that main drug peak was not affected by other plant matrix, impurities and it was carried out by comparing the standard retention time and sample retention time.

System suitability

The system suitability summery indicates the system was suitable for performing the validation after analyzing theoretical plates (USP), capacity factor, asymmetry and RSD (relative standard deviation) for RT (retention time) and AUC (area under curve).

Quantification of quercetin-3-O-rutinoside & quercetin in cassia auriculata extract

Applicability of proposed method for the laboratory-based Cassia auriculata extract was quantified for the marker components quercetin-3-O-rutinoside & quercetin. The content of two markers were determined by injecting the prepared laboratory sample as per proposed chromatographic condition.

Results and discussion

A simple method was developed, optimize and validated by ultra-fast liquid chromatography for the analysis of quercetin-3-O-rutinoside & quercetin in *Cassia auriculata* extracts. The composition of the mobile phase was optimised by varying the percentage of solvents in reverse phase column. The optimization was done by different solvent system and operating system. The analytical method was validated as per International Conference on harmonization guidelines. The concentrations of both markers were determined as discussed in inter day and intraday precision (Table 3).

System suitability parameters

After various trials the mobile phase 0.1 % Formic acid in water and methanol with the gradient program optimise for good resolution and sharp peak. The below mentioned chromatogram passed the system suitability parameters such as tailing factor, theoretical plates, capacity factor, and Resolution.

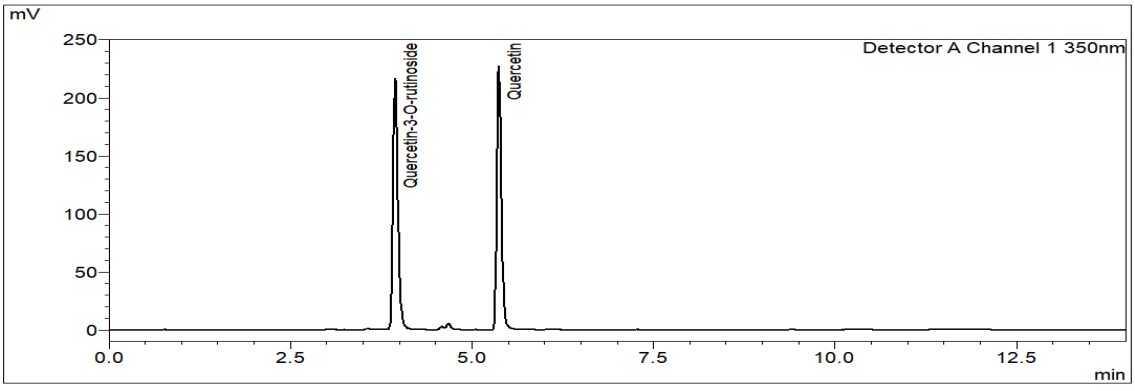


Fig 2: Standard chromatogram of quercetin-3-O-rutinoside and quercetin.

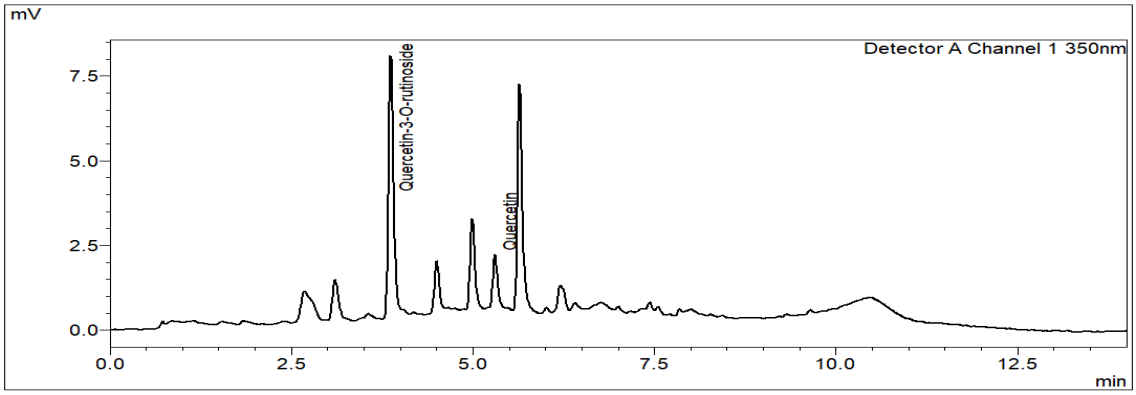


Fig 3: Sample chromatogram of *Cassia auriculata* extract

Table 1: peak symmetry for quercetin-3-O-rutinoside and quercetin.

Name	Retention time	% Area	Tailing factor	Theoretical plate	Resolution
Quercetin-3-O-rutinoside	3.94	51.15	1.266	12967	-
Quercetin	5.37	48.84	1.263	27530	10.65

Linearity, range, LOD & LOQ

Calibration curves were obtained by plotting the peak areas of each analyte against the corresponding concentrations from the mixed standard solutions. Linear regression analysis of the calibration curves shows the assay was linear for all analytes over the concentration range 50–150 µg/mL with correlation coefficients (r^2) >0.994 (Table 2) indicating the response of each analyte is proportional to the concentration of analytes in the test solution. The experimentally determined LOD and LOQ values (Table 2) indicate that the method is sufficiently sensitive for the analysis of extract.

Table 2: Linear regression equation, correlation coefficients, LOD and LOQ of quercetin-3-O-rutinoside & quercetin.

Markers	Retention time	Range	Linear regression equation	r^2	LOD (µg/mL)	LOQ (µg/mL)
Quercetin-3-O-rutinoside	3.95	50-150	9857.4x - 242007	0.9947	0.487	1.476
Quercetin	5.37	50-150	9038.7x - 210694	0.9973	0.619	1.876

Precision

Intra- and inter-day accuracies and precisions (as relative standard deviation RSD) were determined by injecting six replicate samples on the same day and on two different days. Intra-day and inter-day precisions were of quercetin 3-O-rutinoside and quercetin ≤2.0% &< 4.0% in intraday & inter day is ≤2.0% &< 3.0%. The precision values indicate the method is suitable for routine simultaneous quantification of the analytes.

Table 3: Inter day and Intraday precision data.

Marker	Concentration (µg/ml)	Intraday (n=3)	Inter day (n=3)
		Content (µg/ml) ± SD	% Content (µg/ml) ± SD
Quercetin-3-O-rutinoside	50	0.168 ± 0.00035	0.167 ± 0.0025
	100	0.168 ± 0.00090	0.166 ± 0.00065
	200	0.168 ± 0.00035	0.167 ± 0.00055
Quercetin	50	0.0585 ± 0.00026	0.0584 ± 0.0010
	100	0.0591 ± 0.00027	0.059 ± 0.00053
	200	0.0587 ± 0.00024	0.058 ± 0.00024

Accuracy

The recovery of the three analytes at three different concentrations were in the range 93.0–100.0% (Table 4) indicating the method is suitable for routine analysis. No carry-over of quercetin-3-O-rutinoside and quercetin was detected during the validation of the developed method.

Table 4: Recovery of quercetin-3-O-rutinoside and quercetin.

Markers	Concentration Levels (%)	Average recovery %
Quercetin-3-O-rutinoside	50	93.31±0.78172
	100	98.44±0.69255
	150	99.39±0.97166
Quercetin	50	97.76±0.47247
	100	99.20±0.59632
	150	99.37±0.77565

Robustness

For changes in flow rate, column oven temperature, extraction time and detection wavelength, the results showed that the system suitability parameter such as tailing factor, theoretical plates, resolution and% relative standard deviation of RT & Peak area passed the specified limit as per ICH Guideline. Therefore, method is robust.

Conclusion

The developed RP-UFLC method will assist in the standardization of *Cassia auriculata* extract as well as quantification of these markers in raw material and extracts, which may address to inferior quality or adulteration of raw material in it. The proposed UFLC method for simultaneous estimation of quercetin 3-O-rutinoside and quercetin seems to be accurate, precise, reproducible and repeatable. The

validation data indicated that the method was reliable. Here quantification of both markers was done in laboratory-based extract. With the growing demand for botanical drugs and with increased belief in its usage, this standardization tool will help in ensuring the quality and batch to batch consistency of fractions and extracts.

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List of Abbreviations

S. No	Abbreviation	Title
1	CA	*Cassia auriculata*
2	DM	Diabetes mellitus
3	RP-UFLC	Reverse phase Ultra-Fast Liquid Chromatography
4	CA-PE	Petroleum Ether extract
5	CA-NB	n-Butanol extract
6	CA-AM	Acetone Methanol extract
7	CA-MW	Methanol water extract
8	USP	United State pharmacopeia
9	RT	Retention Time
10	AUC	Area under Curve
11	ICH	International Conference on Harmonisation
12	SD	Standard deviation
13	g	gm/Gram

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