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Development and Validation of Liquid Chromatography-Tandem Mass Spectrometric Method for Simultaneous Quantitation of Mangiferin and Isoquercetin from Dried Leaf Powder Extract of *Bombax ceiba* Linn

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Abstract: ***A rapid, sensitive, and accurate liquid chromatography-tandem mass spectrometric (LC-MS/MS) method is developed for simultaneous quantitation of mangiferin and isoquercetin from dried leaf powder extract of Bombax ceiba Linn. The chromatographic separation was carried out using a Shim-pack XR ODS (75 mm L x 3 mm ID; 2.2 µm) column. The mobile phase used was 0.1% formic acid in water and acetonitrile at a flow rate of 0.5 mL/min in a gradient elution mode. Nexera UHPLC system coupled with LCMS-8040 triple quadrupole mass spectrometer with Electro Spray Ionization (ESI) source was used as a detector in Multiple Reaction Monitoring (MRM) mode. The method was found to be simple, precise, accurate, fast, specific, and sensitive that can be used for routine quality control of dried leaf powder extract of Bombax ceiba Linn.***

Keywords: LC-MS/MS, mangiferin, isoquercetin, MRM, *Bombax ceiba* Linn.

1. Introduction

Herbal drugs are chief fundamental in traditional medicinal system such as Ayurveda, homeopathic, neuropathic, and other medicinal systems. The toxic side effects of the drugs of modern medicine and the lack of medicines for many chronic diseases have led to re-emergence of the herbal medicines, with possible treatment for many health problems. In herbal medicine the therapeutic effect varies as the phytochemical constituent varies which is due to distinction in geographical region, time of collection, environmental factors, etc. For this purpose, it is very important that a system of standardization is established for every plant medicine in the market because the scope of variation in different batches of medicine is enormous. In order to get constant composition of herbal preparation, adequate analytical methods have to be applied. [1]

Chromatographic techniques such as high-performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC), and gas chromatography (GC), liquid chromatography-mass spectrometry (LC-MS) are used to efficiently determine the quality of the herbs by developing fingerprints and estimation of biomarkers. In the present research work, liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and validated for simultaneous quantitation of the mangiferin and isoquercetin from dried leaf powder extract of *Bombax ceiba* Linn.

Bombax ceiba is commonly known as silk cotton tree and semal which belongs to family Bombacaceae. It is one of the important medicinal plants in tropical and subtropical India. According to Ayurveda, it has stimulant, astringent, hemostatic, aphrodisiac, diuretic, antidiarrhoeal, cardiotonic, emetic, demulcent, antidysentery, alterative, and antipyretic properties. [2] Many chemical compounds have been isolated from different parts of *B. ceiba*. Leaves of *B. ceiba* are reported to have crude protein, crude fiber, calcium, phosphorus, shamimin, mangiferin. Flowers contain β-sitosterol, β-sitosteril-β-D-glucoside, polysachharide-Dgalactose, hentriacontane, hantriacontanol, traces of essential oil, kaempferol, quercetin, Larabinose, L-rhamnose and isoquercetin [3,4].

Number of HPLC and LC-MS/MS methods have been reported for quantification of mangiferin and isoquercetin alone or in combination with other constituents from various medicinal plants and other matrices. [5,6,7] After a systematic review of literature, it was found out that no validated LC-MS/MS method has been reported for simultaneous quantitation of the mangiferin and isoquercetin from any matrix to the best of our knowledge. Hence, the aim of the present research work was to develop and validate LC-MS/MS for simultaneous quantitation of the mangiferin and isoquercetin from dried leaf powder extract of *Bombax ceiba* Linn.

2. Materials and Methods

2.1 Experimental reagents

Methanol and acetonitrile (LCMS grade) were purchased from J.T. Baker (Mumbai, India). Formic acid (purity 99.9 %) was purchased from Fluka (Steinheim, Germany). High

purity deionized water was obtained using a Milli-Q water purification system from Millipore (Bangalore, India).

2.2 Reference standards

Reference standards of mangiferin and isoquercetin (purity 98 %) were procured from Sigma- Aldrich (Aldrich Division; Steinheim, Federal Republic of Germany).

2.3 Plant material

The plant material of *Bombax ceiba* Linn. was collected from Mumbai, Maharashtra, India. The plant material was authenticated from Botanical Survey of India, Pune, India (voucher specimen no. DB-2). Leaves of *Bombax ceiba* Linn. were dried at room temperature, under shade and then ground in a mixer to a fine powder. Powder was then passed through an ASTM BSS mesh (size 85) and stored in an airtight container at room temperature.

2.4 Preparation of stock solution

About 10 mg of mangiferin was a weighed and transferred to 10 mL volumetric flask. Five mL of methanol was added and the contents of the flask were sonicated in an ultrasonic bath for 5 minutes for complete dissolution of mangiferin. The contents were then diluted up to the mark with methanol to obtain stock solution of mangiferin of 1000 µg/mL. Similarly, about 10 mg of isoquercetin was weighed and transferred to 10 mL volumetric flask. Five mL of methanol was added and the contents of the flask were sonicated for 5 minutes for complete dissolution of isoquercetin. The contents were then diluted up to the mark with methanol to obtain stock solution of isoquercetin of 1000 µg/mL. These stock solutions were further diluted using methanol to prepare mix standard solutions of mangiferin and isoquercetin.

2.5 Preparation of sample solution

About 1 g of dried leaf powder of *Bombax ceiba* Linn. was weighed and transferred in a 50 mL centrifuge tube. Fifteen mL of methanol was added to it and the flask was sonicated in an ultrasonic bath for 15 minutes. The contents of the tube were then centrifuged at 5000 rpm and supernatant was collected in a separate tube. Same procedure was repeated with the remaining residue and filtrates were combined. Hence, 1 g of sample was extracted in overall 30 mL of methanol. Sample solution was then filtered using nylon syringe filter (0.22 µ). Filtered sample was then diluted in ratio of 1:1000 using methanol and analyzed using developed LC-MS/MS method.

2.6 Instrumentation and analytical conditions

LC-MS/MS analysis was performed using Nexera UHPLC chromatograph coupled with LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corporation, Japan). Nexera system was equipped with binary gradient pump (LC-30AD), auto sampler (SIL-30 AC) and column oven (CTO-20 AC). A reversed phase, Shim-pack XR ODS column (75 mm L x 3 mm I.D., 2.2 µ particle size) was used for the chromatographic separation and column temperature was maintained at 40° C. Mobile phase used for analysis was 0.1 % formic acid in water (MP A) and acetonitrile (MP B) in gradient elution mode (refer to Table 1) and flow rate used was 0.5 mL/min. Injection volume used was 5 µL. LCMS-8040 was equipped with ESI source and analysis was carried out in negative mode. Nitrogen was used as nebulizing as well as drying gas and flow rates used were 3 L/min and 10 L/min respectively. Desolvation line and heat block temperatures were maintained at 250 ° C and 400 ° C respectively. Argon was used as Collision Induced Dissociation (CID) gas at a pressure of 230 kPa. Capillary voltage used was – 3.5 kV. Quantification was performed using MRM mode with transition of 421.20 >301.05 for mangiferin and sum TIC of transitions of 463.20 > 300.05 and 463.20 >301.05 for isoquercetin. The peak widths of precursor and product ions were maintained at 0.7 Da at FWHM in the MRM mode.

Table 1: HPLC gradient program

Time (min)	*MP A conc. (%)*	*MP B conc. (%)*
0.01	90	10
1.00	30	70
1.30	0	100
2.50	0	100
3.00	90	10
4.50	90	10

3. Optimization of MS parameters

Full scan MS analysis was carried out to determine precursor ion m/z of selected phytochemicals. As shown in Figures 1 and 2, the most intense m/z of 421.15 and 462.95 in the full scan MS spectrum of ESI negative mode corresponded to the deprotonated species $[(M\text{-}H^{+})^{-}]$ of mangiferin and isoquercetin respectively.

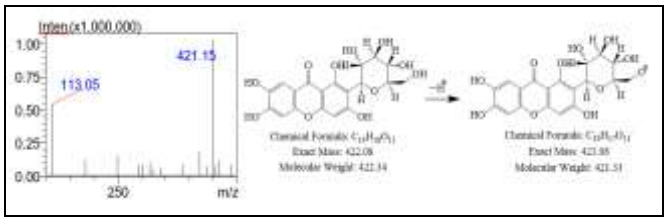

Figure 1: Full scan MS spectrum of mangiferin in ESI negative ionization mode

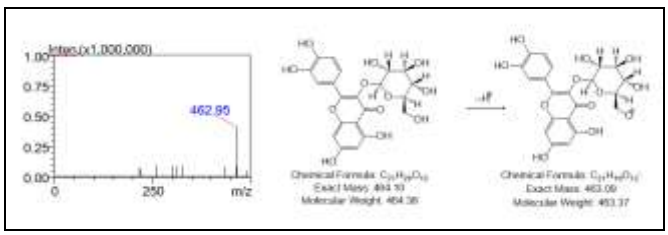

Figure 2: Full scan MS spectrum of isoquercetin in ESI negative ionization mode

Selection of most abundant and consistent product ions was carried out using 'Automatic MRM Optimization' tool of LabSolutions software. Representative product ion scan spectra of mangiferin and isoquercetin are shown in Figures 3 and 4 respectively.

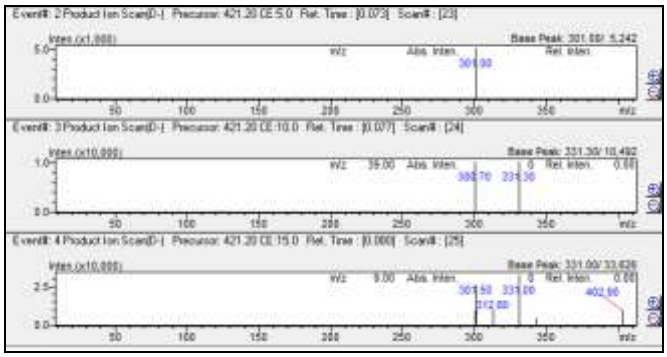

Figure 3: Representative product ion scan spectra of mangiferin

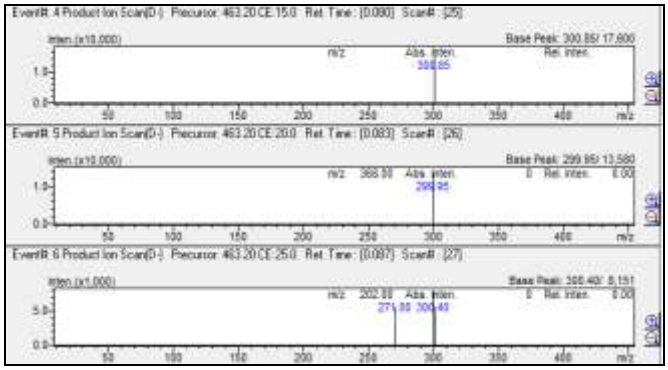

Figure 4: Representative product ion scan spectra of isoquercetin

Mangiferin is xanthone C-glycoside, while isoquercetin is quercetin-3-O-glucoside. C-glycosides do not generate abundant aglycone ions in the CID mass spectrum, but give characteristic fragments of the C-glycoside unit itself. Losses of 120 and 150 Da from the cross-ring cleavage of C-sugar of are relatively easy and can be directly observed with strong abundance in the product ion spectra of C-glycosides. As shown in Figure 3, highly abundant product ion m/z 301 corresponds to the loss of C-glycoside fragment (120 Da) from deprotonated mangiferin. On the other hand, product ion m/z 301 obtained after loss of a glucose unit (−162 Da) from isoquercetin as shown in Figure 4. The LC-MS/MS spectrum of quercetin-3-O-glucoside also showed a fragment ion at m/z 300, which is characteristic of the deprotonated radical aglycone ion [quercetin-H^+]$^-$, formed by the homolytic cleavage of the O-glycosidic bond, and has been proposed as indicative of quercetin glycosides. For further quantitative analysis, MRM transition of 421.20 >301.05 was used for mangiferin whereas sum TIC of two MRM transitions 463.20>300.05 and 463.20>301.05 were used for isoquercetin. Selected MRM transitions are in accordance with the MRM transitions reported in the literature for the given phytochemicals. [8,9] Furthermore, MS/MS parameters including fine adjustments of precursor and product ions, collision energies and, Q1 & Q3 pre-bias voltages were carried out to improve the overall sensitivity of selected phytochemicals.

4. Method validation

4.1 Linearity

Linearity of method for simultaneous quantitation of mangiferin and isoquercetin was determined by analyzing mix standard solutions of concentration levels of 2 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL and 500 ng/mL on LC-MS/MS system in triplicates, under the optimized analytical conditions. The peak areas obtained for each analyzed concentration were noted. The calibration curves of both the analytes were obtained by plotting graphs of mean peak areas of each standard versus corresponding concentration (Figures 5 and 6). The values of correlation coefficient, intercept and slope were determined from the corresponding graphs of mean peak area of mangiferin and isoquercetin (Y axis) against injected concentrations of mangiferin and isoquercetin (X axis) by applying weighted ($1/A^2$) least-squares linear regression analysis. The results, listed in Table 2, show that within the concentration range indicated, there was a good correlation between mean peak area and concentration of standards.

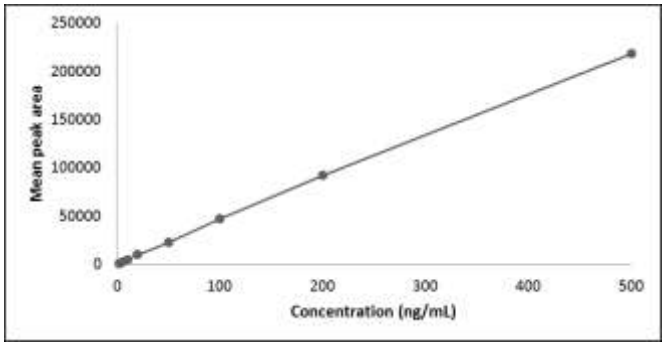

Figure 5: Graph of mean peak area v/s concentration for mangiferin

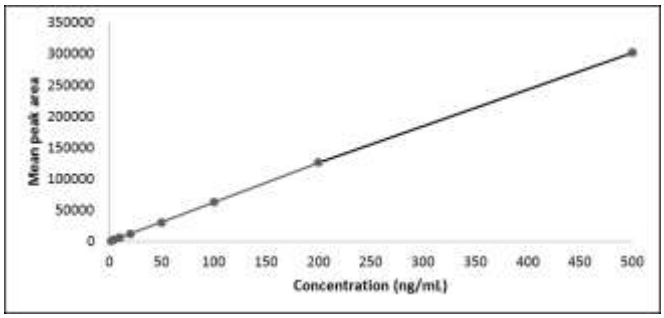

Figure 6: Graph of mean peak area v/s concentration for isoquercetin

4.1 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD is defined as the lowest concentration of an analyte that can be reliably detected but not necessarily quantified, while LOQ is taken as the lowest concentration that can be determined with acceptable accuracy and precision, under the optimized experimental conditions. The LOD and LOQ of the method for both the analytes were estimated by measuring the signal to noise ratio corresponding to 3 and 10, respectively. The results are listed in Table 2.

4.2 System suitability

System suitability was carried out to verify that reproducibility of the system was acceptable for the analysis. System suitability was determined by injecting, mix standard solution of mangiferin and isoquercetin at concentration level of 200 ng/mL into the LC-MS/MS system, under the optimized analytical conditions in six replicates. The system suitability parameters like retention time reproducibility, peak area reproducibility, peak tailing factor, and column efficiency were evaluated. Results of the system suitability are presented in Table 2.

4.3 Specificity

The specificity of the proposed LC-MS/MS method was ascertained by injecting diluent (methanol) to observe for interference at the retention times of peaks of interest. Figures 7 and 8 show overlay of MRM chromatograms of methanol and standard solution of mangiferin and isoquercetin at LOQ level (2 ng/mL) respectively, to check that the interference, if any, is below LOQ level for both the analytes. No interference from the diluent was observed for both the analytes.

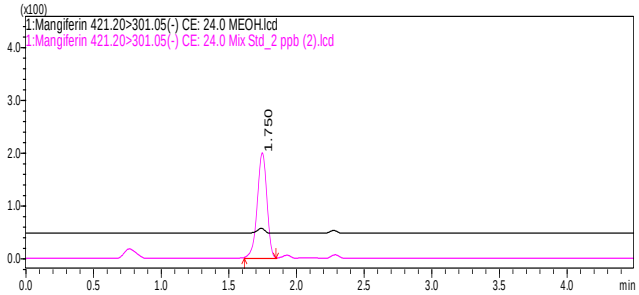

Figure 7: Overlay of MRM chromatograms of methanol and standard solution of mangiferin at LOQ level

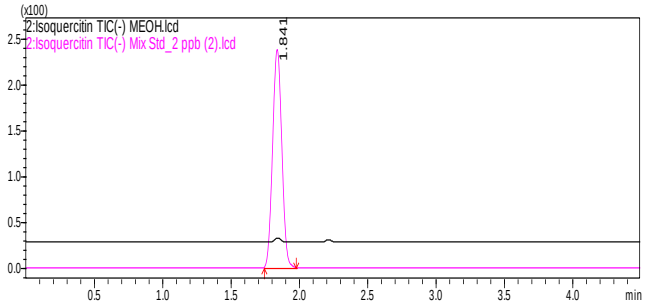

Figure 8: Overlay of MRM chromatograms of methanol and standard solution of isoquercetin at LOQ level

4.4 Precision

The precision of the method was studied by determining repeatability and intermediate precision. Repeatability was evaluated by analyzing sample solution of dried leaf powder of *Bombax ceiba* Linn. in six replicates, on the same day. The intermediate precision of the method was evaluated by analyzing the sample solution in six replicates on three different days, under the specified chromatographic conditions. The precision results were expressed as percentage relative standard deviations of peak areas for mangiferin and isoquercetin. The results, listed in Table 2, indicate that the proposed method is precise and reproducible.

4.5 Standard stability

The stabilities of mangiferin and isoquercetin standards were determined by comparing the peak areas of mix standard solution of mangiferin and isoquercetin of concentration level of 200 ng/mL at different time intervals, for a period of minimum 48 hours, at room temperature. Values of percentage relative standard deviation for area counts (less than 5 %) indicates no significant degradation for mangiferin and isoquercetin for 48 hours.

4.6 Assay

The developed and validated LC-MS/MS method was used for quantitation of mangiferin and isoquercetin from dried leaf powder extract of *Bombax ceiba* Linn.

The identities of peaks of mangiferin and isoquercetin in the sample solution were confirmed by comparing the retention times of peaks found in sample solution of with that of the standard solution of mangiferin and isoquercetin. Figure 9 shows MRM chromatogram of mix standard solution of mangiferin and isoquercetin at concentration level of 50 ng/mL. Figure 10 shows MRM chromatogram of sample solution. Amount of mangiferin and isoquercetin present in the sample solution was determined from their respective calibration curves, by using the peak area of mangiferin and isoquercetin present in the sample solution. To ascertain the repeatability of the method, the assay experiment was repeated six times. The results of assay experiment are shown in Table 2.

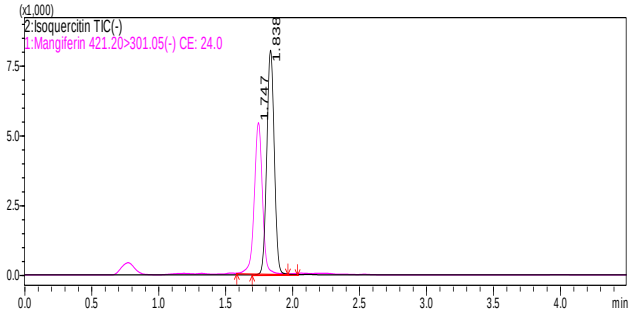

Figure 9: MRM chromatogram of mangiferin and isoquercetin mix standard of 50 ng/mL

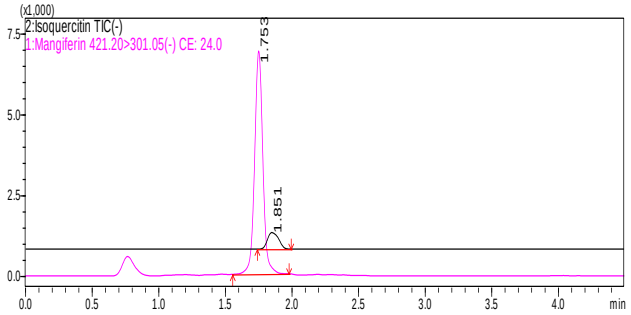

Figure 10: MRM chromatogram of mangiferin and isoquercetin present in dried leaf powder sample solution of *Bombax ceiba* Linn.

4.6 Recovery

Recovery of the method was ascertained by spiking the pre-analyzed samples with known concentration of standards and then analyzing samples under optimized analytical conditions. The spiking was done at three different concentration levels at 80 %, 100 % and 120 % of the assay concentration level of sample. The average percentage recovery at each concentration level was evaluated and tabulated in Table 2.

Table 2: Method validation results for simultaneous quantification of mangiferin and isoquercetin from dried leaf powder extract of *Bombax ceiba* Linn.

Method validation parameters	*Mangiferin*	*Isoquercetin*
Linear range (ng/mL)	2-500	2-500
Correlation coefficient (r^2)	0.9984	0.9995
Slope (m)	454.310	622.153
Intercept (c)	44.1755	-125.232
LOD (ng/ mL)	1	1
LOQ (ng/ mL)	2	2
Stability of standard solution	48 hours	48 hours
System suitability (% RSD area counts)	1.92	1.96
System suitability (% RSD retention time)	0.06	0.05
Column efficiency	26810	31730
Peak tailing	0.958	1.026
Repeatability (% RSD area counts)	3.70	6.04
Intermediate precision (% RSD area counts)	3.70	8.54
Assay (mg/1 g)	1.953	0.147
% Recovery	Level 1-98.26 Level 2-102.86 Level 3-99.06	Level 1- 96.15 Level 2- 96.55 Level 3-96.88

5. Discussion

In the present research work, a LC-MS/MS method was developed and validated for simultaneous quantitation of the mangiferin and isoquercetin from dried leaf powder extract of *Bombax ceiba* Linn.

This developed and validated LC-MS/MS method for simultaneous quantification of mangiferin and isoquercetin over concentration range 2-500 ng/mL has significant advantages in terms of sensitivity (LOQ- 2 ng/mL) and selectivity (use of MRM mode), shorter run time (4.5 mins).
An introduction of tandem mass spectrometric detection (MS/MS) has led to a significant improvement in detection ability and selectivity by employing MRM data acquisition in recent years. Due to the high selectivity of MRM mode, sample pre-treatment and optimization of chromatographic separation are not essential or crucial any more. This could be considered a major advantage over conventional HPLC methods, which usually require additional time for chromatographic method development and results in substantial losses of analytes due to multiple steps involved in sample pre-treatment.

6. Conclusion

A precise, accurate and reproducible LC-MS/MS method has been developed for simultaneous quantitation of the mangiferin and isoquercetin from dried leaf powder extract of *Bombax ceiba* Linn. The developed method has been validated according to the US FDA guidelines. This method can be used as an analytical tool for quality evaluation of plants and formulations containing mangiferin and isoquercetin as chemical markers. It is an efficient method to assess quality and authenticity dried leaf powder of *Bombax ceiba* Linn. Hence, it is demonstrated that LC-MS/MS is a powerful practical tool for comprehensive quality control of plant raw materials and its formulations.

7. Acknowledgement

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