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SHORT COMMUNICATION

Rapid Development and Validation of Improved Reversed-Phase High-performance Liquid Chromatography Method for the Quantification of Mangiferin, a Polyphenol Xanthone Glycoside in *Mangifera indica*

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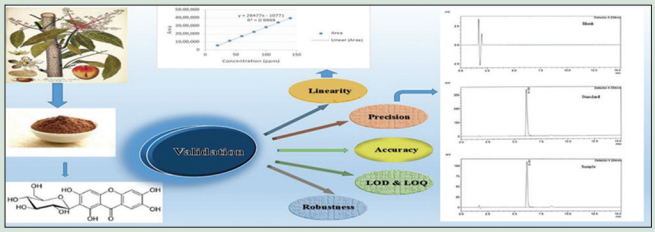
ABSTRACT

Mangiferin, a polyphenolic xanthone glycoside from *Mangifera indica,* is used as traditional medicine for the treatment of numerous diseases. The present study was aimed to develop and validate a reversed-phase high-performance liquid chromatography (RP-HPLC) method for the quantification of mangiferin from the bark extract of *M. indica*. RP-HPLC analysis was performed by isocratic elution with a low-pressure gradient using 0.1% formic acid: acetonitrile (87:13) as a mobile phase with a flow rate of 1.5 ml/min. The separation was done at 26°C using a Kinetex XB-C18 column as stationary phase and the detection wavelength at 256 nm. The proposed method was validated for linearity, precision, accuracy, limit of detection, limit of quantification, and robustness by the International Conference on Harmonisation guidelines. In linearity, the excellent correlation coefficient more than 0.999 indicated good fitting of the curve and also good linearity. The intra- and inter-day precision showed <1% of relative standard deviation of peak area indicated high reliability and reproducibility of the method. The recovery values at three different levels (50%, 100%, and 150%) of spiked samples were found to be 100.47, 100.89, and 100.99, respectively, and low standard deviation value <1% shows high accuracy of the method. In robustness, the results remain unaffected by small variation in the analytical parameters, which shows the robustness of the method. Liquid chromatography–mass spectrometry analysis confirmed the presence of mangiferin with M/Z value of 421. The assay developed by HPLC method is a simple, rapid, and reliable for the determination of mangiferin from *M. indica*.

Key words: Liquid chromatography–mass spectrometry, *Mangifera indica*, mangiferin, reversed-phase high-performance liquid chromatography, validation

SUMMARY

The present study was intended to develop and validate an RP-HPLC method for the quantification of mangiferin from the bark extract of *M. indica*. The developed method was validated for linearity, precision, accuracy, limit of detection, limit of quantification and robustness by International Conference on Harmonization guidelines. This study proved that the developed assay by HPLC method is a simple, rapid and reliable for the quantification of the mangiferin from *M. indica*.



Abbreviations Used: *M. indica*: *Mangifera indica*, RP-HPLC: Reversed-phase high-performance liquid chromatography, M/Z: Mass to charge ratio, ICH: International conference on harmonization, % RSD: Percentage of relative standard deviation, ppm: Parts per million, LOD: Limit of detection, LOQ: Limit of quantification.

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INTRODUCTION

Plant-based medicaments had served from the onset of human civilization as the most important therapeutic weapon available to fight various human and animal diseases. In the last few years, there has been an exponential growth in the field of herbal medicine, and these drugs are gaining popularity both in developing and developed countries because of their natural origin and less side effects.[1] Standardization and identification of phytochemicals are always very challenging era in polyherbal formulations because of complex nature and inherent variability of the chemical constituents of plant-based drugs. Quantitative determination of individual phytochemicals in the polyherbal preparation required optimal separation technique by which each phytoconstituents are separated with the highest resolution and the least interferences from each other. The World Health Organization has emphasized the need to ensure the quality of medicinal plant products using modern controlled technique and applying suitable standards.[2]

Mangifera indica (L.), belonging to the family *Anacardiaceae*, is one of the second largest tropical fruit crops in the world.[3] *M. indica*, also known as mango, has been an important herb in the ayurvedic and indigenous medical systems for over 4000 years. Phytochemical studies on different parts of *M. indica* have demonstrated that it contains polyphenols, flavonoids, phytosterols,[4,5] and mangiferin.[6,7] Mangiferin, a polyphenolic antioxidant and stable a xanthone glycoside, is reported to be present in stem bark, heartwood, leaves, and dried roots of *M. indica*.[8] Mangiferin has been reported to possess antioxidant,[3,9,10] antitumor,[11,12]

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immunomodulatory,[11,13] anti-HIV,[11] antiviral,[14] anti-inflammation,[15] cardioprotective,[16] antipyretic activity,[17] anticancer,[18,19] antidiabetic,[20,21] radioprotection,[22] neuroprotective,[23] antimicrobial,[24] hepatoprotective,[9] and hypocholesterol activity.[25] However, there have been limited studies on the quantification of mangiferin from *M. indica*. In the present investigation, we have developed a simple, optimized, and validated reversed-phase high-performance liquid chromatography (RP-HPLC) method for quantitative determinations of mangiferin, a major bioactive polyphenol in the stem bark of *M. indica*. The developed method was validated on the basis of its linearity, repeatability, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and robustness as per the International Conference on Harmonisation (ICH) guidelines.[26]

MATERIALS AND METHODS

Materials

Mangiferin standard (98%) was obtained from Sigma-Aldrich (India). All reagents and solvents were analytical and HPLC grade. The powdered bark material of *M. indica* was procured from Ess Kay Herbs, Lucknow, India.

Preparation of the extract

Hydroalcoholic extract of mangiferin was prepared by Soxhlet extraction of 100 g of powdered bark material of *M. indica* using 70% ethanol at 75°C for about 3 h in three successive batches. The first batch extraction by adding 800 ml solvent for 2 h and further two successive extractions were done by adding 500 ml per batch for 1 h. After completion of extraction, the cooled liquid extract was concentrated by evaporating its liquid contents in rotary evaporator, with an approximate yield of 12% w/w.

Instrumentation and chromatographic conditions

HPLC was performed on a Shimadzu LC-2030 C Prominence-i (Japan) system equipped with a quaternary low-pressure gradient Solvent Delivery LC-2030 pump with high-pressure switching valves, online LC-2030 degasser unit, a high sensitivity LC-2030 ultraviolet (UV)-detector, high-speed drive LC-2030 autosampler with a 100 µl loop, and it accommodates 216 samples at a time with direct access rack system and large capacity column oven. The system controlled and data analyzed by LabSolutions software. A separation was carried out in Kinetex XB-C18 column (100 Aº, 250 mm × 4.6 mm, 5 µm pore size). The mobile phase consists of isocratic elution with a low-pressure gradient using 0.1% formic acid: Acetonitrile (87:13) with a flow rate of 1.5 ml/min and the injection volume of 10 µl. All solutions were degassed and filtered through 0.45 µm pore size filter. The column was maintained at 26°C throughout analysis, and the UV detector was set at 254 nm. Nearly 70% methanol used as a diluent for assay by HPLC analysis and the total liquid chromatography (LC) run time was 15 min. The instrument was calibrated and qualified before the analysis. Using these chromatographic conditions, it was possible to confirm the retention time of mangiferin by injection of corresponding standard separately. The hydroalcoholic extract of *M. indica* was also analyzed by LC–mass spectrometry (LC-MS) (Shimadzu LC-MS 8040) and identified by comparison of their M/Z value with the reference standard mangiferin.

Preparation of standard solution

Accurately weighed known weight of mangiferin reference standard to 100 ml volumetric flask and was dissolved in 70% methanol to obtain a stock solution of 500 ppm. Working standard solutions were obtained by diluting the 5.0 ml standard stock solution to 25 ml volumetric flask and made up with 70% methanol to achieve the final concentration of 100 ppm. Before analysis, the solutions were filtered through 0.20 µm nylon membrane filters.

Preparation of sample

The dried hydroalcoholic bark extract of *M. indica* was prepared in 70% methanol to achieve the final concentration of 100 ppm. Before analysis, the solutions were filtered through 0.20 µm nylon membrane filters.

Preparation of spiked sample

Three different volumes (2.5, 5.0, and 7.5 ml) of standard stock solution were added to the sample solution (100 ppm) separately. The standard stock solution was spiked into the samples to determine recovery. Before analysis, the solutions were filtered through 0.20 µm nylon membrane filters.

Validation of the method

The validation of the developed method was done according to the ICH guidelines.[26] The method is validated for linearity, precision, accuracy, LOD, LOQ, and robustness.

Linearity

Linearity was determined by different known concentrations of mangiferin standard (20–140 ppm) solution in triplicate by diluting the standard stock solution. The standard solutions were injected, and the peak area was measured. Calibration curve was constructed for mangiferin by plotting peak areas against concentration and linear regression equations. The correlation coefficient was also computed.

Precision

Precision is a measure of the reproducibility of the whole analytical method. It is determined using the method to assay a sample for a sufficient number of times to obtain statistically valid results. Precision was determined by studying the intra- (repeatability) and inter-day precision. The intra- and inter-day precision was determined at three different concentration levels of mangiferin standards 60, 80, and 100 ppm and the sample solution (100 ppm). The intra-day precision was examined by 6 times within 1 day, whereas the inter-day precision was examined for 3 consecutive days. The precision was expressed as percentage relative standard deviation (% RSD).

Accuracy

Accuracy is a measure of closeness of test results obtained by a method to the true value. The accuracy of the method was tested by performing the recovery studies at three different levels of standard stock solution added to the samples. The standard stock solution was spiked into the samples to determine recovery. Three different volumes (2.5, 5.0, and 7.5 ml) of standard stock solution (500 ppm) were added to the sample solution (100 ppm). Triplicate injections were made with all the spiked samples.

% of Recovery= $(b - a)/c \times 100$

where, 'a' is the amount of drug found in the sample before addition of standard drug

'b' is the amount of drug found after addition of standard drug

'c' is the amount of standard drug added

Limit of detection and limit of quantification

Detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated under the stated experimental conditions. Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with

accuracy. LOD and LOQ were determined based on the signal-to-noise ratio response. For LOD, it should not be not <3. For LOQ, it should not be not <10.

Robustness

The robustness is evaluated by varying the analytical parameters. The robustness is evaluated by varying the analytical parameters such as changing the wavelength parameter from 254 to 256 nm and by changing the mobile phase concentration of formic acid from 0.1% to 0.08%. The robustness of the method was done at three different standard concentration levels 60, 80, and 100 ppm and sample concentration of 100 ppm at triplicate injections. The corresponding peak areas were recorded and expressed in terms of % RSD.

Statistical analysis

Results were expressed as mean ± standard deviation. The data were submitted to statistical analysis using Excel software.

RESULTS AND DISCUSSIONS

An RP-HPLC method was developed and validated for the determination of mangiferin in *M. indica*. The chromatographic conditions were optimized to provide a good performance of the assay. During optimization of the method, different mobile phases, different stationary phases, and organic modifiers were tried to get a best separation of peaks. The Kinetex XB-C18 column (100 Aº, 250 mm × 4.6 mm, 5 µm pore size) provided better separations of mangiferin in the extract using combination of acetonitrile and 0.1% formic acid. The maximum absorption of mangiferin was detected at 254 nm and this wavelength is chosen for the assay method. The retention time of mangiferin standard (100 ppm) and sample (100 ppm) solution was at 6.129 and 6.151, respectively [Figure 1]. The chromatographic parameters such as column efficiency and peak symmetry were done to mangiferin standard according to the ICH guidelines.[27] The theoretical plates and tailing factor were 11763 and 1.307, respectively, which indicated column efficiency is satisfactory. LC-MS showed M/Z value of 421 $[M - H]^-$ for hydroalcoholic extract of *M. indica* and reference standard mangiferin. In literature, same M/Z value observed for mangiferin.[28]

Linearity was evaluated by the peak area against concentrations of mangiferin standard in the range of 20–140 ppm, and the calibration plots were linear are summarized in Figure 2. The correlation coefficient (r) of mangiferin is 0.999 (n = 7). This indicates good fitting of the curve and the method is good linearity corresponds to peak area on concentrations. The minimum acceptable correlation coefficient is 0.990.[26,27] The calibration curve was represented by the linear equation y = 28477x – 10771 for mangiferin (where y is the response as peak area and x is the concentration).

The data [Table 1] pertaining to precision test revealed that in intraday, the % RSD of peak area of mangiferin standard (60, 80, and 100 ppm) and sample (100 ppm) concentration was found to be 0.05, 0.05, 0.06, and 0.10, respectively. In interday, the % RSD of peak area of mangiferin standard (60, 80, and 100 ppm) and sample concentration (100 ppm) was found to be 0.42, 0.52, 0.30, and 0.83, respectively. The method developed in the present study shows the acceptable intra- and inter-precision with RSD value <1.0%. This indicated the method was found to be precise and reproducible.

The recovery study was determined by the method of standard addition. The excellent recovery values at three different levels (50%, 100%, and 150%) of spiked samples were found to be 100.47%, 100.89%, and 100.99%, respectively, and low standard deviation value <1% shows the high accuracy of the method. Therefore, this HPLC method can be regarded as selective, accurative, and precise. The results of recovery study are summarized in Table 2.

LODs at signal-to-noise ratio of (3:1) the smallest concentration of mangiferin standard that gives a measurable response was found to be 0.48 ppm, and the LOQs, at signal-to-noise ratio of (10:1), was found to be 1.95 ppm for mangiferin. The robustness of an analytical procedure is its ability to remain unaffected by small variation in the analytical parameters. This indicates the proposed method was stable, precise, and reproducible.

CONCLUSION

The developed assay by HPLC method is a simple, rapid, and reliable for the determination of mangiferin from the bark extract of *M. indica*. This method was proved to be useful for the determination of the purity of the drug available from various extracts by detecting the related impurities in quality control laboratories. It has advantages over all available methods in the literature because it is faster and a mobile phase composition is simpler. With the growing demand for herbal drugs, the development of standardization tool will help in maintaining the quality control and herbal preparations of *M. indica*. Further explorations are needed to investigate the standardization of individual phytoconstituents of *M. indica*.

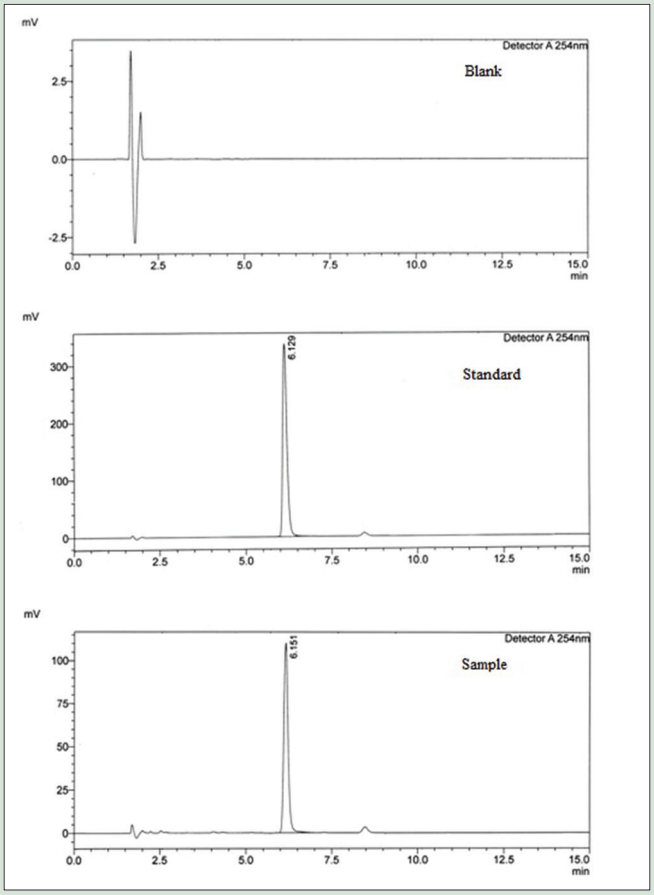

Figure 1: High-performance liquid chromatography chromatograms of blank, standard, and sample. The retention time of mangiferin standard and sample peak were found at 6.129 at 6.151, respectively. The theoretical plates and tailing factor of standard peak are 11763 and 1.307 were observed

Table 1: Intra- and inter-day precision study for the developed method for mangiferin

Concentration (ppm)	Intra-day precision			Inter-day precision		
	Mean area	SD	Percentage RSD	Mean area	SD	Percentage RSD
Standard 60	1,696,775	795	0.05	171,8271	7214	0.42
Standard 80	2,253,572	1066	0.05	2,301,559	12,038	0.52
Standard 100	2,829,830	1625	0.06	2,866,490	8623	0.30
Sample 100	931,188	973	0.10	945,449	7854	0.83

Values are expressed as mean±SD (n=6). SD: Standard deviation; RSD: Relative standard deviation

Table 2: Recovery study for the developed method for mangiferin

Recovery study of mangiferin					
	Volume of reference standard stock solution added (ml)	Mean area	SD	Percentage RSD	Percentage of recovery
50% spiked sample	2.5	2,359,028	1592	0.07	100.47
100% spiked sample	5.0	3,818,602	1679	0.04	100.89
150% spiked sample	7.5	5,277,383	13,543	0.25	100.99

Values are expressed as mean±SD (n=3). SD: Standard deviation; RSD: Relative standard deviation

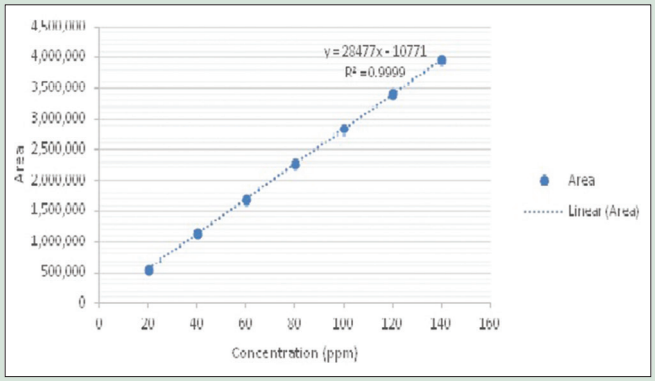

Figure 2: Calibration curve for mangiferin by plotting peak area against concentration and regression equation

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Nil.

Conflicts of interest

The authors declare no conflicts of interest.

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