Identification and quantification of cinnamic acid derivatives in *Cichorium intybus* seed and its extract by High-Performance Liquid Chromatography with Diode-Array Detector (HPLC-DAD) and Electrospray Ionization Mass Spectrophotometry (LC-MS/MS)

Devendra Reddy, Chennu Surendra, I. Bindu, L. Sharath,
***N. S. Prakash., R. Sundaram and U.V Babu**
R&D Centre, Himalaya Wellness Company, Makali,
Bengaluru – 562162, India
*E-mail: devendra.reddy@himalayawellness.com

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Abstract- A sensitive method coupling high-performance liquid chromatography (HPLC) with diode –array detector (DAD) and electrospray ionization mass spectrometry (MS) was optimized for separation, identification, and quantification of cinnamic acid derivatives in *Cichorium intybus* seed and its extract. Cinnamic acid derivatives such as chlorogenic acid, caffeic acid and Chicoric acid were quantified using respective standards. Apart from 4-o-Caffeoylqunic acid, other cinnamic acid derivative such as 3-o-caffeoylquinic acid was also identified and quantified by UV and MS/MS spectra and calculated as total caffeoylquinic acids using 4-o-caffeoylqunic acid as standard in the seed and its extract. Other cinnamic acid derivatives such as 1,3-dicaffeoylqunic acid, 1,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3, 4-dicaffeoylquinic acid, dicaffeoylquinic acid-1 and dicaffeoylquinic acid-2 (two unknown) were identified and quantified by UV and MS spectra and calculated as total dicaffeoylquinic acids using chlorogenic acid standard in the seed and its extract. The total cinnamic acids were quantified by calculating the sum of chlorogenic acid, caffeic acid, chicoric acid, total caffeoylquinic acids(4-o-caffeoylquinic acid and 3-o-caffeoylqunic acid) and total dicaffeoyl-quinic acids(1,3-dicaffeoylqunic acid, 1,4-dicaffeoylquinic acid, 3,5-dicaffeoyl-quinic acid, 3, 4-dicaffeoylquinic acid, dicaffeoylquinic acid-1 and dicaffeoyl-quinic acid-2). The Phytochemical screening of *C. intybus* seed and its extract revealed that this plant is a rich source of cinnamic acid derivatives so, these markers (cinnamic acid derivatives) can used for routine quality control of *Cichorium intybus* seed and its extract.

Keywords- *Cichorium intybus* L, Cinnamic acid, HPLC-DAD, LC-MS/MS

Introduction

Cichorium intybus L. is commonly known as chicory is an erect woody perennial herb with about1 m height and

fleshy taproot up to 75 cm in length with large basal leaves[1-3]. It belongs to a family of Asteraceae. Ancient Egyptians grew chicory as a medicinal plant, coffee substitute, and vegetable crop. It was used occasionally for many years for animal forage. This plant is used for diabetics due to its negligible impact on blood sugar [4]. Chicory can tolerate extreme temperatures during both vegetative and reproductive growth stages [1]. All parts of the plant exudates milky latex. In Europe, Asia and Africa the *Cichorium intybus* issued as a medicinally important plant. Even though it has been used for many years, there is no monograph is available in the European Pharmacopoeia or in any official Pharmacopoeia of a European Union member state [5]. However, due to its widespread distribution, different parts of the plant have been used globally in traditional medicines[7]. There are many medicinally important compounds such as alkaloids, inulin, sesquiterpene lactones, coumarins, vitamins, chlorophyll pigments, unsaturated sterol, flavonoids, saponins and tannins are reported in this plant[9-11]. Insulin's a naturally occurring polysaccharides produced industrially from chicory[12-15]. The insulin belongs to class of dietary fibers. In plants, Insulin is used as a means of storing energy and is typically found in roots or rhizomes.

C. intybus has been traditionally used for the treatment of fever, diarrhea, jaundice and gallstones [16-21]. The studies revealed that *C. intybus* possesses anti-hepatotoxic,anti-diabetic [22-24] anti-bacterial [25,26,27], anti-inflammatory[28,29], hyperglycemic and anti-ulcerogenic activities. The *C. intybus* seed contain phenolic constituent's mainly cinnamic acid derivatives such as chlorogenic acid, caffeic acid, Chicoric acid, 4-caffeoylquinic acid, other caffeoylquinic acids and dicaffeoylquinic acids [30, 34]. There are few methods are available for the identification of phenolic constituents in this plant. A high-performance liquid chromatography coupled with mass spectrometry in tandem mode method was established for the analysis of various cinnamic acid derivatives. The seeds were first subjected to hydro alcoholic extraction with 2:1ethanol and water followed by evaporation and spray drying. Reversed-phase liquid chromatography coupled by an electrospray (ES) interface to an ion trap mass spectrophotometry (MS) was used for the separation of the various cinnamic acid derivatives. The identification and quantification of cinnamic acid derivatives such as chlorogenic acid, caffeic acid, chicoric acid, caffeoylquinic acids and dicaffeoylquinic acids has been achieved using this chromatographic technique.

Material and methods

Plant materials- *Cichorium intybus* seeds were collected from Bengaluru market, Karnataka, India. The seeds were identified and authenticated at the R & D Center, Himalaya Wellness Company, Bengaluru, and Karnataka, India.

Chemicals and Reagents- All the organic solvents were of LC-MS and HPLC grade and were purchased from Thermo Fisher. The LC-MS grade water was purchased from J. T. Baker. Acetic acid was procured from Rankem, Acetonitrile from Fisher scientific and

methanol from Finer. The standard chlorogenic acid,4-o-caffeoylquinic acid, caffeic acid and chicoric acid were purchased from Sigma-Aldrich. Purified water used for the preparation of mobile phase is from Milli-Q water purification system (Millipore, Pure lab, Classic, ELGA).Filtration membranes of 0.45 µm cellulose acetate/cellulose nitrate mixed esters were purchased from Millipore.

Sample preparation

Powdering of the material- The dried samples of *C. intybus seeds* were ground using a rotary grinder, sieved through 25 mesh sieves and stored in airtight HDPE container at room temperature. The analytical variations can be minimized using the powdered sample which is used throughout the study for further study.

Extraction procedure- Accurately weighed about 100 g of air-dried powdered material into a 1000 ml round bottom flask and extracted by refluxing on water bath at 85^0C to 90^0C using 500 mL ethanol and water in the ratio (2:1).The process was repeated for two more times with of 300 mL ethanol and water in the ratio 2:1.The hydro alcoholic extract was filtered, concentrated and dried at 105 ± 2^0C. The crude extract obtained after drying was used for further analysis.

Standard solutions- Standard solution of chlorogenic acid, caffeic acid, chicoric acid and4-o-caffeoyl quinic acid was prepared at a concentration of 0.05 mg/ml with methanol.

Test solution- The powdered seed and hydro alcoholic extract was extracted with methanol by refluxing on water bath at 80^0C at a concentration of 20 mg/ml and 10 mg/ml respectively. The extract was filtered through a 0.45 µm syringe filter and 10 µl was injected into the HPLC-PDA-ESI/MS system for analysis.

HPLC and LC-ESI-MS analysis

The chromatographic separation was achieved by a high-performance liquid chromatography with photo diode array detector. The HPLC used was Shimadzu Prominence-i equipped with a photo diode array detector, SIL-20ACHT auto sampler, DGU-20A5 degasser, LC-20AD pump, CBM-20 A system controller, CTO-10 ASVP oven and LC solutions software. Many trials were conducted for the separation of the phyto-compounds. The best chromatographic condition was achieved using 0.2 % v/v acetic acid in water (mobile phase A) and 0.2 % acetic acid in acetonitrile and purified water in the ratio of 1:1 (mobile phase B). The linear gradient elution was performed with the following ratio of mobile phase B 15%, 0 minutes, 35%, 20 minutes, 45%, 25-35minutes, 15%, 45 minutes at a flow rate of 1 mL/minute with a column oven temperature of 40°C.

Mass spectrometric method- Mass scans were acquired on API 2000 (Applied biosystem/MDS SCIEX, Canada) mass spectrometer coupled with ESI (Electron spray ionization) source with HPLC chromatographic system. Analyst 1.5 version software was used for the batch acquisition and data processing. The ion trap has been operated in data-independent, full scan (100-1000 m/z), zoom scan, and MS^n mode to obtain fragment ion m/z with a collision energy of 35% and an isolation

with 3 m/z. Data dependent acquisition, where user-specified criteria are applied to select the ion of interest for subsequent fragmentation, are among the most useful approaches employed to identify unknown compounds by MS. Using this approach single stage MS provides the molecular mass that can be obtained by tandem MS analysis via the fragmentation pathway. When greater discrimination was required additional targeted MS^2 experiments were performed on selected pseudo molecular ions.

The MS parameters were optimized with 0.05 mg/mL standard solutions. The sample was run, and intensity was checked in both positive and negative ionization mode. Subsequently, good intense response was observed in the negative mode and other parameters like declustering potential (DP) -60v, nebulizing gas (GS1 and GS2)55 and 65psi, curtain gas (CUR) 30 psi, focusing potential (FP) -400 v, Entrance potential (EP) -10 v and source temperature (TEM) 420°C and Collision energy (CE) for fragmentation of precursor to product ions were optimized through multiple runs using LC in order to reach the each of the most intense precursor to product ion. The negative and positive parameters of the ion mode ESI source has achieved by flow injection analysis, using chlorogenic acid at a concentration of 0.05mg/ml.

For MS analysis the negative ion mode of ESI was selected because it provided extensive structure information for most phenolic acids present in *Cichorium intybus*. In addition to detection of the deprotonated molecular ions, collision induced dissociation was performed in the MS^2 and MS^3, and the resulting product ions were used as fingerprints of each component.

Quantification of total caffeoylquinic acids (3-o-caffeoylquinic acid and 4-o-caffeoylquinic acid) has achieved using standard of 4-o-caffeoylquinicacid. The dicaffeoylquinic acids (1,3-dicaffeoyl quinic acid, 1,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, dicaffeoylquinic acid-1 and dicaffeoylquinic acid-2) were quantified using chlorogenic acid as standard. Identification and quantification of chlorogenic acid, caffeic acid and chicoric acid has been calculated using respective standard. Identification was carried out by UV spectra, retention time with authenticated standards followed by confirmation from mass spectra of the corresponding peaks.

Validation- The developed method was validated as per the International Conference on Harmonization (ICH) guidelines. The validation parameters include specificity, system suitability, precision, accuracy, range and robustness.

System Suitability- System suitability has been executed by injecting six injections of standard chlorogenic acid. The parameters such as theoretical plates (N), peak asymmetry factor (As), % relative standard deviation (% RSD) and retention time were recorded.

Specificity- The specificity study has done by injecting blank, standard and the sample solution to find out the interference at the analyte retention time (RT) of chlorogenic acid with diluents

peak. The peak purity profile was established using diode array detector.

Precision- The precision of the method was performed by injecting three replicates of three different concentrations at 50%, 100% and 150% of working concentration of 10mg/mL sample solution on the same day(intraday precession) and different day (inter day precession)and the % RSD was calculated.

Linearity- Linearity was performed by preparing five different concentrations of the sample ranging from 50% to 150% of working concentration of 10 mg/mL sample solution (100%).

Accuracy- It can be defined as exactness of an analytical method or the closeness of results. The accuracy was derived once the precision, linearity and specificity had been established (option 'c' as per the ICH guideline (Q2 R1 validation). Since accuracy was derived from precision, linearity and specificity,

acceptance criteria should comply as mentioned for the precision, linearity and specificity.

Robustness- Reliability of the method was done with respect to deliberate variations in method parameters like variation in the flow of the mobile phase (± 0.1 ml/min), variation in temperature (38°C to 42° C) and change in column by injecting duplicate injections of single preparation.

Results and discussion

The Hydro alcoholic extract obtained from *C. intybus* seed was submitted to HPLC analysis. The chromatographic method was developed to explore various phytoconstituents from *C. intybus* seed and its extract. Different methods have been designed by changing column, mobile phase, organic modifier, and with different concentration of samples to separate the different derivatives of cinnamic acid from the seed powder and its extract. The best separation was achieved using 0.2 % v/v acetic acid in water (mobile phase A) and 0.2 % acetic acid in acetonitrile and purified water in the ratio of 2:1 (mobile phase B). **Figure-1**.

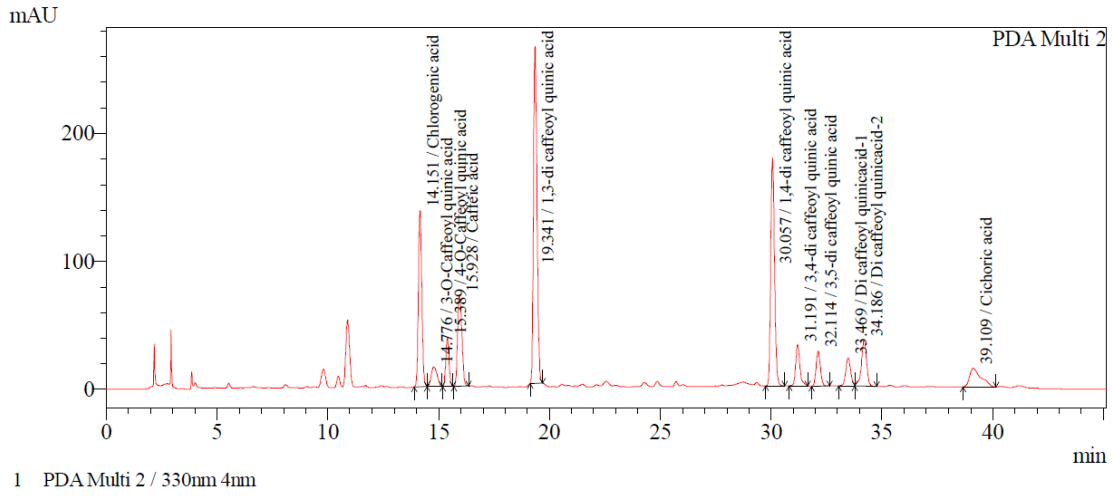

Figure-1 HPLC chromatogram of *C. Intybus* extract at 330 nm

The optimized HPLC-PDA method was validated for the simultaneous analysis of cinnamic acid derivatives such as chlorogenic acid, caffeic acid and chicoric acid using respective standard and 3-o-caffeoylquinic acid and 4-o-caffeoylquinic acid as total caffeoylquinic acids using 4-o-caffeoylquinic acid and 1,3 di caffeoylquinic acid, 1,4 di caffeoylquinic acid, 3,4 di caffeoylquinic acid, 3, 5 di caffeoylquinic acid and two di caffeoylquinic acids-1 & 2 (unknown di caffeoylquinic acids) quantified as total dicaffeoylquinic acids using chlorogenic acid as standard. **Table-1**.

Table-1 Identification and quantification of Cinnamic acid derivatives in *Cichorium intybus* seed and extract.

	Chlorogenic acid (% w/w)	**Caffeic acid (% w/w)**	**Chicoric acid (% w/w)**	**Total caffeoylquinic acids (% w/w)**	**Total di caffeoylquinic acids (% w/w)**	**Total cinnamic acid derivatives (% w/w)**
C. Intybus seed	0.09	0.01	0.062	0.10	0.53	1.38
C. Intybus extract	0.40	0.11	0.21	0.69	1.64	3.22

Mass analysis and identification

In this study,1 1 compounds were identified and characterized by UV-Spectra and MS/MS data and the standard solutions of chlorogenic acid, caffeic acid, chicoric acid and 4-o-caffeoylquinicacid were prepared and injected to compare the retention times.

The HPLC-DAD chromatograms and total ion chromatograms (TIC) in the negative mode of the extracts of *Cichorium intybus* are shown in Figure-2. The application of MS for the analysis of Cinnamic acid derivatives has increased by soft ionization techniques. These compounds are polar nonvolatile and thermally liable.

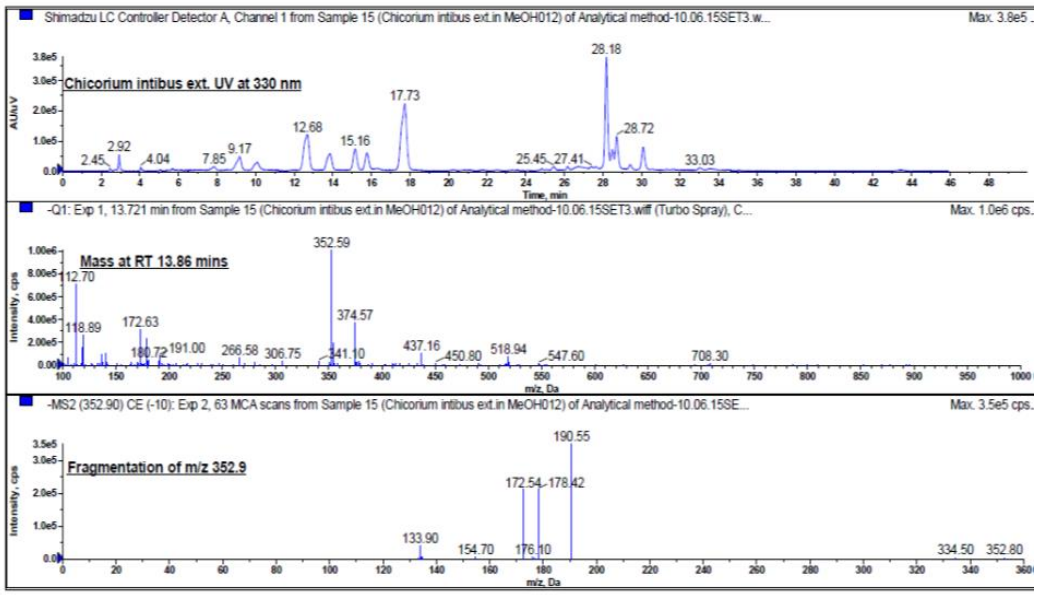

Figure-2 LC-MS/MS data of *C. Intybus* extract

The UV and Mass spectra of phyto-compounds confirmed that they are all mono-caffeoyl quinic acid (CQA) isomers. The mono-CQA has an ion at m/z 191 as the base peak [M-H caffeoyl] _ when the acyl group is linked to the 3-OH or 5-OH position, and an ion at m/z 173 when the acyl group is attached to the 4-OH position. The 3-CQA and 4-CQA were differentiated based on the relative intensity of m/z 179 $[M-H]^-$quinic acid, which is more significant for 3-OH compounds. Based on the retention times, UV–Vis spectra, and MS2 fragments with the standard, a 3-CQA and 4-CQA were identified.

Dicaffeoylquinic acids eluted later in the chromatogram, gave $[M-H]^-$at m/z 515, and MS2 fragment at m/z 353, corresponding to the loss of a caffeoyl moiety $[M-H]^-$162as the base peak. The presence and intensity of MS2 secondary fragments confirms the dicaffeoylquinic acid isomers.The presence of Ion with m/z 299 (intensity 35%) and the less intense fragments at m/z 255 and 203 were unique characteristic of 4-acyl dicaffeoylquinic acid.

Hydro alcoholic extract of *C. intybus* yielded 1,3-dicaffoylquinic acid, 1,4-dicaffoylquinic acid, 3,4-dicaffoyl quinic acid, 3,5-dicaffoyl quinic acid, dicaffeoylquinic acid-1 and dicaffeoyl-quinic acid-2 with parent ion at m/z 515. **Table-2.**

Table-2 LC-MS/MS data of chromatographic peaks

Peak no.	Compound name	UV λmax (nm)	MS $[M-H]^-$ (*m/z*)	MS/MS (*m/z*), (Abundance)
1	Caffeic acid	330nm	179	MS^2[179]: 135 (100)
2	3-O-Caffeoylquinic acid	330nm	353	MS^2[353]: 191 (100), 179 (50), 135 (5)
3	4-O-Caffeoylquinic acid	330nm	353	MS^2[353]: 191 (15), 179 (60), 173 (100)
4	Chicoric acid	330nm	473	MS^2[473]: 311 (100), 293 (20), 179 (45), 149 (30)
5	Chlorogenic acid	330nm	353	MS^2[353]: 191 (100), 179 (2)
6	1,3 dicaffeoylquinic acid	330nm	515	MS^2[515]: 353 (100), 335 (30) MS^3[353]: 191 (100), 179 (50)
7	1,4 dicaffeoylquinic acid	330nm	515	MS^2[515]: 353 (100), 299 (35), 317 (28), 255 (10), 203 (5) MS^3[353]: 191 (24), 173 (100)
8	3,4 dicaffeoylquinic acid	330nm	515	MS2[515]: 353 (100), 299 (25), 255 (18), 203 (8)
9	3,5 dicaffeoylquinic acid	330nm	515	MS^2[515]: 353 (100), 191 (18) MS^3[353]: 191 (100), 179 (50), 173 /2), 135 (10)
10	Dicaffeoylquinicacid-1	330nm	515	MS^2[515]: 353 (100)
11	Dicaffeoylquinicacid-2	330nm	515	MS^2[515]: 353 (100)

Conclusion

The lifestyle diseases and chronic health issues are a major health problem worldwide. The health care management demand to deliver an optimal environment for better treatment and to meet the need of human population. In this context, identification of phytomarkers with therapeutic property are very essential for the ailment of many chronic diseases. The quality of health care can be improved by exploring the medicinal plants which were used in the traditional medicine.

The present study was aimed to identify the maximum phyto constituents in *C. intybus* seed and its extract and establish the method to quantify the same for routine quality control of seed. Many trials were conducted to achieve the well separation of phyto markers. The chromatographic method developed for HPLC, and LC-MS/MS is able to resolve all the phenolic compounds present in *C. intybus* seeds and its extract. There are about eleven cinnamic acid derivatives such as chlorogenic acid, caffeic acid,chicoric acid,3-o-caffeoylquinic acid, 4-o-caffeoylquinic acid, 1,3di caffeoylquinic acid, 1,4 di caffeoylquinic acid, 3,4 di caffeoylquinic acid,3, 5 di caffeoylquinic acid, di caffeoylquinic acid-1 and di caffeoylquinic acid-2 were identified and quantified. The chromatographic method developed for HPLC and LC-MS/MS for the identification and quantification of cinnamic acid derivatives in the seeds of *C. intybus* seed and its extract has shown similar chromatographic profile. The developed method confirms in terms of precision, accuracy, linearity, specificity, and robustness. It was observed that there were no relevant variations attributable to the nature of the detected fragments or their relative intensities were observed.

The present study has attained the chromatographic identification and quantification of different cinnamic acid derivatives in *C.intybus* seeds and its extract. This method can be used for the standardization of *C. intybus* seed and to find out the authenticity and quality of crude drug. This analytical data is an important tool for the development of herbal raw material as a medicinal ingredient and will enable to open a window in search of phyto markers as bio active compounds.

Disclaimer Statement

Authors declare that no competing interest exists. The products used for this research are commonly used products in research. There is no conflict of interest between authors and producers of the product.

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