# Development and Validation of RP-HPLC Method for Simultaneous Determination of Apigenin and Luteolin in Ethanol Extract of *Clerodendrum serratum* (Linn.) Leaves

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**ABSTRACT—** ***A simple, specific, precise, accurate, and sensitive method for separation and quantification of two flavonoids mainly apigenin (API) and luteolin (LUT) by reverse phase high performance liquid chromatography (RP-HPLC) was developed and validated. Flavonoids present in the leaves of Clerodendrum serratum L. (C. serratum) were analyzed and quantified. Analysis was carried out on enable C18G column (250 mm × 4.6 mm i.d, 5 µm) as stationary phase, mobile phase consisting of methanol-acetonitrile-acetic acid-orthophosphoric acid-water (40:20:0.05:0.05:40) at a flow rate of 0.6 mL min$^{-1}$ and detection wavelength at 352 nm. The proposed method was validated by ICH Harmonized Tripartite Guidelines, Validation of Analytical Procedures: Text and Methodology Q2 (R1). In this study, an excellent linearity was obtained with correlation coefficient ($r^2$) higher than 0.999. Besides, the chromatographic peaks also showed good resolution. Other validation parameters including precision, specificity, accuracy, and robustness demonstrated good reliability in the quantification of apigenin and luteolin. Thus the newly developed and validated method can be conveniently used for the quantification of API and LUT in leaves of C. serratum L. leaves and also be applied to standardization of multicomponent herbal remedies containing C. Serratum.***

**Keywords—** *Clerodendrum serratum*, Apigenin, Luteolin, RP-HPLC

## 1. INTRODUCTION

*Clerodendrum serratum* (Linn.) commonly known as '*Bharangi*' belongs to the family Verbenaceae, is being used since ancient period in traditional systems of medicine to alleviate various ailments [1]. Ethno-medicinal importance of the plant has been reported in various indigenous systems of medicines like Ayurveda, Siddha and Unani for the treatment of various life-threatening diseases such as syphilis, typhoid, cancer, jaundice and hypertension [2]. However, the difficulty in the use of medicinal plants is that they are usually used without any standardization. This makes it difficult to document and institute a system of verification or assessment of the efficacy of the treatment.

*C. serratum* is abundantly seen throughout in Indian forests as well as globally in Ceylon, Malay and Peninsula [3]. The major phytochemical constituents reported in leaves of *C. serratum* are flavonoids mainly catechin, luteolin, apigenin, luteolin-7-o-β-D-glucuronide, baicalein, scutellarein, phenolic acids like caffeic acid, ferulic acid, steroids mainly α-spinasterol, stigmasterol and carbohydrates which includes a mixture of glucose, arabinose and glucuronic acid [4]. Previous studies on the roots and leaf extracts of *C. serratum* have revealed its antioxidant, antipyretic and anti-inflammatory, analgesic, anti-allergic, antiasthmatic, hepatoprotective and anticancer activities [5-11].

Flavonoids existing as free aglycones and glycosides are one of the most important groups of bio-active compounds in *C. serratum*, exhibiting a wide range of biological activities. Hence diverse pharmacological activities exhibited by *C. serratum* could be attributed to the presence of flavonoids, namely apigenin (API) and luteolin (LUT) shown below in figure 1 [12]. So both these flavonoids could be reliably used as chemotaxonomic markers for the standardization of *C. serratum* extract that are used in herbal remedies. The only method reported so far for standardization of *C. serratum* is high performance thin layer chromatography (HPTLC), which quantified only stigmasterol, a terpenoid. As per literature survey, no studies have reported on the estimation of flavonoids API and LUT in *C. serratum* plants. These flavonoids

have already been reported in plants like *Marchantia convoluta, Achillea millefolium, Bacopa monnieri, Cardiospermum halicacabum, Caucalis platycarpos* [12-16].

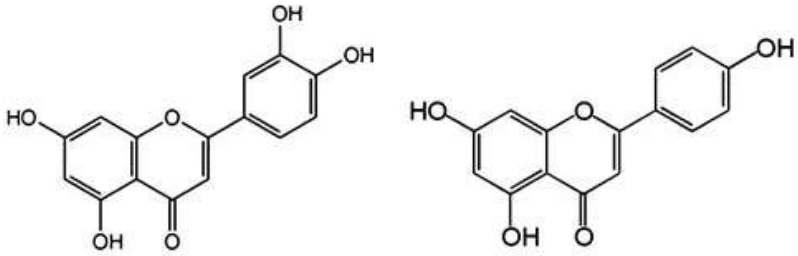


**Figure 1:** Chemical Structure of Apigenin and Luteolin

Plants may contain constituents that can be used to treat various diseases, but the scientific information on most of these medicinal plants in use are lacking. Therefore as part of the efforts to promote the use of medicinal plants either as an alternative or an adjunct to conventional medicine, it is necessary for scientists to carry out investigations on standardizations of herbal medicines. This will help to bridge the gap between conventional and herbal medicines. Hence the present study was undertaken to develop and validate a RP-HPLC method for the simultaneous quantification of API and LUT in *C. serratum* leaves, so that both these flavonoids could be reliably used in future as a chemotaxonomic marker for the standardization of *C. serratum* extract that are used in herbal remedies.

## 2. METHODOLOGY

### *2.1 Materials and Reagents*

API standard (97%) and LUT standard (98%) were purchased from Sigma-Aldrich Laboratories Ltd., (Bangalore, India). HPLC grade methanol, acetonitrile, orthophosphoric acid from Ranbaxy Laboratories Ltd., (Mumbai, India), acetic acid glacial extrapure from Sisco Research Laboratories Ltd., (Mumbai, India) and millipore water were used for HPLC analysis.

### *2.2 Apparatus and Chromatographic conditions*

Analysis was carried out with Shimadzu Japan HPLC system consisting of a solvent delivery pump, UV detector, autosampler and system controller. Data collection and analysis were performed using LC solution. Separation was performed on enable C18G column (250 mm × 4.6 mm i.d., 5µm). The detection wavelength was set at 352 nm. The mobile phase consisted of methanol: acetonitrile: acetic acid: orthophosphoric acid: water (40: 20: 0.05: 0.05: 40) at a flow rate of 0.6 mL $min^{-1}$.

### *2.3 Sample preparation*

The leaves of plant *C. serratum* were collected during the month of September-October from Attapadi, Palakkad district, Kerala, India. It was taxonomically identified by Department of Botany, University of Kerala, Trivandrum and an herbarium of the plant is preserved for future reference [Voucher no: 114 10/3 (UCBD]**.** The leaves were washed and shade dried at room temperature. Dried leaves were coarsely powdered (437 g) and subjected to extraction by cold maceration with 70% ethanol (7.45% w/w yield) at room temperature with continuous stirring for 6 days, after de-fatting with petroleum ether (60-80$^{o}$c). Solvent was allowed to evaporate and the concentrated ethanol extract was then fractionated successively with chloroform, ethyl acetate, n-butanol and water. All the extracts were dried under vacuum and then subjected to various qualitative tests for identification of phytochemical constituents and thin layer chromatography for confirmation. Among all the fractions, n-butanol fraction and ethylacetate fraction were then subjected to RP-HPLC quantification of API and LUT.

12.9 mg of n- butanol fraction and 12.1 mg of ethyl acetate fraction were weighed accurately and transferred separately into 10 mL volumetric flask, dissolved in methanol, sonicated for 2 min and final volume was made up with methanol to produce 1290 µg/mL and 1210 µg/mL respectively.

#### *2.3.1 Preparation of standard solution*

1 mg each of standard LUT and API were weighed accurately and transferred separately into 10 mL volumetric flask, dissolved in methanol, sonicated for 2 minutes and final volume was made up with methanol to produce 100 µg/mL stock solution and working solutions were prepared by stepwise dilution of the stock solution with methanol to get 1.0 µg/mL.

### *2.4 Assay Validation*

Analytical method was validated as per the guidelines of International Conference on Harmonization (ICH) for linearity, accuracy, precision, system suitability, specificity, robustness, LOD and LOQ by the following procedures [17].

#### *2.4.1 Linearity*

Linearity was performed for each API and LUT standards with five different concentrations between the ranges of 0.25 µg/mL-5 µg/mL were analyzed in triplicate for each concentration. Calibration curves were constructed by plotting peak areas against analyte concentrations. The linearity was assessed by calculating the slope, Y-intercept and coefficient of determination.

#### *2.4.2 Precision, Accuracy, Specificity*

The precision of the method was examined by performing the intra-day and inter-day assays of six replicate injections of the mixture of the standard solution of three concentration levels (1, 2.5, and 5 µg/mL). The intraday assay precision test was performed at intervals of 4 h in 1 day, while the interday assay precision test was performed over 3 days. The accuracy of the method was determined by calculating the recoveries of API and LUT by the method of standard addition. Known amount of the standard (5µg/mL) were added to the pre-analyzed sample solutions and the amounts of these standard were estimated by measuring the peak areas and by fitting these values to the straight line equation of calibration curves. Specificity is the ability of an analytical method to differentiate and quantify the analyte in presence of other components in the sample. The separation was performed on enable C18G column (250 mm × 4.6 mm i.d., 5µm).

#### *2.4.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)*

LOD and LOQ of both the analytes were determined using the formula, **k × SD / b**
Where, k is a constant (3.3 for LOD and 10 for LOQ); SD is the standard deviation of the analytical signal and b is the slope of the concentration/response graph.

#### *2.4.4 Robustness*

The robustness of test method was demonstrated by carrying out mobile phase variation $\pm$ 2%, flow variation $\pm$ 10% and column brand variation.

#### *2.4.5 System suitability*

System suitability of the method was performed by calculating the chromatographic parameters namely, asymmetry factor, theoretical plates, peak area and retention time on the repetitive of injection of standard solution.

### *2.5 Statistical analysis*

The data were submitted to statistical analysis using excel software.

## 3. RESULTS AND DISCUSSION

### *3.1 Method development and chromatographic conditions*

RP-HPLC method carried out in this study was aimed at developing a chromatographic system, capable of eluting and resolving flavonoid components in *C. serratum* ethanol extract. During method development, different mobile phase combinations with different buffers such as orthophosphoric acid and formic acid were investigated to obtain chromatograms with good resolution and symmetric peak shapes. Development of RP-HPLC method for the determination of API and LUT in leaves of *C. serratum* involved the use of several solvent systems mainly tetrahydrofuran-acetonitrile-methanol-orthophosphoric acid (0.5%) and methanol-acetonitrile- acetic acid-orthophosphoric acid-water and separation columns mainly Enable C18G, Phenomenex Gemini C8, Phenomenex Gemini C18, and Thermoscientific Betasil C18 column. From the data, it was found that enable C18G column and mobile phase combination containing methanol-acetonitrile-acetic acid-orthophosphoric acid-water (200 mL: 100 mL: 0.75 mL: 0.75 mL: 200 mL) provided better separation of API and LUT from the ethanol extract of *C. serratum* leaves.

The choice of detection wavelength was determined by performing a screening with 10 ppm each of LUT and API in methanol in a UV/VIS spectrophotometer. The UV spectra were recorded from 220 to 380 nm, and exhibited maximum wavelength at 320 nm and 352 nm and wavelength of 352 nm recorded better response for both the compounds.

#### *3.1.1 Quantification of API and LUT in C. serratum leaf extract*

Upon application of the developed method, well separated peaks were obtained for both apigenin and luteolin in *C. serratum* leaf extract as shown in figure 4 and 5, compared to their respective standards as in figure 2 and 3.

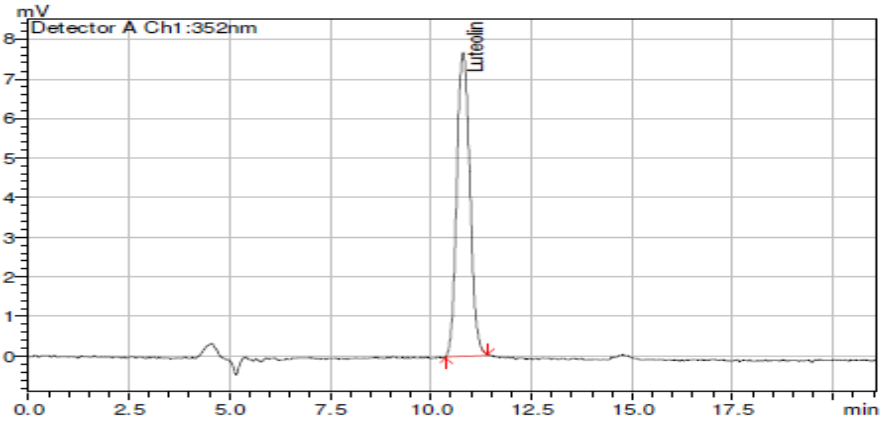


**Figure 2:** RP-HPLC Chromatogram of Luteolin Standard

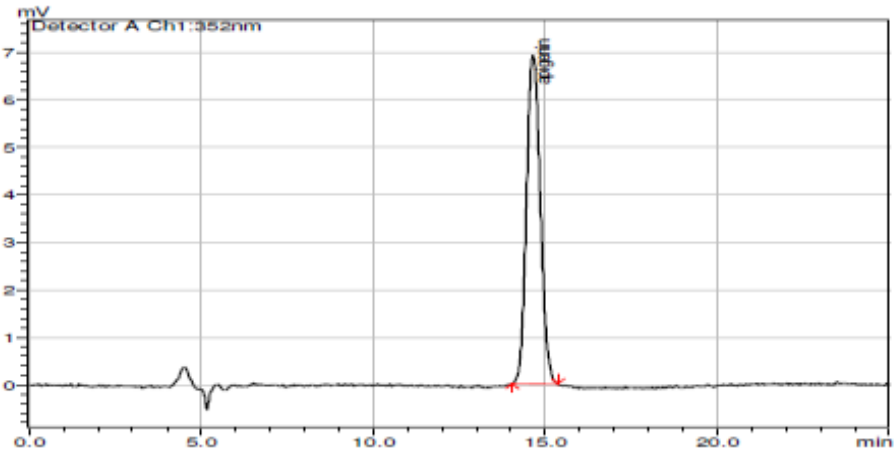


**Figure 3:** RP-HPLC Chormatogram of Apigenin Standard

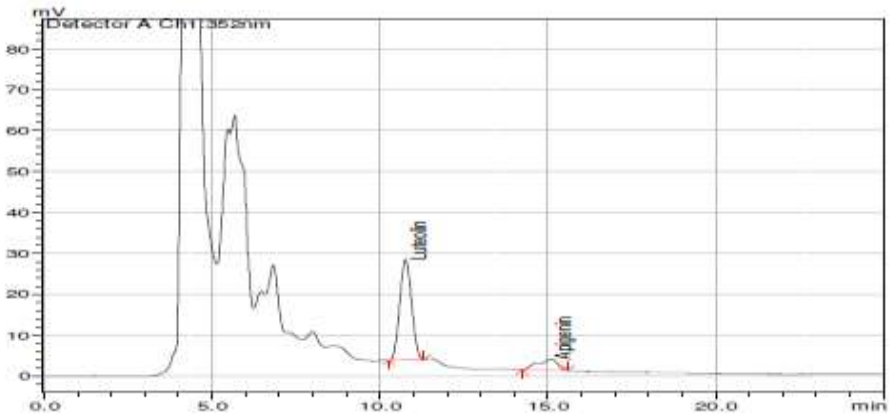


**Figure 4:** RP-HPLC Chromatogram of Luteolin and Apigenin in n-Butanol Fraction of Ethanol Extract of *C. serratum* Leaves

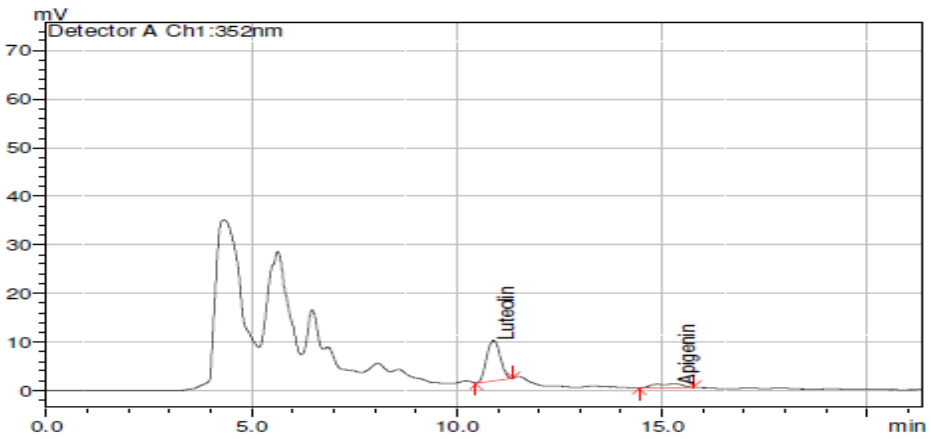


**Figure 5:** RP-HPLC Chromatogram of Luteolin and Apigenin in Ethyl acetate Fraction of Ethanol Extract of *C. serratum* Leaves

The quantitative analysis revealed that apigenin and luteolin were found to be predominant in n-butanol fraction (0.44 mg/g of API and 2.64 mg/g of LUT) compared to ethylacetate fraction (0.07 mg/g of API and 0.37 mg/g of LUT) of ethanol extract of *C. serratum* Linn leaves.

### *3.2 Method validation*

#### *3.2.1 Calibration curve and sensitivity*

Linearity is the ability of the method to obtain test results which are directly proportional to the concentration of the analyte in the sample. Linearity can be assessed by visual inspection of the plot and by statistical calculations namely, correlation coefficient, Y-intercept and slope. Linear regression analysis data confirmed that $r^2$ values for LUT and API, were 0.9999 (>0.999) confirming the linear relationship between the concentration of the drugs and area under the curve as given in table 1 and 2. RP-HPLC method developed for both compounds in *Marchantia convoluta* by Chen X and Xiao J, demonstrated $r^2$ values to be 0.9995 and 0.9991 respectively [12]. Another validated HPLC method for LUT and API in *Achillea millefolium*, illustrated that regression coefficient values for both compounds were 0.997 and 0.992 [13]. All these findings revealed that relationship between concentration of drugs and area under the curve was more linear in *C. serratum* leaf extract and our plant extract could be considered as a standard marker for quantification of LUT and API in other plants.

**Table 1:** Linearity of Luteolin Standard

| Concentration (µg/mL) | Area |
|---|---|
| 0.25 | 44331 |
| 0.5 | 85810 |
| 1 | 173705 |
| 2.5 | 434151 |
| 5 | 891583 |

**Table 2:** Linearity of Apigenin Standard

| Concentration (µg/mL) | Area |
|---|---|
| 0.25 | 63060 |
| 0.5 | 95769 |
| 1 | 193225 |
| 2.5 | 443756 |
| 5 | 871849 |

The calibration curves constructed for both luteolin and apigenin were linear over the concentration range of 0.25–5 µg/mL for each marker as shown in figure 6 and 7. Final optimized method parameters were tabulated in table 3.

**Figure 6:** Calibration Curve of Luteolin Standard

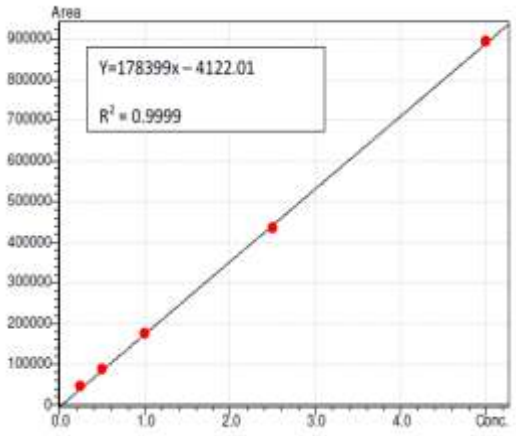


**Figure 7:** Calibration Curve of Apigenin Standard

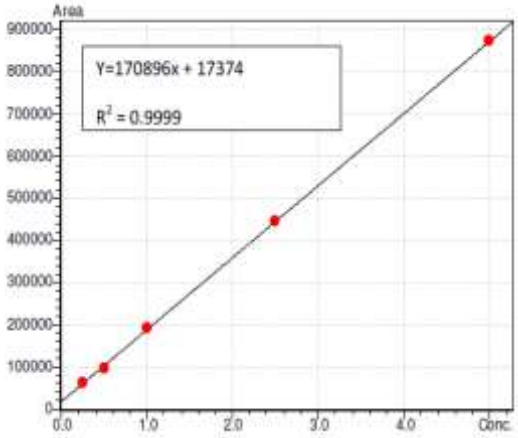


**Table 3:** Linear regression data for calibration curves of luteolin and apigenin

| Parameters | Luteolin | Apigenin |
|---|---|---|
| Retention time (min) | 10.768 ± 0.033 | 14.790 ± 0.118 |
| Detection wavelength (nm) | 352 | 352 |
| LOD (µg / mL) | 0.05 | 0.6 |
| LOQ (µg / mL) | 0.15 | 1.8 |
| Linearity range (µg/ mL) | 0.25-5 | 0.25-5 |
| Correlation coefficient (r) | 0.9999 | 0.9999 |
| Regression equation (area) | Y=178399x + 4122.01 | Y= 170896x + 17374 |

### *3.2.2 Precision*

Precision is the degree of agreement between a series of measurements obtained from multiple sampling of same homogenous sample. The precision results for the solution at the three concentrations (1.0 µg/mL, 2.5 µg/mL and 5.0 µg/mL) were presented in table 4. RSD values for peak areas for LUT and API in *C. serratum* leaf extract were 0.761 and 1.433%. From the results, RSD values for retention time were <1%, while for peak area were <2% for both intra-day and inter-day assay precision, whereas the corresponding values in *M. convoluta* for LUT and API were found to be 1.21 and 2.49% respectively [12]. Since universally accepted RSD limit is not more than 2%, comparison of results indicated that our results were more precise. Also, there were no significant differences between assay results, indicating that the precision of the proposed method was satisfactory.

**Table 4:** Intraday and interday precision results of the developed RP-HPLC method

| Components (µg/mL) | Intraday precision | | | | Interday precision | | | |
|---|---|---|---|---|---|---|---|---|
| | Retention time | | Peak area | | Retention time | | Peak area | |
| | Mean | RSD | Mean | RSD | Mean | RSD | Mean | RSD |
| **Luteolin** | | | | | | | | |
| 1 | 10.768 | 0.313 | 176115 | 0.761 | 10.775 | 0.155 | 176944 | 1.191 |
| 2.5 | 10.797 | 0.272 | 437856 | 0.797 | 10.759 | 0.115 | 444077 | 1.466 |
| 5 | 10.790 | 0.514 | 891469 | 0.812 | 10.783 | 0.165 | 891583 | 1.523 |
| **Apigenin** | | | | | | | | |
| 1 | 14.825 | 0.163 | 176388 | 1.433 | 14.784 | 0.306 | 175215 | 1.820 |
| 2.5 | 14.790 | 0.279 | 438394 | 1.354 | 14.699 | 0.310 | 436342 | 1.297 |
| 5 | 14.811 | 0.590 | 852715 | 0.384 | 14.801 | 0.657 | 865231 | 0.473 |

### *3.2.3 Accuracy*

Accuracy is the degree of agreement between the expected value and observed value. The recovery of the compounds LUT and API was determined by spiking the extracts of *C. serratum* Linn leaves with known amounts of LUT and API standards. Recovery of the luteolin ranged from 99.58-100.23% and that of apigenin varied from 97.61-101.07% and their % RSD values were all <2% as given in table 5. Results from recovery tests showed that the developed method was suitable for quantification of LUT and API. Recovery results of LUT and API in *M. convoluta* were 92.18–95.13% and 98.72–103.19% respectively, which were not as significant as our results [12]. From the results it could be concluded that our method was more accurate.

**Table 5:** Recovery results of luteolin and apigenin from the n-butanol fraction

| Components | Quantity added (µg/mL) | Total quantity present (µg/mL) | Quantity found (µg/mL) | Recovery (%) |
|---|---|---|---|---|
| **Luteolin** | 0.58 | 2.36 | 2.35 | 99.58 |
| | 1.17 | 2.95 | 2.95 | 100.86 |
| | 2.5 | 4.28 | 4.29 | 100.23 |
| **Apigenin** | 0.55 | 0.84 | 0.82 | 97.61 |
| | 1.05 | 1.34 | 1.33 | 99.25 |
| | 2.5 | 2.79 | 2.82 | 101.07 |

### *3.2.4 Specificity*

Retention time and resolution are the important parameters used to assess specificity of the method. Average retention time for LUT was 10.8 min and that of API was 14.8 min. Well resolved peaks were recorded for both flavonoids as asymmetry factor values for both flavonoids were found to be 1.111 and 1.113. Another validated RP-HPLC method developed by Aiyalu R et al. [14] revealed asymmetry factor for both flavonoids as 0.69 and 0.813. Analogizing the results, it could be recommended that our method was more specific for quantifying LUT and API. Also the proposed RP-HPLC method demonstrated high specificity at 352 nm for the detection of both LUT and API from the extracts of *C. serratum* Linn leaves showing reliability in the quantification of LUT and API.

### *3.3 Limit of Detection and Limit of Quantification*

The LOD and LOQ for LUT were found to be 0.05 and 0.15µg/mL, and that of API were 0.6 and 1.8µg/mL respectively as shown in table 5. From the results it might be concluded that the proposed method was more sensitive for detecting luteolin compared to apigenin. Detection limit taken as lowest absolute concentration of analysis in a sample, were found to be 0.46 and 0.6 for luteolin and API in *M. convoluta* [12] and that of *C. serratum* exhibited values as 0.05 and 0.6. Also, quantification limits, taken as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy were 2.02 and 1.46 for LUT and API in *M. convoluta*, whereas LOQ values for both compounds in *C. Serratum* were 0.15 and 1.8. Comparison of the results illustrated that, our method was more sensitive for detection and quantification of both compounds.

**Table 5:** Limit of detection and limit of quantification of luteolin and apigenin

| Parameters | Luteolin | Apigenin |
|---|---|---|
| LOD (µg/mL) | 0.05 | 0.6 |
| LOQ (µg/mL) | 0.15 | 1.8 |

### *3.4 Robustness*

Robustness was evaluated to ensure that the HPLC method is insensitive to small changes in the experimental conditions. In this study, selected parameters like different C18 column, flow rate and mobile phase remained unaffected by small variations as given in table 7 and 8. The recovery data obtained concluded that the methods were consistent for change in column brand, flow rate variation and mobile phase variation.

**Table 7:** Robustness of the developed RP-HPLC method

| Parameters changed | Luteolin | | Apigenin | |
|---|---|---|---|---|
| | Retention time | Area | Retention time | Area |
| Mobile phase -1 | 10.713 | 176115 | 14.848 | 176388 |
| Mobile phase -2 | 10.824 | 177416 | 14.912 | 177848 |
| Flow rate-1 | 10.595 | 176548 | 14.698 | 176498 |
| Flow rate -2 | 10.856 | 177520 | 14.854 | 177865 |

**Table 8:** Robustness of the developed RP-HPLC method

| Colum brand | Luteolin | | Apigenin | |
|---|---|---|---|---|
| | Column Type | Recovery (%) | Column Type | Recovery (%) |
| | Enable $C_{18}$ G | 99.9 | Enable $C_{18}$G | 99.85 |
| | Phenomenex Gemini $C_{18}$ | 101.1 | Phenomenex Gemini $C_{18}$ | 101.2 |
| | Thermo scientific Betasil | 98.9 | Thermo scientific Betasil | 98.8 |

### *3.5 System suitability*

Six replicate injections of the system suitability solution gave % RSD values for retention time and peak area within 2%, indicating low variation of the measured values. The asymmetry factors for all peaks are < 2. The efficiency of the column as expressed by the number of theoretical plates was more than 2000. Good separation between the peaks of LUT and API was achieved, with the retention time, 10.7 min for LUT and 14.8 min for API. Furthermore the chromatographic peaks showed good resolution between LUT and API. In relation to asymmetry, the peaks showed values 1.111 for LUT and 1.138 for API. The results in table 9 indicated that critical parameters such as retention time, area and number of theoretical plates met the acceptance criteria on all the experimental days.

**Table 9:** System suitability parameters of luteolin and apigenin

| Compound | Parameter | Average | %RSD |
|---|---|---|---|
| Luteolin | Retention time | 10.768 | 0.313 |
| | Peak area | 176115 | 0.761 |
| | No. of theoretical plates | 5089.409 | 1.819 |
| | Asymmetry factor | 1.111 | 0.187 |
| Apigenin | Retention time | 14.825 | 0.163 |
| | Peak area | 176388 | 1.433 |
| | No. of theoretical plates | 6650.844 | 2.049 |
| | Asymmetry factor | 1.138 | 0.682 |

## 4. CONCLUSION

The RP-HPLC method developed and validated in the present study enabled the simultaneous estimation of API and LUT from n-butanol fraction and ethylacetate fraction of ethanol extract of *C. serratum* Linn leaves. To the best of our knowledge, this is the first literature on simultaneous estimation of API and LUT in *C. serratum* plant. This method achieved higher specificity and better sensitivity for the analysis of both API and LUT, compared to other methods developed and validated for both compounds in *M. convoluta*, *A. millefolium* and *B. monnieri* [12-14]. Thus from the results obtained, it might be concluded that the proposed method can be conveniently used for the simultaneous analysis of API and LUT in a sensitive, specific, precise, accurate, simple and easy manner in *C. serratum* plant and also for standardization of multicomponent herbal remedies containing *C. serratum* like *Bharangyadi kasayam*, *Dhasmula arista*, *Dhasmula kwatha*, *Chavanprash aveleh*, *Ayaskrti*, *Haritaki avleh*, *Mahamanjistha dikwath*, *Kankasav*, *Visgarbha tail*, *Yograjaguggulu vatic* [18-20].

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