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Simultaneous Estimation of Luteolin and Apigenin in Methanol Leaf Extract of *Bacopa monnieri* Linn by HPLC

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Authors' contributions

This work was carried out in collaboration between all the authors. Author AR designed the study, wrote the protocol and completely corrected and revised the entire manuscript. Author NS managed literature searches and carried out the experimental part. Author MK performed the statistical analysis and author VK wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To develop, validate and quantify the content of flavonoids luteolin and apigenin in aerial parts of methanol leaf extract of *Bacopa monnieri* (*B. monnieri*) by reverse phase liquid chromatography.
Study Design: High Performance Liquid Chromatography
Place and Duration of Study: Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore and Indian Pharmacopoeia Commission, Ghaziabad, India from 1.7.2010 to 30.6.2011.
Methodology: Separation and quantification of flavonoids was performed on a C18 column (5µm, 200mm×4.6mm, id.) using potassium dihydrogen phosphate buffer (20mM, pH 3.5 adjusted with ortho phosphoric acid, v/v) and acetonitrile as mobile phase with a flow rate of 1ml/min. The column effluents were monitored at 348nm with column temperature kept at 30±1ºC.
Results: A validated method for simultaneous estimation of luteolin and apigenin was developed, where limits of detection and quantification was found to be 0.03 and 0.91µg/ml for luteolin, 0.041 and 0.13µg/ml for apigenin respectively. The percentage of

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these phytoconstituents recovered was in the range of 98.07-99.71 (%RSD<2%).
Conclusion: The developed validated HPLC method was found to be accurate, precise and robust and may be used for analysis of luteolin and apigenin in the extracts of B. monnieri.

Keywords: Liquid chromatography; estimation; luteolin; apigenin; B. monnieri.

1. INTRODUCTION

B. monnieri Linn (*Scrophulariaceae*), commonly known as Brahmi is widely distributed throughout India in wet, damp and marshy areas [1,2], was used to improve memory, revitalisation of sensory organs [3,4], cardiac disorders, respiratory [5] and for neuropharmacological disorders [6]. Anti-inflammatory, analgesic, antipyretic, sedative [7], antioxidant [8], anti-tumor [9,10] activities are reported for this plant. Significant antioxidant potential [11] and anticancer activity on mouse sarcoma cell [12,13] was reported for *B. monnieri*.

Phytoconstituents apigenin and luteolin was reported be present in *B. monnieri* [14]. A HPTLC method was reported for the quantification of luteolin and apigenin in *B. monnieri* extract [15]. Simultaneous estimation of luteolin and apigenin by RP-HPLC method was not reported so far in methanol leaf extract of *B. monnieri*. Hence the present study was undertaken develop a validated RP-HPLC method for the estimation of the same in the methanol leaf extract *B. monnieri*.

2. MATERIALS AND METHODS

2.1 Material

Leaves of *B. monnieri* were procured from National Botanical Research Institute (NBRI), Lucknow, India.

2.2 Reagents and Chemicals

Methanol AR grade was used for preparation of extracts, while the AR grade orthophosphoric acid and AR grade potassium dihydrogen orthophosphoric acid (Merck, Mumbai, India) were used in HPLC analysis. Solvents used in HPLC analysis are of HPLC grade. Pure apigenin and luteolin was provided by LGC prochem, Bengaluru, India.

2.3 Chromatographic Instrumentation and Conditions

Agilent HPLC system (CA, USA, Hewlett-Packard 1100 series) with a quaternary pump (HP 1311 A), a degasser (Agilent 1322 A), Oyster ODS 3 column (5µm, 200mm×4.6mm, i.d, Merck), a loop injector (20µl, Rheodyne 7725), column thermostat maintained at 30±1ºC. (HP 1316) was used for analysis. A variable wavelength detector (HP 1314A) used for detection was set at a wavelength of 348 nm. Potassium dihydrogen phosphate, pH 3.5 adjusted with orthophosphoric acid, v/v) and acetonitrile were used as mobile phase with the flow rate of 1ml/min.

2.4 Extract Preparation

B. monnieri (10g, coarsely powdered) was transferred into a beaker (250ml). It was macerated with methanol (3×50ml) for 24h and filtered through Whatman No 1 filter paper. Extract obtained was concentrated in a vacuum rotary evaporator (Prama, Germany).

2.5 Sample Solution

B. monnieri extract (10mg) was weighed, transferred into a volumetric flask (10ml) and dissolved in methanol (5ml). The solution was sonicated for 5min and the volume was made up to the mark with acetonitrile. This solution (0.2ml) was further diluted to 10ml with acetonitrile and filtered through 0.45µm nylon filter paper.

2.6 Method Validation

As per ICH guidelines [16] the developed method was validated.

2.7 Linearity and Range

Five different concentrations of standard apigenin and luteolin solutions were prepared in the concentration range of 1-70µg/ml and the analysis was performed in triplicate for each concentration. A plot between peak area and analyte concentration were constructed, where the linearity was calculated by scheming the slope, y-intercept and correlation coefficient (r^2) using Microsoft Excel software (2007)

2.8 Precision

Intra and inter-day assays of six replicate injections of apigenin and luteolin mixture solutions at three concentration levels (1, 30 and 70µg/ml) was carried out for determining the precision of the developed method. At an interval of 4h a 1 day and for a period of over 3 days intra and interday assay precision test was performed respectively.

2.9 Detection and Quantification Limit

The lowest amount of analyte in a sample which can be detected but not necessarily quantitated is called as LOD that can be calculated by the formula; $LOD=3.3\times\sigma/S$. The lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy is termed as LOQ ($LOQ=10\times\sigma/S$ where, σ is the standard deviation of y intercepts of regression line and S is the slope of the calibration curve).

2.10 Accuracy

Standards, apigenin and luteolin (80, 100 and 120%) were added to the pre-analyzed methanol leaf extract of *B. monnieri* solutions, and the content of the standards were determined from the peak areas obtained.

2.11 Robustness

Robustness of the method was assessed by deliberately varying the pH, extraction time and different columns for quantification.

2.12 System Suitability

System suitability test is an integral part of chromatographic method which is performed to ensure that the complete testing system including instrument, reagents and column is suitable for the intended application. Determination of system suitability parameters and limits depend on the method in the method development process. The criteria to be used for system suitability tests at each stage of method development will vary with the requirements of the method and its intended application.

Column efficiency, resolution, peak asymmetry factor and HETP are chromatographic parameters evaluated for assessing the System suitability of method.

2.13 Specificity

Peak angle (θ_p) and peak threshold angle (θ_{th}) was computed using millineum software for assessing the peak purity of the analytes by the developed method. The degree of similarity of UV spectra across the peak in the range of 190-800 nm was compared for confirming the specificity.

3. RESULTS AND DISCUSSION

3.1 Optimisation of Extraction

Selection of solvent for extraction based on the earlier reports [17], where methanol was found to be effective as extraction solvent and yielded high amount of luteolin and apigenin from plants rich amount of flavonoids. The percentage yield was found to be 1.538%.

3.2 Optimisation of Chromatographic Conditions

Chromatographic conditions were optimised by exploring the use of various composition of and wavelength used for detection. Gradient elution was initially attempted with methanol and acetate buffer pH 4, where poor resolution and tailed peaks was obtained. Second gradient elution trail was attempted with acetonitrile and potassium dihydrogen phosphate buffer (20mM, adjusted to pH 3.5 with orthophosphoric acid), where well resolved peaks with less tailing of peaks was obtained.

3.3 Method Validation

Our previous study reported that the method validation parameters such as system suitability, peak purity, linearity regression data, intra and interday precision of the method were performed by using standard solutions of luteolin and apigenin were in the acceptable limit [18].

3.3.1 System suitability

The %RSD values for retention time and peak area, symmetry of peaks were less than 2%, indicating the method is suitable (Table 1). The suitability of the developed method was further confirmed by N>2000, high resolution and Asymmetry factor less than 1.

3.3.2 Specificity

Retention time, resolution and peak purity are the important parameters to assess the specificity of the method. The average retention time for luteolin and apigenin was found to be 7.024 and 7.614min respectively (Fig. 1). Both the flavonoids were well resolved and the peak purity was confirmed as the peak threshold angle (θ_{th}) was found to be greater than that of peak angle (θ_p) (Table 2). Further, luteolin and apigenin peaks in methanol leaf extract of *B. monnieri* were found to be akin in shape to the peaks of standards, confirming its purity (Fig. 2).

Table 1. System suitability parameters of luteolin and apigenin [18]

Parameters	Luteolin (1µg/ml)	Apigenin (1µg/ml)
Number of theoretical plates (N)	21211	33492
Asymmetry factor	0.69	0.813
Resolution	5.42	3.11
HETP	0.94	0.59

Table 2. Peak purity profile of luteolin and apigenin [18]

Components	Peak angle	Threshold
Luteolin (5µg/ml)	0.669	3.773
Apigenin (5µg/ml)	0.321	4.601

3.3.3 Linearity and range

It is the ability to obtain test results which are directly proportional to the concentration of analyte in the sample. Linearity can be assessed by visual inspection of the plot and by statistical techniques viz. correlation coefficient, y-intercept and slope. Linear regression analysis assessed confirms the linear relationship between the concentration of the flavonoids and peak area as the r^2 values for both the standards were found to be >0.999. The linear regression equation of y-intercept and slope was provided in (Table 3). Range is the interval between upper and lower concentration of the analyte in the sample as it has been demonstrated that the procedure has a suitable level of precision, accuracy and linearity, where the concentration of both the standards were in the range of 1-70 µg/ml.

3.3.4 Precision

Precision is the degree of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample. The %RSD values for retention time and the peak area for both intra and interday assay precision (intraday, 4h, six injections; interday, 3 days; Table 4) was found to be within 1% and 2% respectively.

3.3.5 Accuracy

Accuracy is the degree agreement between observed value and expected value. It was performed by spiking the standard with samples, where the accuracy of the method will be assessed based on the amount recovered. The percentage recovery (Table 5) of the flavonoids is in the range of 98.12-99.74%, and the % RSD values were within 2%, suggesting the method is accurate.

Table 3. Linear regression data for the calibration curves (n=3) [18]

Parameter	luteolin	apigenin
Retention time(min)	7.024±0.118	7.614±0.107
Detection wavelength	348nm	348nm
LOD µg/ml	0.03	0.041
LOQ µg/ml	0.91	0.13
Linearity range µg/ml	1-70	1-70
Correlation coefficient(area)	0.9995	0.9998
Regression equation(area)	y=151.99x+ 26.608	y=124.16x+ 37.51

Table 4. Intra and interday precision of the developed method (n=6) [18]

Components	Intraday				Interday			
	Retention time		Peak area		Retention time		Peak area	
	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
luteolin								
1µg/ml	7.09	0.137	302	0.753	7.06	0.069	297	0.953
30µg/ml	7.05	0.241	4492	1.185	7.01	0.264	4469	1.363
70µg/ml	7.02	0.273	10748	0.935	6.96	0.369	10639	1.274
apigenin								
1µg/ml	7.59	0.182	217	0.581	7.57	0.093	209	0.936
30µg/ml	7.62	0.178	3754	1.184	7.59	0.397	3684	1.425
70µg/ml	7.64	0.358	8649	1.327	7.62	0.274	8598	1.638

Table 5. Recovery study of luteolin and apigenin from the extract of *B. monnieri*

Components	Quantity added %	Total quantity present, µg/ml	Amount quantity found, µg/ml	Recovery,%	%RSD*
	0	0.870	0.862	99.08	0.54
	80	1.566	1.549	98.91	0.99
luteolin	100	1.740	1.71	98.27	1.32
	120	1.914	1.897	99.11	1.71
	0	1.738	1.733	99.71	0.93
	80	3.128	3.094	98.91	1.28
apigenin	100	3.476	3.409	98.07	1.53
	120	3.823	3.791	99.16	1.28

**average of 3 determinations*

3.3.6 Detection and quantification limit

Detection and quantification limit was found to be 0.05 and 0.15µg/ml, respectively, for luteolin; 0.041 and 0.13µg/ml for apigenin respectively.

3.3.7 Robustness

Variation in pH, extraction time and different columns not affected the results of the quantification study (Table 6). The percentage recovery was found to be in the range of 98-101% for both the flavonoids.

Table 6. Robustness of the method (n=6)

Parameters changed		Recovery, % ± SD	
		luteolin	apigenin
Extraction time	20h	99.32±0.92	99.23±0.32
	24h	99.68±1.02	102.53±0.85
	48h	100.08±0.96	99.78±0.23
pH	3.0	98.36±0.85	99.86±0.98
	3.5	99.26±1.20	101.28±0.89
	4.0	99.36±0.91	99.53±1.68
Column brand	Zobrax	99.39±0.84	101.35±0.98
	Oyster	101.52±0.97	99.56±0.56
	Hypersil	99.52±0.98	101.25±0.96

3.3.8 Applicability of the developed method

The proposed method was successfully applied for the simultaneous determination of Luteolin and apigenin in the methanol leaf extract of *B. monnieri* by a validated RP-HPLC method Fig. 2. The analytes were identified by their retention times (t_R) of the sample injected which is in correlation with the retention time of standards. The sample matrix not elicited any response when injected along with the standard flavonoids that the method is specific. The percentage of luteolin and apigenin in methanol leaf extract of *B.monnieri* was found to be 0.22% and 0.45% respectively.

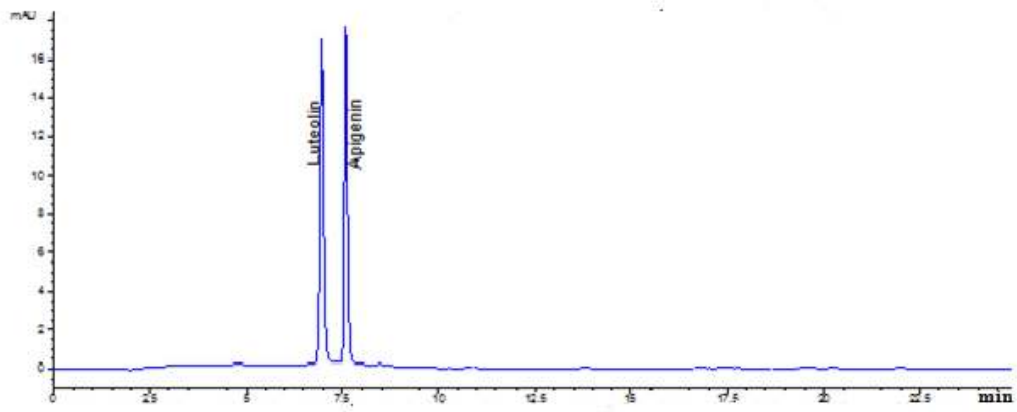

Fig. 1. Chromatogram of luteolin and apigenin standard (0.5µg/ml)

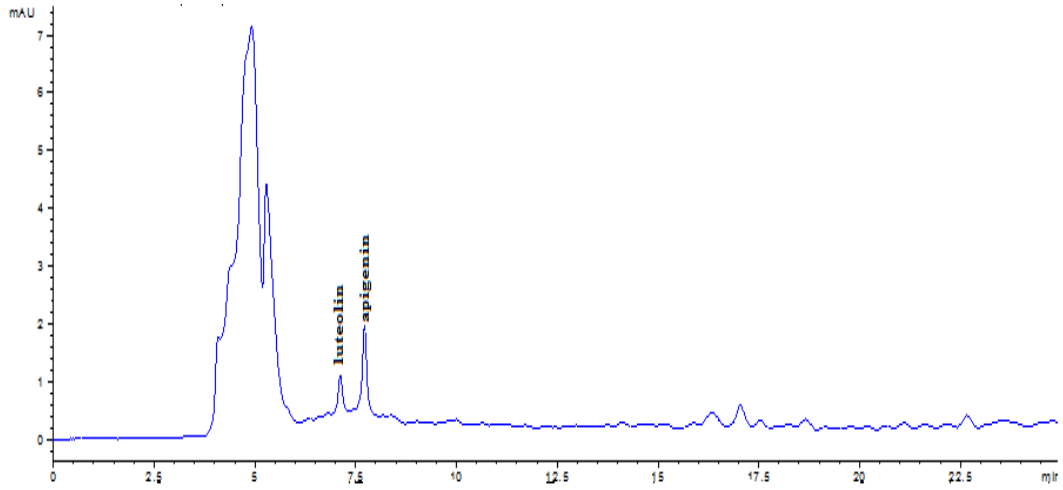

Fig. 2. Chromatogram of methanol leaf extract of *B. monnieri* (20µg/ml)

4. CONCLUSION

A novel method for the simultaneous estimation of luteolin and apigenin in methanol leaf extract of *B. monneri* was developed. Method validation confirmed that the method is simple, accurate, precise and sensitive. The proposed method can be used for routine analysis in quality control laboratories for the estimation of these two flavonoids in the methanol leaf extract of *B.monnieri*.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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