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Original Article

Pharmacognostic Specifications and RP-HPLC Analysis of *Manilkara hexandra* Stembark

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ABSTRACT

Background: *Manilkara hexandra* (Roxb.) Dubard (Sapotaceae) is valued in traditional system of medicine as febrifuge, astringent, tonic, antiperiodic, antidysentric etc. **Objectives:** The current study aimed for establishing quality control parameters accompanied by development of validated Reversed-Phase Liquid Chromatographic (RP-HPLC) method of four bioactive flavonoids (quercetin, luteolin, kaempferol and apigenin) in stembark. **Materials and Methods:** The stembark was evaluated for complete pharmacognostical parameters such as macroscopy, microscopy, ash value and extractive values. Chromatographic separation was performed on a C_{18} column with a mobile phase consisting of 0.5% orthophosphoric acid and 100% methanol (40:60 %v/v), at a flow rate of 1.0 mL/min. The analysis was performed using a UV detector at different wavelengths. The method was validated in terms of selectivity, linearity, accuracy, precision, robustness and recovery. **Results:** Transverse section of the stembark showed presence of rhytidoma, discontinuous rows of phloem fibres associated with idioblasts, ceretanchyma and few latex cells in phloem. In HPLC study, good linearity was observed over the investigated concentration range of 5-30 µg/mL for quercetin and luteolin; 2-64 µg/mL of kaempferol and 2-12 µg/mL of apigenin with correlation coefficient (r^2) values greater than 0.998. The intra- and inter-day precision over the concentration range was <0.57% (relative standard deviation) and the accuracy were between 98.06 and 100.65%. The %RSD of recovery for all the analytes was 0.49-0.81%. **Conclusion:** The information derived on pharmacognostic parameters and validated HPLC method for estimation of four bioactives for *M. hexandra*, would aid as coherent measures for its quality assessment.

Keywords: Flavonoids, *Manilkara hexandra* stembark, RP-HPLC (Reverse Phase High-Performance Liquid Chromatography), Standardization.

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INTRODUCTION

Manilkara hexandra (Roxb.) Dubard [Synonym: *Mimusops hexandra* (Roxb.) Dubard] commonly known as 'Khirni or Rayan', is found in central India and the Deccan Peninsula and cultivated throughout the greater parts of India.[1] Ethnomedicinally, stembark is popularly used as an astringent, aphrodisiac, stomatitis, fever, jaundice, asthma, diseases of gum and teeth as well as vitiated conditions of Pitta.[2] It contains approximately 10% tannin, rendering it valuable for tanning purposes.[2] The plant is reported to exhibit antioxidant, antiulcer, anti-inflammatory, antiviral, antimicrobial, immunostimulant and antidiabetic activities. Methanolic extract of stembark showed the presence of significant amounts of phenolic contents, phlobatannins, alkaloids, flavonoids, steroids, terpenoids, saponins, reducing sugars.[3] Flavonoids playing pivotal roles in a spectrum of health-promoting properties and finding indispensable applications in pharmaceuticals, cosmetics, nutraceuticals and medicinal realms.[4] HPLC is one of the most accurate and widely used analytical method for the quantitative as well as qualitative analysis of crude drugs,[5] optimization of HPLC method is a complex process which include several variables like mobile phase and its pH, buffer concentration, flow rate, column temperature, detector wave length, etc. are to be concurrently controlled in attaining the desired separations.[6] Among all HPLC detectors, the most simple and the most widely used is UV detector.[7]

As the reports on the pharmacognostical research of this plant is lacking till date, the present study was carried out with the objective to perform macro-microscopical studies and RP-HPLC method development for phytochemicals quercetin, kaempferol, luteolin and apigenin in the stembark of *M. hexandra* and its validation as per the International Council for Harmonisation (ICH) guidelines.[8]

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MATERIALS AND METHODS

Plant material

Stembark was freshly collected during the monsoon season from the medicinal garden of authors' institute. The bark was identified and authenticated by the taxonomist of Gujarat University, Gujarat, India. Voucher specimen has been deposited at the department of the authors. Fresh stembark was cleaned, dried in a hot air oven to maintain a 60ºC temperature and powdered to 80# used for further study.

Chemicals and reagents

Standards quercetin, kaempferol, luteolin and apigenin were procured from Sigma Aldrich, India. All the solvents used were of chromatography grade and other chemicals used were of Analytical (AR) grade.

Pharmacognostical studies

Stembark was studied for morphological characters. Microscopical study was performed for both entire (free hand transverse sections) and powdered material. Moisture content, ash values and extractive values were determined.[9]

Estimation of marker compounds in *M. hexandra* stembark by RP-HPLC Method

Chromatographic conditions

Shimadzu HPLC system equipped with an SPD-40V Ultraviolent (UV) detector, SIL-40C autosampler, CTO-10ASVP column oven and LC-20AD pump using LabSolutions software version 6.110. The chromatographic separation for the HPLC method was achieved using a Shimadzu shim-pack solar column (5 µm C18, 4.6×250 mm), with column oven temperature maintained at 25ºC throughout the analysis. The mobile phase consisted of 0.5% orthophosphoric acid (Solvent A) and 100% methanol (Solvent B) (40:60, *v/v*). The mobile phase flow rate was 1.0 mL/min with isocratic elution. The injection volume was 20 µL and samples were run for a total of 20 min. The detector is configured specifically for the detection of various flavonoids quercetin was detected at 370 nm and kaempferol at 367 nm, while luteolin and apigenin exhibited absorption maxima at 350 nm and 340 nm, respectively. The initial analysis of the sample extract involved the setup of a UV detector based on recommendations from reference book[10] for details on the spectral maximum of flavonols and flavones. The high-performance liquid chromatography conditions for *M. hexandra* stembark extract was fine-tuned based on literature data.[11]

Extraction

Precisely weighed 100 g of plant powder was defatted using petroleum ether. Subsequently, the defatted material was refluxed for 8 hr in a Soxhlet apparatus using methanol and dried to yield 26.2 g of extract. Tannins were removed from 20 g of this extract by re-dissolving in methanol to get a saturated solution followed by adding 10% polyvinylpyrrolidone solution. The resulting tannin precipitate was filtered and the extract was dried under vacuum to yield 6.64 g of reddish-brown semisolid consistency that was dissolved in ethyl acetate. The ethyl acetate layer was collected by filtering and evaporated under vacuum and yield was noted as 0.37 g (ESB).

Preparation of standard stock solution

Accurately weighed 1 mg of standards, quercetin, kaempferol, luteolin and apigenin were transferred to a separate 10 mL volumetric flask and dissolved in methanol. Volume in each case was made with methanol to obtain standard stock solutions of concentration 100 µg/mL for each standard. These stock solutions were further diluted for the studies as required.

Preparation of test solution

Precisely weighed at 50 mg Ethyl acetate Stembark extract (ESB) was dissolved in methanol in a 10 mL volumetric flask. Methanol was then added to achieve a sample stock solution with a concentration of 5 µg/mL. Subsequently, these stock solutions were subjected to further studies.

Analytical method validation

The RP-HPLC method underwent validation following ICH guidelines, encompassing assessments of system suitability, linearity, limits of quantitation and detection, precision, accuracy and robustness.

System suitability studies

System suitability was ensured by conducting six replicate injections of a standard solution containing quercetin, kaempferol, luteolin and apigenin. The % Relative Standard Deviation (% RSD) of peak areas, Tailing factor (T) and theoretical plate Number (N) were subsequently determined.

Calibration curve (Linearity)

The contents of the markers were determined using a calibration curve established with six dilutions of each standard, at concentrations ranging from 5-30 µg/mL of quercetin and luteolin; 2 -64 µg/mL kaempferol and 2-12 µg/mL of apigenin. Each concentration was measured in triplicate. The corresponding peak areas were plotted against the concentrations of the markers injected. Peak identification was achieved by comparison of both the Retention Time (RT) and UV absorption spectrum with those obtained for the reference standards.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ values were derived from the calibration curves using the formula k SD/b, where k equals 3 for LOD and

10 for LOQ. Here, SD represents the standard deviation of the response of the minimum detectable drug concentration, while b denotes the slope of the calibration curve.

Accuracy (Recovery)

Accuracy could refer to how well a system can retrieve lost or corrupted data without errors or loss of integrity. The method involves spiking samples at three distinct levels (50%, 100% and 150%) and conducting triplicate analysis. Recovery is then computed by determining the disparity between the spiked and unspiked samples for each recovery level.

Precision (Repeatability)

Intra-day precision was determined by conducting three analyses of the standard on the same day. Inter-day precision, on the other hand, was determined by carrying out the same analysis every day for three consecutive days, selecting low, medium and high concentrations within the range and conducting triplicate analysis.

Robustness

To demonstrate the robustness of the method, intentional variations were made to the chromatographic conditions. This included adjusting the flow rate of the mobile phase from 1.0 to 0.9 mL/min and from 1.0 to 1.1 mL/min. Additionally, alterations were made to the composition of the mobile phase from 60:40 (methanol: 0.5% orthophosphoric acid) to 65:35 (methanol: 0.1% orthophosphoric acid) and from 60:40 (methanol: 0.1% orthophosphoric acid) to 55:45 (methanol: 0.1% orthophosphoric acid), representing a 5% change. Furthermore, variations in the temperature of the column oven from 25°C to 30°C to 25°C to 20°C (i.e., 25±5°C) were introduced. The sample solution for the robustness study was applied to the column in triplicate and the resulting responses were determined.

Quantification

The developed analytical method was applied for simultaneous determination of the four flavonoids in the ESB samples. An aliquot of 20 µL of sample solution (5 µg mL^{-1}) was run along with a range of standard solutions: quercetin and luteolin ranging from 5 to 30 µg mL^{-1}, kaempferol ranging from 2 to 64 µg mL^{-1} and apigenin ranging from 2 to 12 µg mL^{-1} on the HPLC system. The peak areas were noted and quantification of flavonoids in the ESB sample was performed using linear regression equations of the respective compound.

RESULTS

Pharmacognostical evaluation

M. hexandra is a large evergreen glabrous tree about 50-60 ft in height with a shady head and erect trunk. Bark is blackish grey and deeply furrowed (Figure 1). Results of transverse section of stem bark and powdered drugs are shown in Figures 2 and 3.

Physico-chemical parameters like the moisture content, ash and extractive values were determined (Table 1).

Estimation of marker compounds by HPLC Method

Chromatography

A favourable separation was achieved using a mobile phase composed of methanol and 0.5% Orthophosphoric Acid (OPA) (60:40, v/v) with a flow rate set at 1 mL^{-1}. Quercetin, luteolin, kaempferol and apigenin were eluted at retention times of 9, 10.6, 13.7 and 15.2 min, respectively. These flavonoids are identified in the plant extract through HPLC and based on literature; these phytoconstituents have been shown to possess various pharmacological activities (Table 2).

Analytical method validation

The selectivity of the method was determined by comparing certain parameters of the chromatographic profile, such as retention time, structure of the UV spectrum and λ_{max} of the reference standards and the plant extract sample. The chromatographic profiles of the standard mixture and the extracted sample were identical concerning the parameters mentioned above (Figure 4). In addition, no interference was observed at the retention times of any analytes in the chromatogram of the ESB sample.

Representative calibration curves (Figure 5) obtained from mean data (n=5) for quercetin and luteolin (5-30 µg/mL); kaempferol (2-64 µg/mL) and apigenin (2-12 µg/mL) were found to be linear

Table 1: Physicochemical evaluation.

Parameter	%w/w
Total Ash value.	7.50
Acid insoluble ash.	1.00
Water insoluble ash.	6.00
Water soluble ash.	1.50
Moisture content.	50.79
Water soluble extractive.	6.72
P. Ether soluble extractive.	2.37

Figure 1: *Manilkara hexandra* (Roxb.) Dubard.

Table 2: Activity of phyto-components identified in *M. hexandra* stembark by HPLC.

Sl. No.	Flavonoid subclass	Name of compound	Reported activity
1.	Flavonols	Quercetin	Anti-SARS-CoV-2,[12,13] Antioxidant,[14] Anticancer,[15,16] Antiaging,[17] Antiviral,[18] Anti-inflammatory activities,[19] Preventing photobiologic damage.[20]
		Kaempferol	Anti-inflammatory,[21] Anticoagulant effect,[22] Antiviral,[23] Antimalarial,[24] Antioxidant,[25] Osteoprotective effect,[26] gastric tumour growth inhibitory,[27] Antimicrobial activities.[28]
2	Flavones	Luteolin	Anti-inflammatory,[29] Antiviral,[30] Anti-allergic,[31] Cancer preventive,[32,33] Antibacterial,[34] Antitumor,[35] Antithrombic activities,[36] Protect against Alzheimer disease.[37]
		Apigenin	Therapeutic agent for the treatment of Triple-negative breast cancer[38] Anti-leukemic,[39] Anti-inflammatory,[40] Antioxidant,[41] Antibacterial,[42] Anti-obesity activities,[43] Regulate cholesterol metabolism.[44]

Table 3: Data of intra- and inter-day precision.

Standards	Concentration (µg/mL)	Intra-day (*n*=3)		Inter-day (*n*=3)	
		Area Mean±SD	RSD (%)	Area Mean±SD	RSD (%)
Quercetin	5	167084±514.84	0.308	175047±657.80	0.375
	15	604048±699.65	0.115	614816±1396.35	0.227
	30	1234915±2268.08	0.184	1249951±2978.26	0.237
Luteolin	5	267806±2377.77	0.887	270701±1479.44	0.546
	15	787428±2492.57	0.316	807954±7193.37	0.290
	30	1642146±8721.91	0.531	1639852±3304.33	0.201
Kaempferol	2	97536±894.76	0.917	98579±169.77	0.172
	8	413034±1750.00	0.423	414433±595.52	0.143
	64	3363221±33148.09	0.985	3344987±4073.69	0.121
Apigenin	2	80564±511.76	0.635	81094±353.32	0.435
	6	303551±438.90	0.144	305066±1766.81	0.579
	12	524550±841.04	0.160	524848±1091.02	0.207

*S.D.=Standard deviation; RSD=Relative standard deviation.

Table 4: Results of system suitability study.

Standards	Area (mean±SD)	% RSD	Retention time	Theoretical plate number (N)	Tailing Factor (T)
Quercetin	1846526±2380.79	0.128	8.862±0.050	5507	1.064
Luteolin	2608635±2318.71	0.123	10.538±0.042	6815	1.066
Kaempferol	2704874±839.40	0.031	13.695±0.048	8559	1.051
Apigenin	2430387±3491.12	0.143	15.259±0.094	9730	1.019

*S.D.=Standard deviation; RSD=Relative standard deviation.

with good coefficients of regression (>0.99). LOD and LOQ for quercetin were found to be 0.3128 and 0.9480 µg/mL; for luteolin 0.4589 and 1.3908 µg/mL; for kaempferol 0.4077 and 1.2356 µg/mL while found to be 0.1494 and 0.4530 µg/mL The results suggested that developed method was appropriately sensitive to estimate the respective markers in stem bark extract. RSD of measured peak area (*n*=3) for quercetin, luteolin, kaempferol and apigenin in repeatability study, interday and intraday precision were found to be <2% which suggest that methods are precise for the estimation of respective marker compounds (Table 3).

System suitability was established by injecting six replicate injections (50 µL mL^{-1}) of standard solution the % Relative

Table 5: Results of the robustness study.

Standards	Parameters	Mean±S.D.	% RSD
Flow rate mL/min (±0.1)			
Quercetin (100 µg/mL)	0.9	4256811±30900.20	0.725
	1.0	3846597±20664.58	0.537
	1.1	3508035±5475	0.156
Luteolin (100 µg/mL)	0.9	5205570±23395.41	0.449
	1.0	4736725±21121.02	0.445
	1.1	4031637±12744.07	0.316
Kaempferol (100 µg/mL)	0.9	5256684±20516.59	0.390
	1.0	4751031±9315.91	0.196
	1.1	4340756±5796.78	0.133
Apigenin (100 µg/mL)	0.9	5436149±6228.7	0.114
	1.0	4920505±19191.54	0.390
	1.1	4473496±9659.55	0.215
Column oven temperature (±5°C)			
Quercetin (100 µg/mL)	20°C	3838663±4058.48	0.105
	25°C	3845830±7808.47	0.203
	30°C	3835790±22565.41	0.588
Luteolin (75 µg/mL)	20°C	3549095±9274.13	0.278
	25°C	3565169±9074.20	0.254
	30°C	3549095±14806.80	0.416
Kaempferol (100 µg/mL)	20°C	4761063±12949.52	0.271
	25°C	4735677±38556.42	0.814
	30°C	4717078±35750.07	0.757
Apigenin (75 µg/mL)	20°C	3719263±6617.29	0.177
	25°C	3711624 ±21008.94	0.566
	30°C	3678650±24851.36	0.675
Mobile phase (±5mL)			
Quercetin (100 µg/mL)	55:45	3819504±5440.73	0.177
	60:40	3843866±4809.70	0.566
	65:35	3850773±5008.41	0.675
Luteolin (75 µg/mL)	55:45	3333617±9274.63	0.278
	60:40	3565169±9074.13	0.254
	65:35	3552428±14806.36	0.416
Kaempferol (100 µg/mL)	55:45	4761063±12949.52	O.271
	60:40	4735677±38556.42	0.814
	65:35	4717078±35750.07	0.757
Apigenin (75 µg/mL)	55:45	3719263±6617.29	0.17792
	60:40	3711624±21008.94	0.566031
	65:35	3678650±24851.36	0.675557

*S.D.=Standard deviation; RSD=Relative standard deviation.

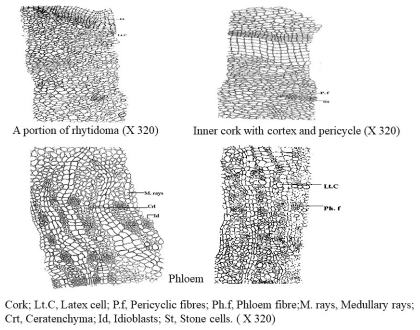

Figure 2: Microscopy of *M. hexandra* stembark.

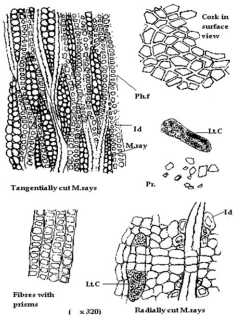

Figure 3: Powder characters of *M. hexandra* stembark.

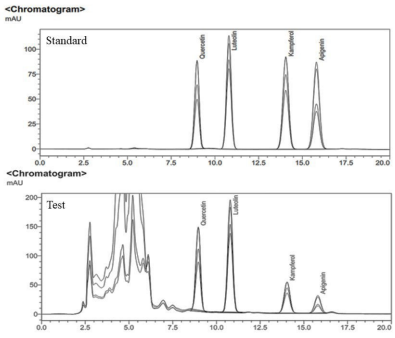

Figure 4: Representative HPLC chromatograms of the standard mixture and sample.

Standard Deviation (% RSD) of retention time, tailing factor and theoretical plates were determined (Table 4).

The percent recovery (n=3) of quercetin from accuracy study were obtained in the range of 100.8-99.08 %; for luteolin 100.65-99.82 %; for kaempferol 99.69-100.24 % and 98.84-100.3 % in apigenin respectively which indicated that method was accurate for estimation of quercetin, luteolin, kaempferol and apigenin in the stem bark extract. RSD for measurement of peak area and R_f values were found to be < 2% after making small deliberate changes in method parameters like flow rate mL/min (±0.1 mL), column oven temperature (±5°C) and amount of mobile phase (±5 mL) for analysis. Results of robustness study (Table 5) suggested that methods were robust for measurement of respective markers.

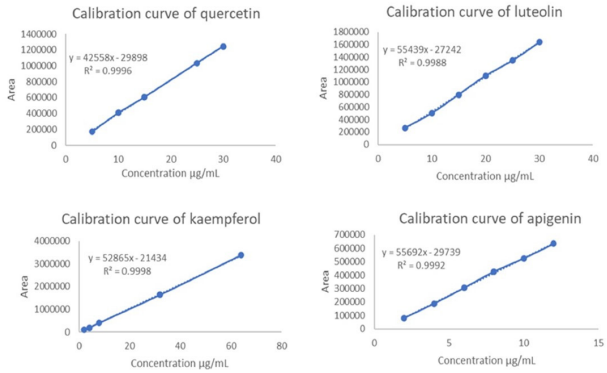

Figure 5: Calibration curves.

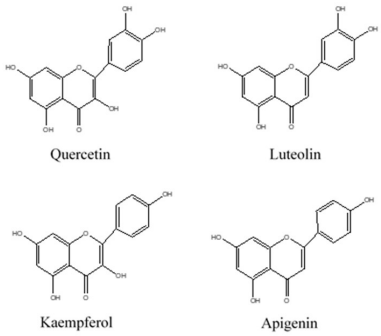

Figure 6: Structures of quercetin, luteolin, kaempferol and apigenin.

Quantification

The Ethyl acetate extract of Stembark (ESB) was found to contain 0.8928% *w/w* of quercetin, 0.8944% *w/w* of luteolin, 0.3115% *w/w* of kaempferol and 0.1863% *w/w* of apigenin. Quercetin and luteolin were found to be the most abundant flavonoids. Structures of quercetin, luteolin, kaempferol and apigenin are shown in Figure 6.

DISCUSSION

The raw material quality is expected to be dependent on geographical location, time of harvesting, improper processing and storage. Thus, economic and precise techniques to measure qualitative specifications of the raw materials used as in process materials and the finished goods are required to be employed. *Manilkara hexandra* (Roxb.) Dubard stembark is commonly used in traditional medicine as an astringent and in treatment of stomatitis, fever, jaundice, asthma and dental disorders.

Bark showed rhytidoma composed of a wide tangential layer of cork, alternating with dead parenchyma consisting of secretory canal, fibres and fibres associated with small groups of stone cells and occasionally idioblasts containing prisms of calcium oxalate; periderm composed of phellum made up of outer thick-walled lignified cells and compressed strongly suberized cells, outer 8-9

rows of secondary cork composed of thin-walled cells followed by strongly thickened tangential cells, well-developed 3-4 layers of tangentially elongated parenchymatous phellogen and narrow parenchymatous phelloderm that is traversed by wide and wedge shaped groups of medullary rays and circular latex cells; wide phloem composed of narrow tangential strips of ceratenchyma, groups of fibres associated with idioblasts containing prisms of calcium oxalate along with few stone cells and uni-tetra seriate radially elongated medullary rays.

Powdered bark showed group of fibres with calcium oxalate sheath; medullary rays in radial and tangential view; thick-walled lignified polygonal cells of cork in surface view and latex cells.

The simultaneous RP-HPLC method for estimation quercetin, luteolin, kaempferol and apigenin in *M. hexandra* was developed and validated according to ICH guidelines. All analytes exhibited satisfactory absorbance at their respective wavelengths, ensuring well-resolved peaks with baseline separation. The proposed HPLC method for estimation of flavonoids was precise, accurate and selective. The method was rapid, economical, sensitive and reproducible.

Thus, the present study reports complete pharmacognostical parameters along with development of RP-HPLC method for the stembark.

CONCLUSION

This is the first report on the pharmacognostic study corroborated with HPLC analysis for *Manilkara hexandra* stembark. The collective of data on standard parameters is useful for the endorsement of quality control and for documenting a monograph on this stem drug. The validated HPLC method for simultaneously identifying and quantifying four predominant flavonoids (quercetin, luteolin, kaempferol and apigenin) in *M. hexandra* is precise, easy and time saving. The validation outcomes demonstrated the method's sensitivity, accuracy and reproducibility. Subsequently, the developed method was effectively employed to determine the flavonoid contents in the ESB sample obtained through different extraction methods. As a result, this method holds promise for the quality assessment of formulation products containing *M. hexandra* extract.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

Manilkara hexandra (Roxb.) Dubard is popularly known as 'Khirni' or 'Rayan' in India. In traditional medicine, stembark is frequently used as an astringent, aphrodisiac, treatment for stomatitis, fever, jaundice, asthma, gum and tooth problems. This is the first report on the pharmacognostic investigation for *M. hexandra* stembark. The simple, precise and time-saving validated RP-HPLC methodology can be used to simultaneously identify and quantify the four main flavonoids (quercetin, luteolin, kaempferol and apigenin) in *M. hexandra*. The ensemble of data on standard parameters is valuable for the approval of quality control and for standardization of this crude drug.

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