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SIMULTANEOUS ESTIMATION OF APIGENIN AND LUTEOLIN IN *ACHILLEA MILLEFOLIUM* L. FROM NILGIRIS BY HPLC METHOD

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ABSTRACT
The objective of this study was to develop and validate a precise and sensitive high-performance liquid chromatographic method for the simultaneous estimation of apigenin and luteolin in the extract of Achillea millefolium Linn. herb. The chromatographic separation was achieved by using Hibar Lichrospher C_8 (150 x 4.6mm i.d., 5µ) column. The system was operated using methanol and 0.5% trifluroacetic acid (80:20% v/v) as mobile phase, with flow rate 1 ml/min and UV detection at 259 nm. The retention time for apigenin and luteolin was 2.947 and 3.691 min respectively. The proposed method was validated in terms of linearity, precision and accuracy, robustness, detection and quantitation limit. A sensitive, accurate and specific analytical method was developed and validated for the simultaneous estimation of apigenin and luteolin in the aqueous Ethanolic and methanolic extract of A. millefolium L. This method can be successfully employed for simultaneous estimation of apigenin and luteolin in Achillea millefolium L. from Nilgiris.

Keywords: *Achillea millefolium L., Yarrow, Apigenin, luteolin, HPLC*

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INTRODUCTION

Achillea millefolium Linn. is a well-known medicinal plant, widely used in folk medicine for centuries. The presence of flavonoids is of great importance as these substances are known to have a strong spasmolytic, choleretic, anti-oxidative and antimicrobial action.[1]

It has been shown that the anti-diabetic and gastro protective properties of extracts from *Achillea* plants may be linked to their antioxidant potential, therefore, it is of high importance to investigate their antioxidant effectiveness. Recent reports indicate that the *Achillea* genus displays a relevant antioxidant activity that is associated or correlated well with its flavonoid and total phenolic contents.[2] Flavonoids are one of the most important groups of bioactive compounds in plants, which exist in the free aglycones and the glycoside forms showing a diverse structure and a broad range of biological activities. Flavonoids include several classes of compounds with similar structure having a C6-C3-C6 flavone segment. They are differentiated on the degree of unsaturation and oxidation of the three carbon segment. Flavonoids represent an important bioactive component in Achillea millefolium.[3]

Since 1975, several studies on the phytochemical composition of *A. millefolium* L. have been reported and led to the identification of flavonoids.[4,5] All these studies increased the knowledge on the chemical composition of this species but, to date, a complete characteristic of its phenolic compounds is not yet available. Concerning the bioactivity of this plant, recent studies reported antimicrobial, inflammatory and spasmodic gastrointestinal complaints, hepato-biliary disorders, as an appetite enhancing drug, against skin inflammations and for wound healing due to its antiphlogistic, choleretic and spasmolytic properties.[6] Chromatographic methods such as HPLC and thin layer chromatography have been used for flavonoid identification and quantification in the genus *Achillea.*[7,8]

OBJECTIVE

The literature survey showed that there was no report about the simultaneous estimation of flavonoid constituents of the Indian species. In the present study, the quantification of main flavonoid which was found to be characteristic for *A. millefolium* L. was reported. Hence, the objective of this study is to develop simple and sensitive methods for the simultaneous quantification of apigenin and luteolin in the hydro alcoholic and methanolic extract of *A. millefolium* L.

EXPERIMENTAL

Materials and reagents

Apigenin (97.8%) and luteolin (98.1%) were purchased from Natural Remedies Ltd., Bangalore. Methanol and ethanol were of HPLC grade from Qualigens fine chemicals. All the reagents and chemicals were of analytical grade. Water (HPLC grade) was obtained from Milli Q RO system.

Plant Material

The whole plant of *A. millefolium* L. was collected during the month of February 2012, from Emerald, Ooty, The Nilgiris. The plant was botanically identified, confirmed and authenticated by Dr. S. Rajan, Field botanist, Department of Ayush, Ministry of Health and Family welfare, Govt. of India, Emerald, Ooty.

Extraction

The whole plant of *A. millefolium* (70 g) were dried in shadow and cut into small pieces. Powdered crude material (75 g) was extracted exhaustively with hydro alcohol and methanol in a Soxhlet apparatus. The liquid extract was evaporated and dried under vacuum to give a gummy extract.

Chromatographic conditions

The simultaneous estimation of apigenin and luteolin was performed on Waters isocratic HPLC system equipped with Waters 1515 isocratic solvent delivery system, Waters 2487 dual wavelength UV absorbance detector and Rheodyne 7725i injector with 50 µl loop volume. Waters Breeze 3.3 data station was used to record the chromatograms and to calculate the chromatographic parameters. Separation of both the components was achieved using C_8 column, 150 x 4.6mm i.d., 5µ Hibar Lichrospher. The mobile phase, methanol and 0.5% trifluroacetic acid (80:20), was pumped with a flow rate of 1 ml/min. The elution was monitored at 259 nm. Peak identity was confirmed by spectrum and retention time comparison. All the analysis was performed at room temperature.

Preparation of standard solution

The standard stock solution (1 mg/ml) of apigenin and luteolin were prepared in methanol. These stock solutions were stored in light resistant containers. Aliquots of apigenin and luteolin (1-5 mcg/ml) were prepared in the mobile phase.

Preparation of sample extracts solution

About 5 mg of each extract was weighed and transferred into a 10 ml volumetric flask. To this 5 ml of mobile phase was added and sonicated for 10 mins. The resulting solution was made up to 10 ml with mobile phase and filtered through Whatmann filter paper No.42. Aliquots of the sample were prepared in the mobile phase. The standard and sample solutions were analyzed by the optimized chromatographic conditions and the chromatograms were recorded.

RESULTS AND DISCUSSION

Qualitative and quantitative composition of apigenin and luteolin in Achillea extracts

Upon application of the developed method, well-separated peaks were obtained for both apigenin and luteolin (*Figure 1*). Apigenin and luteolin were identified in *A. millefolium* herb extract. The quantitative analysis revealed that apigenin (14.73 ± 0.22 mg/g) predominated in the hydro alcoholic extract and 12.50 ± 0.28 mg/g in the methanolic extract of *A. millefolium* L., whereas luteolin were determined in lower quantities (12.02 ± 0.14 mg/g) in hydro alcoholic extract and 11.10 ± 0.17 mg/g in the methanolic extract of *A. millefolium* L. herb. The chromatograms of hydro alcoholic and methanolic extracts containing apigenin and luteolin contents were given (*Figure 2 and 3*).

Method validation

For validation of analytical methods, the guidelines of the International Conference on the Harmonization [9, 10] have recommended the accomplishment of linearity, accuracy tests, precision, detection and quantitation limit and robustness of the method.

Linearity

For linearity study, five solutions in the range of 1-5 µg/ml for apigenin and luteolin were analyzed. Each concentration was made and analyzed in triplicate. The peak areas obtained against each concentration of the analytes were used to build a linear regression equation and to determine value of correlation coefficient (table 1). Good linearity was observed over the above-mentioned range with linear regression equation Y= 163491x-38452 for apigenin and Y=48642x-6253.7 for luteolin (x is concentration of analytes in µg/ml and Y is peak area). The value of correlation coefficient was found to be 0.992 for apigenin and 0.997 for luteolin. The results indicate that the method is linear over the concentration range studied (fig. 4, 5).

Accuracy

This study was performed by adding known amounts of apigenin and luteolin to the placebo solution. Three level of solutions were made having concentrations of 10, 20 and 30 µg/ml for apigenin and luteolin. The recovery range for apigenin and luteolin was found to be 99.70 to 100.11% and 99.63% to 99.80% respectively (limit 98 to 102%). The relative standard deviation ranged from 0.135% to 0.8% for apigenin and from 0.35% to 0.73 % for luteolin (table 2).

Precision

Repeatability was studied by calculating the relative standard deviation (RSD) for six determinations of the concentration of about 1 mg/mL, performed on the same day and under same experimental conditions. The results of apigenin and luteolin determinations in the working standard solution with the relative standard deviation were calculated as 0.94% and 1.26% respectively. Intermediate precision studies include the estimation of variations in analysis when a method is used within laboratories, on different days. The RSD values obtained for apigenin and luteolin were 0.97 and 1.268% respectively (Table 3).

Limit of detection and quantification

LOD were calculated by using the following equations.

LOD = 3.3 x SD/S and LOQ = 10 x SD/S, where SD = the standard deviation of the response, S = Slope of the calibration curve. The LOD values were found to be 26 µg/ml for apigenin and 48 µg/ml for

luteolin. The LOQ values were 79 µg/ml and 145 µg/ml for apigenin and luteolin respectively.

Robustness

The robustness of the proposed method was evaluated by deliberately changing the chromatographic conditions. The results showed that varying the chromatographic conditions had no appreciable effects on the chromatographic parameters table 4.

System suitability

The results of system suitability tests are given in table 5 showing that the parameters are within the suitable range.

CONCLUSION

This study established a quantitative method for the simultaneous determination of apigenin and luteolin from *A. millefolium* L. in Nilgiris. The results shows that *A. millefolium* L. contains considerable amounts of flavonoids, demonstrates that *A. millefolium* L. could be considered as a potential source of natural health-promoting antioxidants for medicinal and food applications. The proposed analytical method for simultaneous estimation of apigenin and luteolin in the extracts of *A. millefolium* L. is accurate, precise, linear, robust, reproducible and within the range. Hence the present RP-HPLC method is suitable for the quality control of the raw materials, extracts and assay of the markers in *A. millefolium* L.

Fig. 1. Typical HPLC Chromatogram of Apigenin and luteolin standard solution

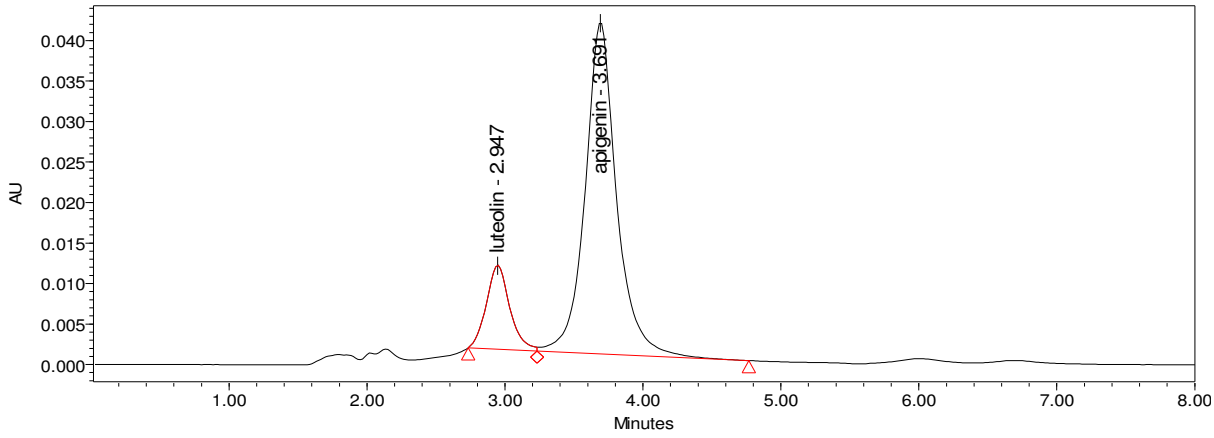

Fig. 2. Typical HPLC Chromatogram of aqueous ethanol extract containing apigenin and luteolin

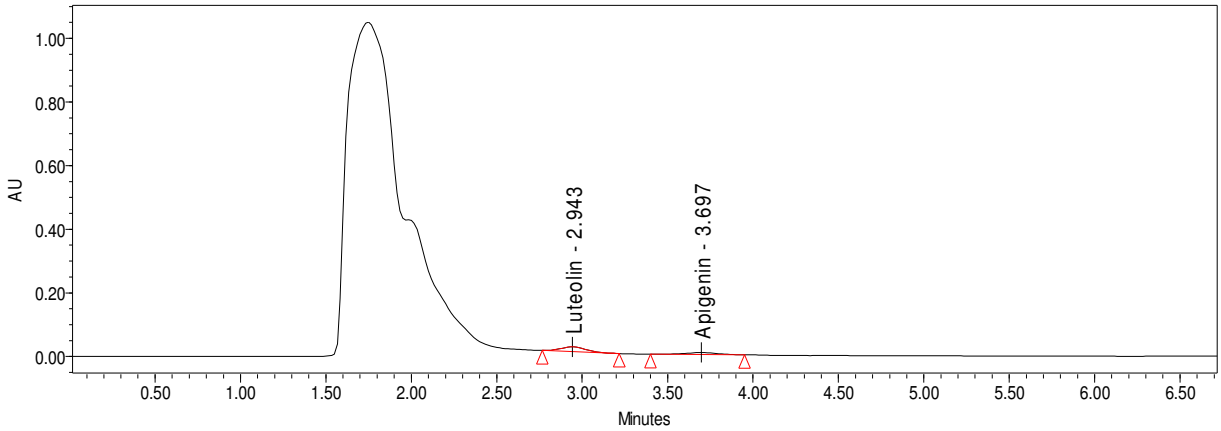

Fig. 3. Typical HPLC Chromatogram of methanolic extract containing apigenin and luteolin

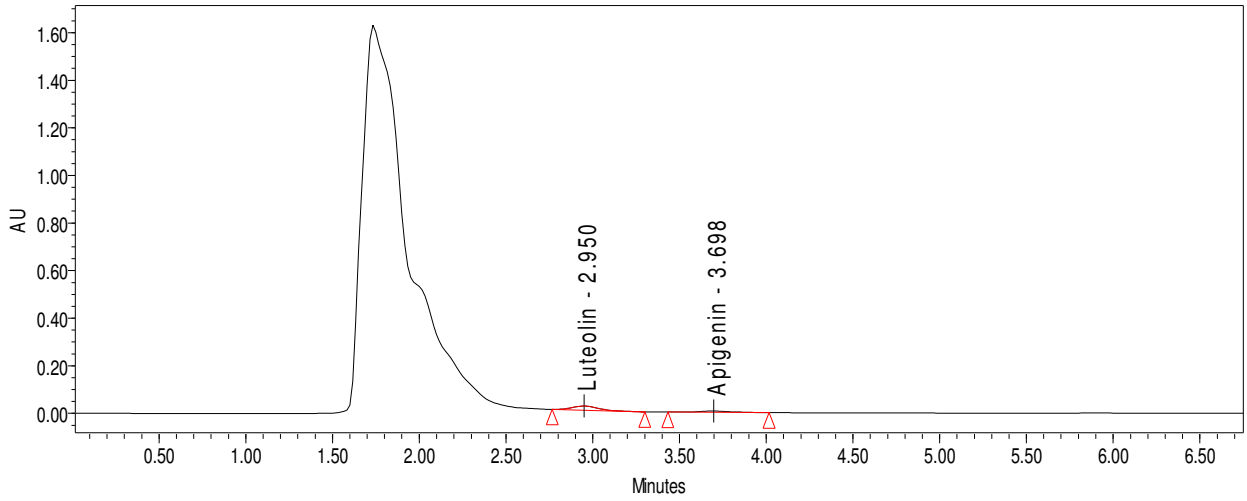

Fig. 4. Calibration curve of Apigenin by HPLC

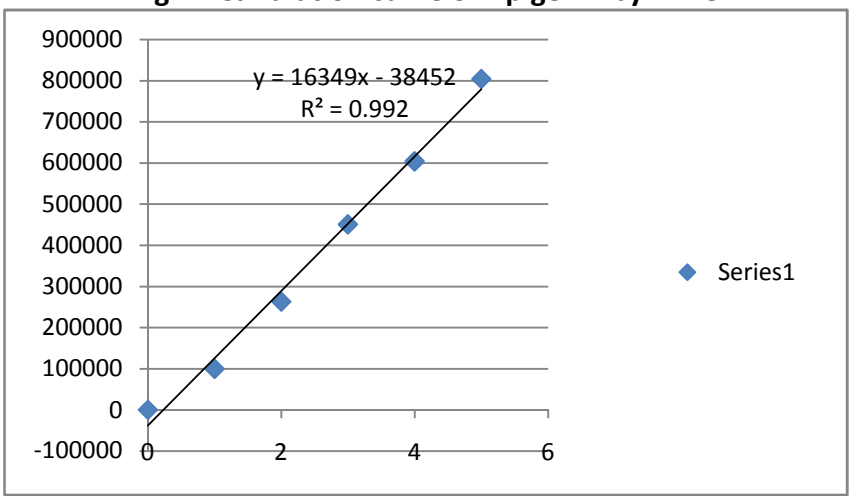

Fig. 5. Calibration curve of Luteolin by HPLC

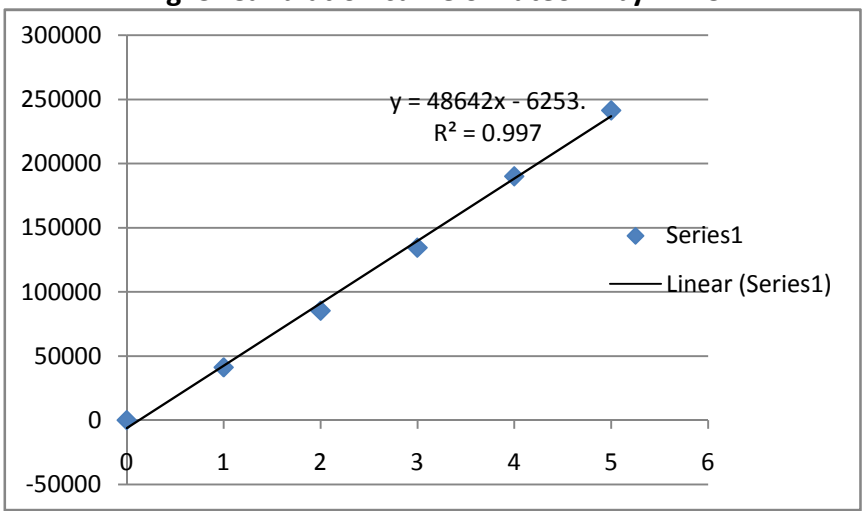

Table 1: Linearity and Range for apigenin and luteolin by HPLC

Sl.No.	Concentration of Apigenin and Luteolin (mcg/ml)	Peak area	
		Apigenin	Luteolin
1	01	99608	41118
2	02	262981	85286
3	03	450812	134360
4	04	604030	189978
5	05	804216	241361

Table 2: Recovery and accuracy data

Compounds	Recovery		
	Amount Added (mg/ml)	Recovery (%)	RSD (%)
Apigenin	0.01	100.11	0.135
	0.02	100.08	0.80
	0.03	99.70	0.42
Luteolin	0.01	99.8	0.66
	0.02	99.75	0.36
	0.03	99.63	0.73

Table 3: Precision studies for apigenin and luteolin

Compound	Conc. (mg/ml)	n	Intra day		Inter day	
			Mean	%RSD	Mean	%RSD
Apigenin	1	6	1.003	0.94	1.003	0.97
Luteolin	1	6	0.990	1.26	0.993	1.268

Table 4: Robustness study of the proposed HPLC method

Parameter	Conditions	Retention Time	
		Apigenin	Luteolin
Flow Rate (ml/min)	0.9	4.35	3.48
	1.0	3.69	2.94
	1.1	3.60	2.90
Mobile Ratio (v/v)	72:28	3.62	3.00
	70:30	3.69	2.94
	68:32	4.1	2.60

Table 5: System suitability studies for estimation of apigenin and luteolin by HPLC

S.No.	Parameters	Apigenin	Luteolin
1	Linearity range	1-5 mcg/ml	1-5 mcg/ml
2	Regression equation	Y= 163491 x -38452	Y= 48642 x -6253
3	Correlation coefficient	0.992	0.997
6	Asymmetric factor	1.0	1.1
7	LOD (mcg/ml)	26	48
8	LOQ (mcg/ml)	79	145

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