Vol. 11, No. 4, 2020
ISSN 2233-4203/ e-ISSN 2093-8950
ARTICLE www.msletters.org | Mass Spectrometry Letters

# Simultaneous Determination of Anthraquinone, Flavonoids, and Phenolic Antidiabetic Compounds from *Cassia auriculata* Seeds by Validated UHPLC Based MS/MS Method

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*Received October 8, 2020; Revised November 28, 2020; Accepted December 6, 2020*
*First published on the web December 31, 2020; DOI: 10.5478/MSL.2020.11.4.82*

**Abstract :** A systematic isolation and characterization study for *Cassia auriculata* (CA) seeds resulted in identifying antidiabetic compounds 1,3,8-trihydroxyanthraquinone and quercetin, quercetin-3-*O*-rutinoside, gallic acid, caffeic acid, ferulic acid, and ellagic acid. The ultra-high-performance liquid chromatography based triple quadrupole mass spectrometry methodology was developed and validated for simultaneous identification and confirmation of these compounds from CA seeds. Multiple reaction monitoring (MRM) based quantification method was developed with MRM optimizer software for $MS_1$ and $MS_2$ mass analysis. The method was optimized on precursor ions and product ions with the ion ratio of each compound. The calibration curves of seven bioactive analytes showed excellent linearity ($r^2 \geq 0.99$). The quantitation results found precise (RSD, < 10 %) with good recoveries (84.58 to 101.42%). The matrix effect and extraction recoveries were found within the range (91.66 to 102.11%) for the CA seeds. This is the first MS/MS-based methodology applied to quantifying seven antidiabetic compounds in CA seeds and its extract for quality control purposes.

**Keywords :** Avartaki, Ayurveda, bioprospecting, emodin, ellagic acid, gallic acid, quercetin-3-O-rutinoside

## Introduction

Bioprospecting is systematic research for the products from nature, having applications in medicine and nutrition. The traditional Ayurveda and Siddha Indian medicines have plant-based remedies which can develop into commercial products with an organized research methodology.[1] In the current regulatory scenario for botanical medicines, standardization plays a crucial role.[2,3]

*Cassia auriculata* (family: Caesalpiniaceae) is an herb potentially identified as Avartaki in Ayurveda texts and Avirai in Siddha traditional medicine from India, used for the treatment of diabetes mellitus (DM).[4-7] The seeds have been quoted in Ayurvedic texts to benefit DM.[8-10] Several other studies have confirmed its antidiabetic potential along with cardioprotective, anti-hyperlipidemic, antioxidant, anti-inflammatory, and anti-cancer properties.[11-17] The phytochemical studies of CA have confirmed anthraquinones, flavonoids, and triterpenes in it.[18-20] However, there is no analytical methodology studying chemical fingerprinting and quantitation of bioactive responsible for DM activity. The accuracy and validation data are necessary for specific and bioactive analytical markers for these plant-based drugs for consistency in the formulation, preclinical/clinical studies. Mass spectrometry is a well-known technique that holds applications in analyzing complex natural products like traditional medicines with its confirmative and quantitative criteria.

Therefore, in this research, a systematic bioprospecting study of CA seeds was carried out. The extraction, fractionation, and identification of seven antidiabetic molecules were identified, and a quantitative UHPLC-MS/MS methodology was developed for the simultaneous determination of these bioactive compounds. The presence of compounds was confirmed with novel triggered MRM (tMRM) data in different extracts of seeds in CA. This

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method further validated linearity, precision, recovery with matrix effect, and extraction recoveries in CA seeds extract as per the guidelines.

## Experimental

### Plant material

The whole, healthy, CA seeds from the dried pods were collected from the Shrirampur area (MH, IN), the herbarium was deposited in the Botanical Survey of India, Pune. (BSI/WRC/IDEN.CER/2016/53).

### Isolation of bioactive compounds

The isolation and identification of bioactive compounds from the CA seeds were carried out. For the isolation study, the 500 g seeds were separated, shade dried, pulverized, and used for fractionation. Powdered seeds were extracted with solvents at room temperature by successive extraction in order; petroleum ether, *n*-butanol, acetone: methanol (1:1), and methanol: water (1:1). The solvents were chosen for more extensive delivery of bioactive compounds, polar and mid-polar.[19] The four individual successive fractions were filtered and concentrated under reduced pressure to yield four different fractions as; petroleum ether (yield, 40.5 g), *n*-butanol (yield, 5.8 g), acetone: methanol (1:1) (yield, 26.8 g), and methanol: water (1:1) (yield, 21.23 g). Further, these fractions are used for column chromatography (CC) for the isolation of compounds.

### Column chromatography and identification of compounds in *Cassia auriculata* seeds

The CC was performed on the four fractions collected from the previous section using gradient elution followed by preparative TLCs, if required, to isolate and confirm the compounds. The CC was performed using silica gel mesh 230–400 (Thomas Baker, Ltd., Mumbai, India), TLC plates purchased from Merck Ltd. (Whitehouse Station, NJ, USA).

The *n*-butanol fraction (5.8 g) was separated by CC using 25% methanol in ethyl acetate as an eluent and finally washing with 100% methanol to collect 20 fractions. Fraction 15 (0.260g) with successive preparative TLCs in 5% methanol in chloroform as an eluent used to isolate 6-methyl-1,3,8-trihydroxyanthraquinone (emodin, EM) (23.3 mg). Fraction-16 (0.360g) was further separated by CC using 7% methanol in chloroform as an eluent to obtain six subfractions with subfraction (16-2) as quercetin (QT) (13.3 mg).

The methanol: water (1:1) fraction (21.2 g) was separated by CC using 1% to 10 % methanol in ethyl acetate as an eluent to collect 15 fractions. Fraction 5 (0.532g) was further separated by CC using 5% methanol in ethyl acetate with 0.1 mL water per 100 mL as an eluent to obtain six fractions. Fraction (5-3) was found to be quercetin-3-*O*-rutinoside (rutin, RT) (7.8 mg). Later, fractions 4-6 yield gallic acid (GA) (5.4 mg), caffeic acid (CF) (6.4 mg), ferulic acid (FA) (2.3 mg). Fraction 7-8 yields ellagic acid (EA) (3.5 mg) in 30% methanol in ethyl acetate elution

The $^{1}H$ and $^{13}C$ NMR spectra were measured using a Bruker Avance III Ultra Shield NMR instrument ($^{1}H$: 400 MHz, $^{13}C$: 100 MHz, Billerica, MA, USA) and AVANCE 500 MHz Bruker NMR ($^{1}H$: 499.99 MHz,$^{13}C$:124.99 MHz). LC-ESI/MS were recorded with a Waters Acquity LCMS instrument and Agilent LC-MS/MS-6495 instrument. The UV and IR spectra were recorded on Shimadzu 1700 UV spectrophotometer and FTIR-ECO-ATR (Bruker, optic).

## UHPLC-MS/MS Analysis

### Reagents and chemicals

The reference standards of GA, CF, FA, EA, RT as rutin hydrate were purchased from Sigma-Aldrich (USA), QT from USP (USA), EM from TCI (India), and water, formic acid with methanol (MS grade) were purchased from JT Baker (India)

### Preparation of CA extract

The seeds of CA were washed, dried, and crushed. The grounded raw material (100g) was extracted with methanol and water (1:1) (0.6 L) (CA-MW) at 55 ± 5°C using the reflux method for 3 hrs. After filtration, the obtained filtrate was concentrated and dried using a rotary evaporator at 60 ± 5°C and weighed. The extract was stored at 4-8°C for further use.

### Sample Preparation and standard solutions

An accurately weighed amount of 3.0 mg/mL of CA-MW extract and 0.1 mg/mL of reference standards solutions were dissolved in methanol. The solutions were dissolved by sonication, centrifuged at 3000 rpm for 15 min, and passed through a membrane filter of 0.45 μm before injecting into the LC system.

### Instrumentation

The analysis of CA seeds was performed on the Agilent 1290 Infinity II UHPLC system consisting of a high-pressure binary pump (G7120 A), a multi sampler with a multi wash option (G7167B), multi-column thermostat (G7116B), and DAD detector (G7117B). The DAD detector was serially connected to the nebulizer of the MS source. The MS analysis was performed on Agilent LC-MS/MS-6495 in ESI mode using the Agilent AJS-ESI source. The multiple reaction monitoring (MRM) method was developed with MRM optimizer software with MassHunter Acquisition software (Version B.07.01).; the unit mass resolution was set for both MS1 and MS2 mass analyses. The mass resolution was set at 0.05 FWHM and resolving power Rp (1,000-3000). Retention time

scheduled dynamic MRM mode was used to activate triggered MRM (tMRM) features to acquire up to ten MS/MS transitions for each analyte, generating a product ion spectrum of all analytes of interest. The acquisition was helpful for a complete set scan for the primary transitions.[22]

Nitrogen from the nitrogen generator was used as a nebulization gas, sheath gas, drying gas, and collision gas. The capillary voltage was set at 2000 V, and nebulizing gas, sheath gas flow, and heated gas flows were 50 L/min, 10 L/min, and 12 L/min. Sheath gas temperature and heated gas temperature were kept at 250 and 225 °C, respectively.

**Chromatographic conditions**

UHPLC separation was carried out on a Shimadzu C18, Shim-pack (150 mm long; 4.6 mm internal diameter; 5 μm particle size) column with temperature 30°C. The mobile phase consisted of 0.1% formic acid solution (A) and acetonitrile (B) with a flow rate of 0.8 mL/min under the gradient program from 5-55% (B), 40 min, 55-100% (B), 50-52 min, 100-55% (B), 52-54 min, 5% B, 56-60 min with injection 5 μL volume. The DAD recorded UV spectra in the range 190–640 nm and was set to monitor at 272, 320, 438, and 210 nm. Confirmation criteria were source parameter optimization and the flow rate, and the eluent composition.[22]

**UHPLC-MS/MS optimization and validation**

The sample matrix extraction process was optimized with the solvents like dimethyl sulfoxide, acetonitrile, water, and methanol for the analysis. The parameters chosen for optimization were solubility, sonication, centrifuge speed (rpm), and time (min) for rapid extraction. Finally, the rapid liquid extraction method with methanol followed by 10-15 min sonication and centrifuged at 3000 rpm for 15 min was optimized to reduce non-analytes concentration and noise. Different systems of mobile phases using different compositions of solvents at different flow rates and column temperatures experimented with obtaining well-separated and appropriate ionization with improved peak shape and restraining the peak tailing.

For the optimization of MS conditions, each targeted CA analyte was infused into the MS, and MS spectra were recorded in both positive and negative ionization modes. During tuning (Q1 and Q3 scan), compound dependent MRM parameters: precursor ion (PRE) (*m/z*), product ion (PRO) (*m/z*), collision energy (CE) (eV), PRE to PRO ratio were optimized for each targeted analyte. Therefore, along with retention time and UV spectra, this information is used as a confirmative criterion to achieve the most abundant and stable MRM (Table 1). The targeted CA analytes in the samples were identified with the comparison of reference compounds, which further validated according to the guidelines of the International Conference on Harmonization (ICH, Q2R1).[23]

**Application of UHPLC-MS/MS method in the quantification of bioactive compounds in CA seeds extracts**

The different CA seeds extracts were prepared and quantified by this UHPLC-MS/MS method to compare with the traditional form of ayurvedic extract (hydroalcoholic, CA-MW). The seeds were separately extracted with petroleum ether ([CA-PE], *n*-butanol, [CA-NB], and acetone: methanol [CA-AM] as per the material and methodology section. An accurately weighed amount of 3.0 mg/mL of CA -NB, CA-AM, CA-PE extract solutions were prepared in methanol. The solutions were dissolved by sonication, centrifuge at 3000 rpm for 15 min and passed through a membrane filter of 0.45 μm before injecting into the LC system. Further, the dilution was done

**Table 1**. UHPLC-MS/MS optimization in positive (+) mode as retention time (rT), UV maximum spectrum (nm), mono-isotopic Mass (Da), precursor ion (PRE) (*m/z*), product ion (PRO) (*m/z*), MS/MS transitions (*m/z*) induced by t-MRM, collision energy (CE) (eV) and PRE to PRO ratio.

| Analyte | Molecular Formula | rT | UV (nm) | Mass (*m/z*) | PRE | PRO | MS/MS transitions | CE | PRE to PRO ratio |
|---|---|---|---|---|---|---|---|---|---|
| Gallic acid | $C_7H_6O_5$ | 6.339 | 270 | 170.2 | 171.1 | 153.1 | 153.1, 127.1, 109.2, 81.1, 51.1 | 20.0 | 45.3 |
| Caffeic acid | $C_9H_8O_4$ | 14.627 | 319 | 180.57 | 181.1 | 162.9 | 162.9, 145.0, 117.0, 89.2, 77.1, 63.2, 51.2, 39.3 | 30.0 | 61.1 |
| Ellagic acid | $C_{14}H_6O_8$ | 18.332 | 262 | 302.00 | 303.1 | 229.1 | 285.0, 256.9, 229.1, 201.0, 172.9, 144.9, 89.2, 63.0 | 40.0 | 46.8 |
| Quercetin-3-*O*-rutinoside | $C_{27}H_{30}O_{16}$ | 18.337 | 362 | 610.15 | 611.1 | 302.9 | 464.9, 302.9, 129.0, 84.9 | 20.0 | 65.9 |
| Ferulic acid | $C_{10}H_{10}O_4$ | 19.649 | 310 | 194.05 | 195.1 | 176.9 | 176.9, 145.0, 136.9, 117.0, 108.1, 89.1, 77.0, 63.1, 45.2 | 10.0 | 69.4 |
| Quercetin | $C_{15}H_{10}O_7$ | 28.117 | 375 | 302.23 | 303.1 | 69.0 | 228.9, 153.1, 136.9, 127.0, 77.0, 69.0, 51.2, 41.2 | 40.0 | 46.3 |
| Emodin | $C_{15}H_{10}O_5$ | 45.159 | 253 | 270.05 | 271.3 | 115.1 | 225.1, 196.9, 168.9, 140.9, 115.1, 91.2, 69.0 | 40.0 | 48.0 |

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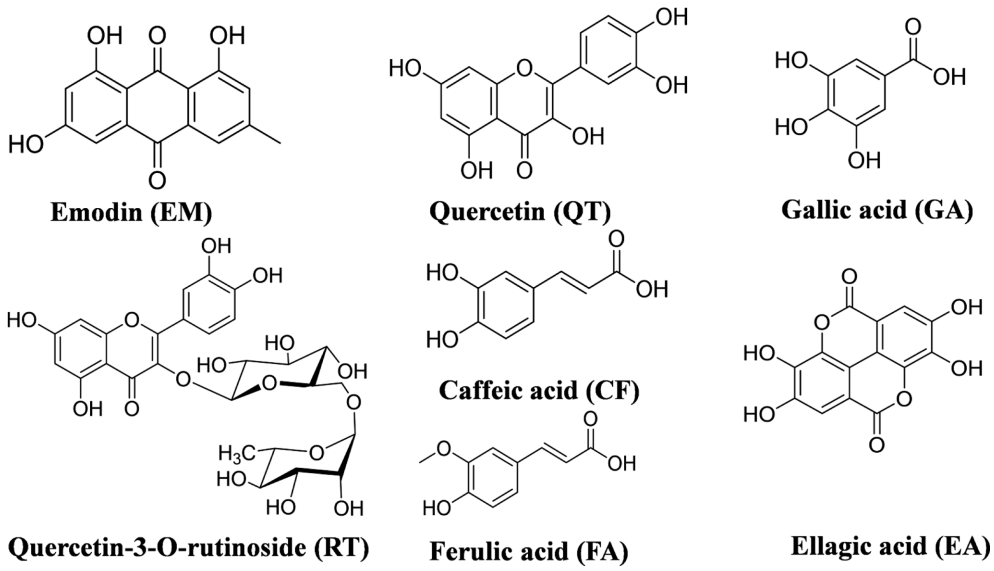


**Figure 1.** Active compounds of *Cassia auriculata* extract.

as per need in the working range of concentration. The quantification of EM, QT, RT, GA, CF, FA, and EA in it along with CA-MW extract by a validated method.

## Results and Discussion

### Bioactive compounds from CA seeds

The CC and preparative TLC has yield two compounds from *n*-butanol fraction as EM (23.3 mg) and QT (13.3 mg).[21] The hydroalcoholic (methanol: water, 1:1) fraction resulted in the isolation of RT (7.8 mg), GA (5.4 mg), CF (6.4 mg), FA (2.3 mg), and EA (3.5 mg).[18,21] Chemical structures are shown in Figure 1. Further, the identification of the compounds performed with the supports of $^{1}H$ and $^{13}C$ NMR, MS/MS, UV, and IR data.

### Determination of anthraquinone, flavonoids, and phenolic antidiabetic compounds in CA seeds

The importance of analytical science and its required application in the evidenced-based Ayurveda is often stressed.[21] The techniques like TQ-MS/MS have applicability in identifying, confirming, and quantifying chemical or bioactive markers from ayurvedic medicines. The selection of these specific analytical or chemical markers plays a key role in the plant-based MS/MS fingerprinting. Thus, systemic bioprospecting research is a guide to isolate, identify, and quantify the bioactive plant compounds.

The isolated compounds from CA have been found to have moderate to potent antidiabetic potential as a single component and in combination. EM, QT, RT, GA, CA, FA, and EA are biochemical markers, exhibited significant antidiabetic activity.[24-30] Thus, this isolation data confirms the possible antidiabetic activity and potential of seeds of CA.[1,9,10,13]

The regulations of plant-based therapeutics in Ayurvedic and herbal medicines suggest the requirements of quantitative and confirmative data for analytical or bioactive markers for the development of standardized products.[2,3] In this study, we have developed and optimized UHPLC-MS/MS methodology for CA using multiple reaction monitoring (MRM) technique for isolated biomarkers.[31-35]

The tMRM data was collected as multiple transitions with retention for each analyte showed a product ion spectrum with the primary transitions (Figure 2).[22] The confirmation criteria were retention time, precursor ion, product ion, and a precursor and product ion ratio with the UV spectrum (Supplementary information 1).

### Identification and confirmation of analytes by MS/MS

For MS analysis, an Agilent LC-MS/MS-6495 mass spectrometer coupled with an ESI mode using an Agilent AJS-ESI source utilized to quantify EM, QT, RT, GA, CF, FA, and EA. In the optimization of mass spectrometer conditions, analytes were analyzed by a full scan in the ESI mode, and the ion intensity was relatively high in positive mode. In the $[M+H]^+$ mode, the most abundant *m/z* was 171.1, 181.1, 303.1, 611.1, 195.1, 303.1, and 271.3 for GA, CF, EA, RT, FA, QT, and EM, respectively. The most abundant and stable product ions in the Q3 MS spectra were observed at *m/z* 153.1, 162.9, 229.1, 302.9, 176.9, 69.0, and 115.1, respectively. The product ion spectra of analytes have been shown in Figure 2. In conclusion, the predominant transitions at *m/z* 171.1/153.1 for GA, *m/z* 181.1/162.9 for CF, *m/z* 303.1/229.1 for EA, *m/z* 611.1/

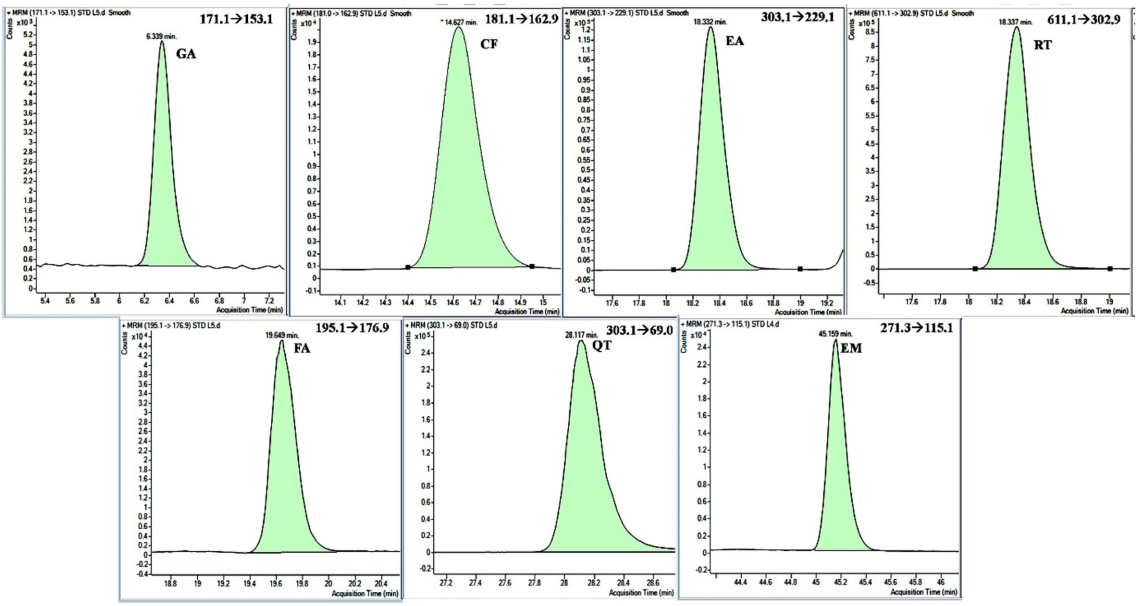


**Figure 2.** Typical Multiple Reaction Monitoring (MRM) chromatograms and MS/MS spectrum of seven components in *Cassia auriculata* seeds extract.

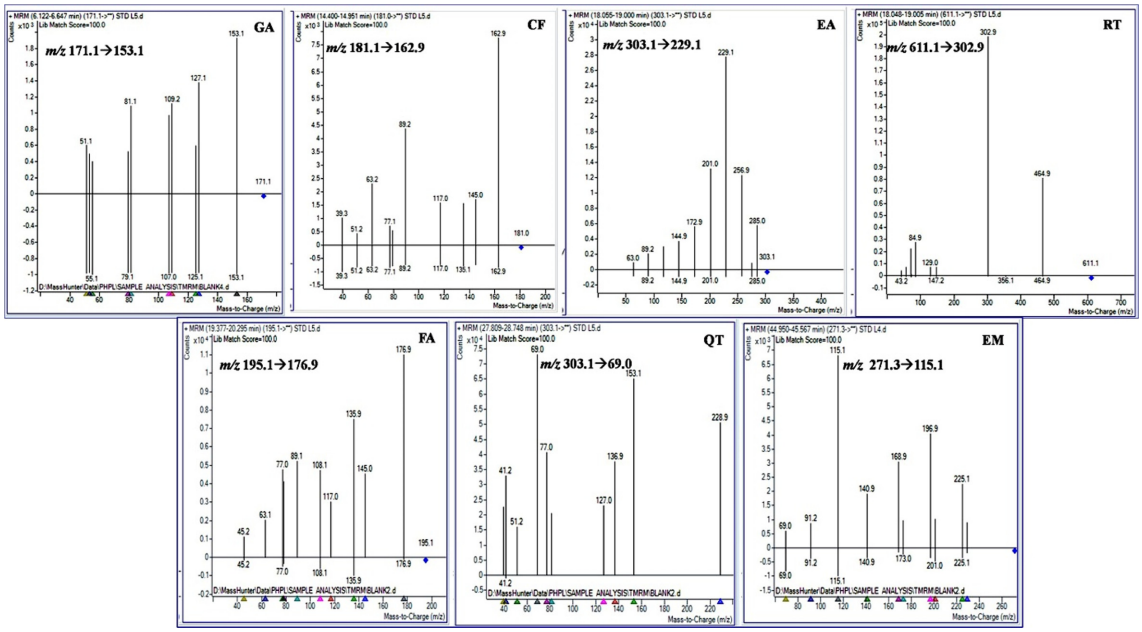


**Figure 3.** Product ion scan mass spectra of isolated compounds from *Cassia auriculata* extract using triggered MRM (t-MRM).

302.9 for RT, *m/z* 195.1/176.9 for FA, *m/z* 303.1/69.0 for QT, *m/z* 271.3/115.1 for EM were utilized in MRM scan mode (Table 1).[36-39]

## Method validation

The instrumental parameters were optimized, as shown in Table 1 for seven phytochemicals from CA seeds. The specificity and maximum response were achieved in a positive mode with retention time (rT), and PRE (*m/z),* PRO (*m/z*). The MS/MS transitions (*m/z*) induced by t-MRM were also generated, and compound databases were created (Figure 3).

In the validation of the developed method, calibration curves with linearity, LOD, and LOQ were studied in the range of 0.15–20 mg/kg. The LOD and LOQ values were determined based on the calibration curve method in which

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**Table 2**. UHPLC-MS/MS method validation parameters of the limit of detection (LOD), the limit of quantification (LOQ), linearity, intra-day day precision, and inter-day precision ($n$ = 6) (%, RSD) with recovery ($n$ = 6) mean ± SD.

| Analyte | LOD (mg/kg) | LOQ (mg/kg) | Linearity co-efficient ($r^2$) | Intra-Day precision (%) | Inter-day precision (%) | Mean recovery ±SD (%) |
|---|---|---|---|---|---|---|
| Gallic acid | 0.513 | 1.538 | 0.9930 | 1.2 | 2.6 | 101.42±2.13 |
| Caffeic acid | 0.174 | 0.522 | 0.9979 | 2.5 | 5.3 | 87.57±2.95 |
| Ellagic acid | 0.224 | 0.672 | 0.9986 | 3.9 | 4.2 | 95.87±1.86 |
| Quercetin-3-*O*-rutinoside | 0.155 | 0.465 | 0.9976 | 4.7 | 4.2 | 98.12±1.89 |
| Ferulic acid | 2.009 | 6.027 | 0.9920 | 5.8 | 5.6 | 84.58±3.26 |
| Quercetin | 0.201 | 0.603 | 0.9930 | 5.6 | 4.5 | 98.14±2.74 |
| Emodin | 1.882 | 5.646 | 0.9916 | 4.5 | 2.4 | 96.15±2.45 |

**Table 3**. Matrix effect, and extraction efficiency, mean ± SD with precision (%, RSD) ($n$ = 6) with accuracy ($n$ = 6).

| Analyte | Nominal concentration (mg/kg) | Matrix Effect | Precision (RSD, %) | Accuracy (RE, %)* | Extraction efficiency | Precision (RSD, %) | Accuracy (RE, %)* |
|---|---|---|---|---|---|---|---|
| Gallic acid | 0.15 | 96.33 ± 2.08 | 2.84 | -5.21 | 99.81 ± 4.50 | 5.23 | -11.19 |
| | 20 | 100.83 ± 3.33 | 5.32 | -6.27 | 97.32 ± 2.54 | 8.69 | -10.65 |
| Caffeic acid | 0.15 | 96.47 ± 3.69 | 6.51 | 2.95 | 91.78 ± 3.65 | 2.98 | 2.19 |
| | 20 | 98.33 ± 3.85 | 7.52 | 5.32 | 94.47 ± 2.84 | 4.78 | 4.79 |
| Ellagic acid | 0.15 | 92.96 ± 1.84 | 6.21 | -11.52 | 99.37 ± 3.92 | 4.44 | 5.46 |
| | 20 | 92.18 ± 3.11 | 5.27 | -10.50 | 95.12 ± 4.18 | 6.32 | -1.18 |
| Quercetin-3-*O*-rutinoside | 0.15 | 92.45 ± 3.44 | 8.42 | 5.14 | 99.23 ± 1.74 | 8.65 | -4.90 |
| | 20 | 95.46 ± 4.01 | 8.21 | 8.65 | 95.60 ± 4.00 | 9.12 | 1.04 |
| Ferulic acid | 0.15 | 93.28 ± 4.14 | 4.69 | 1.12 | 95.18 ± 3.13 | 9.35 | 6.637 |
| | 20 | 97.10 ± 2.49 | 4.89 | 2.58 | 91.66 ± 2.80 | 6.25 | 9.1 |
| Quercetin | 0.15 | 98.77 ± 3.53 | 5.21 | 1.98 | 102.11 ± 2.80 | 10.25 | 6.38 |
| | 20 | 97.39 ± 3.78 | 5.98 | 7.12 | 93.46 ± 4.55 | 8.25 | 5.98 |
| Emodin | 0.15 | 99.55 ± 3.40 | 6.24 | -5.12 | 93.63 ± 3.82 | 10.65 | -4.09 |
| | 20 | 94.59 ± 3.23 | 6.98 | -3.98 | 99.60 ± 1.57 | 7.98 | -9.15 |

*Pre- and post-spike set of experiment matrix effect with blank.

LOD was calculated as 3.3 σ /S, where σ is SD of response, and S was the slope of a calibration curve as per the ICH guidelines. The analytes showed excellent linearity with correlation coefficients ($r^2$) from 0.99155-0.99860 with a sensitive LOQ (0.465 to 6.027 mg/kg).

The interday and intraday precisions indicate the variations to determine the reproducibility of the method. This reproducibility was investigated by quantifying seven analytes in the six replications during a single day and repeating the experiments on three consecutive days. The percentage relative standard deviations (RSDs) were calculated ($n$ = 6), which were found not more than 5.8 %. The recoveries were applied to evaluate the accuracy of quantification for the method by adding a known concentration of the seven reference standards at the (50-150%) levels into the CA-MW sample in triplicates, and the mean average recoveries were calculated. This validated method showed good recovery in the range from 84.58 ± 3.26 to 101.42 ± 2.13 % (Table 2).

A simple and stable liquid extraction method using methanol was used to minimize the CA matrix's interference. The accuracy and matrix effect was achieved with methanol as a solvent for MS/MS analysis. The use of methanol enhanced target analytes recovery and efficiency in the current analytical investigation. The matrix effect was demonstrated by using a matrix factor calculated by dividing the analyte's peak response in the presence of a matrix by the peak response in the absence of a matrix ($n$ = 6). The results were found between 92.18 ± 3.11 to 100.83 ± 3.33%.

The carryover was studied by comparing the response alternatively in a blank following a high concentration standard ($n$ = 3). The total response was noted and monitored for not exceeding 20% of LOQ. The results of the extraction recoveries of the seven compounds are listed in Table 3. The mean extraction recoveries of the analytes in the solvent at three concentration levels were calculated to be 91.66 ± 2.80 to 102.11 ± 2.8 %.

**Table 4**. Quantitation (n=3) data for seven chemical markers mean±SD (mg/kg) by UHPLC-MS/MS methodology of CA extracts in petroleum ether n-butanol, acetone: methanol, and methanol: water as CA-PE, CA-NB, CA-AM, CA-MW.

| Analyte | CA -NB | CA -MW | CA-AM | CA-PE |
|---|---|---|---|---|
| Gallic acid | 356.34 ± 8.082 | 1500.28 ± 18.487 | 1471.36 ± 14.589 | 356.34 ± 10.427 |
| Caffeic acid | 17.31 ± 1.014 | 57.63 ± 2.587 | 95.14 ± 10.518 | 17.31 ± 0.985 |
| Ellagic acid | 123.3 ± 5.482 | 534.15 ± 8.789 | 601.28 ± 20.451 | 123.3 ± 10.248 |
| Quercetin-3-o-rutinoside | 78.84 ± 3.578 | 56.38 ± 2.658 | 45.15 ± 2.687 | ND |
| Ferulic acid | 18.32 ± 1.047 | 87.69 ± 3.874 | ND | 18.32 ± 1.248 |
| Quercetin | 2.46 ± 0.274 | 29.12 ± 1.984 | 30.58 ± 3.245 | ND |
| Emodin | 15.1 ± 0.985 | 100.25 ± 2.134 | 112.65 ± 12.458 | ND |
| Total | 611.67 ± 12.62 | 2365.5 ± 100.258 | 2356.16 ± 110.58 | 515.27 ± 80.562 |

ND= Not detected at LOD level.

**Quantification results of CA seeds extracts**

Amongst CA -NB, CA-AM, CA-PE, and CA-MW extract, the CA-MW extract showed the highest total content of all bioactive compounds. The CA-PE extract showed no presence of anthraquinones and flavonoids but only phenolic acids. Similarly, CA-NB extract showed less content of all seven phytochemicals compared to polar extracts. The highest EM content was found in CA-AM extract, while phenolic acids and flavonoids were found prominently present in CA-MW extract (Table 4).

The quantification results could not distinguish the different non-polar and polar fractions of CA seeds. Still, amongst all results, EM was linked to differentiate these fractions in a calculable manner. Therefore, this methodology can be used for products, enriched extracts, or formulations based on this Ayurvedic plant to quantify these seven antidiabetic compounds.

## Conclusions

The regulations require robust analytical standardization in any Ayurvedic or herbal product with bioactive or chemical markers from known or novel identity.[40] Therefore, the complex and multicomponent traditional and herbal extracts and products need validated analytical methodology to connect the presence and quantification of bioactive compounds.[2,3]

This research has reported the isolation of seven antidiabetic compounds from anthraquinone, flavonoids, and phenolic class in CA seed extract. A sensitive and robust UHPLC-MS/MS was developed on the CA matrix for these seven antidiabetic compounds' simultaneous determination. The method performance showed the selectivity, reliable sensitivity (LOQ from 0.465 to 6.027 mg/kg), an acceptable range of precision (lower than 10.0%), and recoveries (from 84.58 to 101.42%) with novel tMRM data. The MRM based assay provided high selectivity and sensitivity, which was successfully applied for the simultaneous quantitative analysis of seven bioactive constituents in CA. Thus, this research discloses the comparative quantitative analysis and the first identification study of these bioactive compounds present in CA seeds extract. The results indicated that phenolics (GA, EA), and EM were the major constituents in all extracts. The traditional Ayurvedic hydroalcoholic extract showed the highest content of seven antidiabetic bioactive compounds, indicating its potency in DM. [9-10,13] This method could help to develop standardized *Cassia auriculata* seeds and its extract for antidiabetic applications under the regulatory requirements.

## Supporting Information

Supplementary information is available at https://drive.google.com/file/d/1Fkl6x2oeuSjvFLiAQwfBcYBLbP8vhELs/view?usp=sharing.

## Acknowledgments

This research was partially conducted at CSIR-NCL, Pune. We thank our colleagues Dr. Roshan Kulkarni (Chungnam National University, South Korea), Mr. Tophik Naikwadi, and Mr. Madhukar Said (CSIR-NCL, India), for providing insight and expertise that greatly assisted the research. The authors would like to acknowledge Agilent Technologies, Bangalore, for the data processing for the MS-MRM study. Also, the authors acknowledge Dr. Vijay Thawani (Director, Centre for Scientific Research and Development (CSRD), India) for expert assistance in reviewing the manuscript.

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