ANNEXURE II

HERBAL FORMULATION USED FOR THE STUDY

Each 5ml of Liv.52 syrup contains:
- Himsra (*Capparis spinosa*) 34mg
- Kasani (*Cichorium intybus*) 34mg
- Kakamachi (*Solanum nigrum*) 16mg
- Arjuna (*Terminalia arjuna*) 16mg
- Kasamarda (*Cassia occidentalis*) 8mg
- Biranjasipha (*Achillea millefolium*) 8mg
- Jhavuka (*Tamarix gallica*) 8mg

Each Liv.52 tablet contains: Liv.52 tablet contains:
- Himsra (*Capparis spinosa*) 65mg
- Kasani (*Cichorium intybus*) 65mg
- Mandura bhasma 33mg
- Kakamachi (*Solanum nigrum*) 32mg
- Arjuna (*Terminalia arjuna*) 32mg
- Kasamarda (*Cassia occidentalis*) 16mg
- Biranjasipha (*Achillea millefolium*) 16mg
- Jhavuka (*Tamarix gallica*) 16mg
ANNEXURE-III
LIST OF PAPERS PRESENTED

1. A part of research work titled “Development of HPLC method for the simultaneous estimation of apigenin and luteolin from Achillea millefolium Linn.” was presented at two days national seminar on ‘Advanced chromatographic techniques” held at JSS College of Pharmacy, Mysore (March 2013).
LIST OF PAPERS PUBLISHED


DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR THE SIMULTANEOUS ESTIMATIONS OF QUERCETIN AND RUTIN IN AGANOSMA DICHTOMA [ROTH] K. SCHUM

GOMATHY SUBRAMANIAN1, SUBRAMANIA NAINAR MEYYANATHAN2, YAMJALA KARTHIK2, ANJANA KARUNAKARANAIR2 AND DHANABAL S PALANISAMY3

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Received: 14 Dec 2013, Revised and Accepted: 10 Feb 2014

ABSTRACT

Objective: A simple, specific, accurate and precise high performance liquid chromatography method was developed for the simultaneous estimation of quercetin and rutin in Aganosma dichotoma.

Methods: The chromatographic separation was achieved by using C8 column, 150 x 4.6mm i.d., 5µ Hlb Lichrospher, mobile phase containing acetonitrile:25mM ammonium acetate pH 3 (40:60 v/v). The flow rate was 1 ml/min and the absorbance was monitored at 259 nm. The retention time of quercetin and rutin was found to be 4.30 min and 1.71 min respectively.

Results: The proposed method was validated in terms of the analytical parameters such as accuracy, linearity, precision, robustness, limit of detection (LOD), limit of quantification (LOQ) were determined based on the International Conference on Harmonization (ICH) guidelines. The detector response was linear in the range of 1-5 µg/ml 0.1-0.5 µg/ml for quercetin and rutin respectively.

Conclusion: The proposed method was successfully applied for the simultaneous estimation of both the constituents in Aganosma dichotoma. This study established a quantitative method for the simultaneous determination of quercetin and rutin from Aganosma dichotoma.

Keywords: Aganosma dichotoma, Simultaneous estimation, Flavonoids, Quercetin, Rutin.

INTRODUCTION

Flavonoids are a group of polyphenolic compounds, which are widely distributed throughout the plant kingdom. To date about 300 varieties of flavonoids are known. Flavonoids, as a major active constituent, display a remarkable role in various pharmacological activities including anti-allergic, anti-inflammatory and anti-oxidant effects [1-3]. Quercetin and rutin possess antioxidant activity and reduce low density lipoproteins oxidation [4].

Quercetin and rutin are some important flavonoids known for its anti-inflammatory, anti-allergic, anti-thrombic, hepatoprotective, anti-spasmodic and anti-cancer properties [5, 6].

Identification of major and unique compounds in herbs as markers and development of analytical methodologies for monitoring them are the key steps involved in marker-based standardization [7]. Many flavonoid containing plants are diuretics or antispasmodics and some flavonoids have antitumor, antifungal and antibacterial properties as well as antihypotensive activity [8]. High performance layer chromatography (HPLC) has recently emerged as a preferred analytical tool for fingerprints and quantification of marker compounds in herbal drugs because of its simplicity, sensitivity, accuracy, suitability for high throughput screening. HPLC method is a suitable method for estimation of chemical constituents present in plant materials [9-12]. The proposed method is optimized and validated as per the International conference on harmonization (ICH) guidelines [13].

The literature survey showed that there was no report about the simultaneous estimation of flavonoid constituents of Aganosma dichotoma. Quantitative estimation of these compounds is important for current research and a variety of methods are required for this and in the present study the quantification of main flavonoids which was found to be characteristic for Aganosma dichotoma was reported. A sensitive, accurate and specific HPLC method was developed and validated for the simultaneous estimation of quercetin and rutin in the hydro alcoholic and methanolic extract of Aganosma dichotoma.

MATERIALS AND METHODS

Materials and reagents

Quercetin 96% and rutin (98%) were purchased from Sigma-Aldrich, Bangalore, India Methanol and Acetonitrile were of HPLC grade from Qualigens fine chemicals, Mumbai, India. All the reagents and chemicals used were of analytical and HPLC grade. Water (HPLC grade) was obtained from Milli Q RO system.

Plant Material

The whole plant of Aganosma dichotoma was collected during the month of March 2013, from the Western Ghats, Kottayam region, Kerala. The plant was botanically identified, confirmed and authenticated by Dr. Gunasekaran, Field botanist, Coimbatore, Tamilnadu, India. The plants were cut and dried in tray drier at 50°C for 48 hrs. The dried samples were powdered and used for the study.

Preparation of standard solution

The standard stock solution (1 mg/ml) of quercetin and rutin were prepared by dilution in methanol. These stock solutions were stored in light resistant containers. The dilute standard solutions of concentration (1-5 µg/ml of quercetin and 0.1-0.5 µg/ml of rutin) were prepared from above stock solution and used for calibration curve of quercetin and rutin.

Preparation of sample solution

About 50 g of the powdered sample was weighed and extracted with the selected solvents by Soxlet apparatus for 24 h. The extract was collected and filtered; the filtrate was dried at 50°C under reduced pressure in a rotary evaporator (Buchii Rota vapor). The dried extract (1 mg/ml) was dissolved in the mobile phase. After filtering through Whatmann filter paper No.42, the extract was injected directly.

Instrumentation and Chromatographic conditions

The simultaneous estimation of quercetin and rutin was performed on a Shimadzu liquid chromatographic system equipped with LC-2010AT VP solvent delivery system (pump), SPD M-10A photodiode array detector and Rheodyne 7725i injector with 20 µl loop volume,
Class VP 6.01 data station for data collection and processing (Shimadzu technologies, Japan). The mobile phase, acetonitrile and ammonium acetate (40:60) 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 ambient temperature.

RESULTS AND DISCUSSION

Method development and validation

Upon application of the developed method, well-separated peaks were obtained for both quercetin and rutin (Figure 1). Quercetin and rutin were identified in Aganosma herb extracts. The quantitative analysis revealed that rutin (72.08 ± 0.18 mg/g) predominated in the hydro alcoholic extract and 57.8 ± 0.22 mg/g in the methanolic extract of A. dichotoma whereas quercetin were determined in lower quantities (4.87 ± 0.16 mg/g) in hydro alcoholic extract and 3.60 ± 0.20 mg/g in the methanolic extract of A. dichotoma. The chromatograms of hydro alcoholic and methanolic extracts containing quercetin and rutin contents were given (Figure 2, Figure 3). For validation of analytical methods, the guidelines of the International Conference on the Harmonization have recommended the accomplishment of linearity, accuracy tests, precision, detection and quantitation limit and robustness of the method.

Linearity and range of the developed method

For linearity study, five solutions in the range of 1-5 µg/ml for quercetin and 0.1-0.5 µg/ml rutin 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 $Y=88980x-5306$ for quercetin and $Y=41670x+333.33$ for rutin (x is concentration of analytes in µg/ml and Y is peak area). The value of correlation coefficient was found to be 0.997 for quercetin and 0.995 for rutin. The results indicate that the method is linear over the concentration range studied (Figure 4, Figure 5).

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

Accuracy of the developed method

This study was performed by adding known amounts of quercetin and rutin to the placebo solution. Three level of solutions were made having concentrations of 1, 2 and 3 µg/ml for quercetin and 0.1, 0.2 and 0.3 µg/ml for rutin. The recovery range for quercetin and rutin were found to be 99.3 to 101.1 % and 101.3 to 102 % respectively (limit 98 to 102%). The relative standard deviation ranged from 0.185 to 0.529 % for quercetin and from 0.054 to 0.075 % for rutin (Table 2).

Table 1: Linearity and range for quercetin and rutin by HPLC

<table>
<thead>
<tr>
<th>SL.No.</th>
<th>Concentration of Quercetin (µg/ml)</th>
<th>Concentration of rutin (µg/ml)</th>
<th>Peak area Quercetin</th>
<th>Rutin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.01</td>
<td>0.1</td>
<td>88915</td>
<td>4167</td>
</tr>
<tr>
<td>2</td>
<td>0.02</td>
<td>0.2</td>
<td>154416</td>
<td>9334</td>
</tr>
<tr>
<td>3</td>
<td>0.03</td>
<td>0.3</td>
<td>262559</td>
<td>13501</td>
</tr>
<tr>
<td>4</td>
<td>0.04</td>
<td>0.4</td>
<td>355976</td>
<td>16668</td>
</tr>
<tr>
<td>5</td>
<td>0.05</td>
<td>0.5</td>
<td>440992</td>
<td>20835</td>
</tr>
</tbody>
</table>

Fig. 1: Typical HPLC Chromatogram of Quercetin and Rutin

Fig. 2: Typical HPLC Chromatogram of methanolic extract containing Quercetin and Rutin

Fig. 3: Typical HPLC Chromatogram of methanolic extract containing Quercetin and Rutin

Fig. 4: Calibration curve of Quercetin by HPLC

Fig. 5: Calibration curve of Rutin by HPLC
The results show that the plant P. r. demonstrates that the plant contains considerable amounts of flavonoids which promotes the potential source of natural health-promoting antioxidants for medicinal and food applications. This study established a quantitative method for the simultaneous determination of quercetin and rutin from Aganosma dichotoma.

### Table 4: Robustness study of the proposed HPLC method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Conditions</th>
<th>Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Quercetin</td>
</tr>
<tr>
<td>Robustness of the developed method</td>
<td></td>
<td></td>
</tr>
<tr>
<td>The robustness of the proposed method was evaluated by deliberately changing the chromatographic conditions such as solvent ratio, flow rate and absorbance. The results showed that varying the chromatographic conditions had no appreciable effects on the chromatographic parameters (Table 4).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### CONCLUSIONS

Using this method, quercetin and rutin could be determined simultaneously, and the validity of the method was also verified. The proposed analytical method for simultaneous estimation of quercetin and rutin in the extracts of Aganosma dichotoma is accurate, precise, linear, robust, reproducible and within the range. The results show that Aganosma dichotoma contains considerable amounts of flavonoids which demonstrates that the plant could be considered as a potential source of natural health-promoting antioxidants for medicinal and food applications. This study established a quantitative method for the simultaneous determination of quercetin and rutin from Aganosma dichotoma.
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 C₈ (150 x 4.6mm i.d., 5µ) column. The system was operated using methanol and 0.5% trifluoroacetic 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

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]

Available online on www.ijprd.com
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, hepatobiliary disorders, as an appetite enhancing drug, against skin inflammations and for wound healing due to its antiphlogistic, choleretic and spasmyloytic 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 Hiar Lichrospher. The mobile phase, methanol and 0.5% trifluoroacetic 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.

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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.

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*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

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Concentration of Apigenin and Luteolin (mcg/ml)</th>
<th>Peak area</th>
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<tr>
<td></td>
<td></td>
<td>Apigenin</td>
</tr>
<tr>
<td>1</td>
<td>01</td>
<td>99608</td>
</tr>
<tr>
<td>2</td>
<td>02</td>
<td>262981</td>
</tr>
<tr>
<td>3</td>
<td>03</td>
<td>450812</td>
</tr>
<tr>
<td>4</td>
<td>04</td>
<td>604030</td>
</tr>
<tr>
<td>5</td>
<td>05</td>
<td>804216</td>
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</table>

### Table 2: Recovery and accuracy data

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<th>Compounds</th>
<th>Recovery</th>
<th>RSD (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Amount Added (mg/ml)</td>
<td>Recovery (%)</td>
</tr>
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<td>Apigenin</td>
<td>0.01</td>
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</tr>
<tr>
<td></td>
<td>0.02</td>
<td>100.08</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
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</tr>
<tr>
<td>Luteolin</td>
<td>0.01</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>99.75</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>99.63</td>
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</table>

### Table 3: Precision studies for apigenin and luteolin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. (mg/ml)</th>
<th>n</th>
<th>Intra day</th>
<th>Inter day</th>
</tr>
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<tr>
<td></td>
<td>Mean %RSD</td>
<td></td>
<td>Mean %RSD</td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>1</td>
<td>6</td>
<td>1.003</td>
<td>0.94</td>
</tr>
<tr>
<td>Luteolin</td>
<td>1</td>
<td>6</td>
<td>0.990</td>
<td>1.26</td>
</tr>
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</table>

### Table 4: Robustness study of the proposed HPLC method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Conditions</th>
<th>Apigenin Retention Time</th>
<th>Luteolin Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Rate (ml/min)</td>
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<td>3.48</td>
</tr>
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<td></td>
<td>1.0</td>
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<td>2.94</td>
</tr>
<tr>
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<tr>
<td>Mobile Ratio (v/v)</td>
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<td>3.00</td>
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<tr>
<td></td>
<td>70:30</td>
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</tr>
<tr>
<td></td>
<td>68:32</td>
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<td>2.60</td>
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</table>

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

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameters</th>
<th>Apigenin</th>
<th>Luteolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Linearity range</td>
<td>1-5 mcg/ml</td>
<td>1-5 mcg/ml</td>
</tr>
<tr>
<td>2</td>
<td>Regression equation</td>
<td>Y= 163491 x -38452</td>
<td>Y= 48642 x -6253</td>
</tr>
<tr>
<td>3</td>
<td>Correlation coefficient</td>
<td>0.992</td>
<td>0.997</td>
</tr>
<tr>
<td>6</td>
<td>Asymmetric factor</td>
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<td>1.1</td>
</tr>
<tr>
<td>7</td>
<td>LOD (mcg/ml)</td>
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<td>48</td>
</tr>
<tr>
<td>8</td>
<td>LOQ (mcg/ml)</td>
<td>79</td>
<td>145</td>
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ACKNOWLEDGEMENT
The authors are grateful to His Holiness Jagadguru Sri Sri Shivarathri Deshikendra Mahaswamigalavaru of Sri Suttur Mutt, Mysore, for the facilities provided to complete the research work successfully.

REFERENCES