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Development of Simultaneous LC–MS/MS Method for the Quantitation of Apigenin, Luteolin and Quercetin in *Achillea millefolium* Extract

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ABSTRACT

Introduction: *Achillea millefolium, family Asteraceae commonly known as Biramjasif is an erect, pubescent, perennial herb, 15-90 cm in height, commonly distributed in the Himalayas. The plant is known to be reported for traditional uses for the treatment of astringent, diaphoretic, anti-spasmodic, etc. The plant is reported to contain salicylic acid, β-sitosterol, inositol, flavonoids, viz. apigenin, luteolin, cosmosin and sesquiterpenic lactone, austricin.*

Method: *A simple and rapid liquid chromatography–tandem mass spectrometry method was developed and validated for simultaneous determination of apigenin, luteolin and quercetin in Achillea millefolium extract. The compounds were eluted using Gemini C18, (50 mm × 2.0 mm, 3 µm) with the Isocratic mobile phase consisting of water and 0.1% formic acid, Acetonitrile: Methanol (50: 50 v/v) and 0.1% formic acid, flow rate of 0.400 ml/min.*

Result: *The assay exhibited a linear dynamic range of 1.25–5000 ng/ml for apigenin, luteolin and quercetin. The values for Intraday and Interday precision and accuracy were well within the generally accepted criteria for analytical methods. Selectivity, linearity, limit of detection and quantification, accuracy and precision were evaluated.*

Conclusion: *The proposed method is more accurate and sensitive can be used for the routine quantification of the apigenin, luteolin and quercetin in the herbal extracts.*

Keywords: *Achillea millefolium*, Apigenin, Luteolin, Quercetin.

INTRODUCTION

Achillea millefolium, family Asteraceae commonly known as Biramjasif is an erect [1], slightly aromatic, pubescent, perennial herb with stoloniferous roots, 15-90 cm in height, commonly distributed in the Himalayas from Kashmir to Kumaun at altitudes of 1,050-3,600 m; it has been seen growing in Bombay and Belgaum areas [2]. The plant is known to be reported for traditional uses for the treatment of astringent, stimulant, tonic, diaphoretic, anti-spasmodic, vulnerary and styptic properties, and is prescribed for the treatment of cold, flatulent colic, heartburn, hysteria, epilepsy and rheumatism[3-15]. The plant is reported to contain salicylic acid, β-sitosterol and its acetate, inositol, dulcitol, mannitol, betaine, choline, trigonelline, betonicine and stachydrine. Flavonoids, viz. luteolin-7-D-glucoside, apigenin, luteolin, cosmosin and luteolin-7-O-β-D-glucopyranoside and sesquiterpenic lactone, austricin. The presence of folic acid, rutin, queretin and ascorbic acid in abundance has been reported in leaves [16-21].

Different methods have been reported in the literature for simultaneous estimation of apigenin, luteolin and quercetin using reverse phase HPLC and preparative HPLC-MS purification, UV but these are not sensitive. Since there is no specific method available in literature for the quantification using LC-MS/MS system, the aim of the study was the development and validation of simple, sensitive, rapid and specific method.

Compared with other detection techniques, mass spectrometry is considered as the most specific and sensitive technique. In this study, a rapid, simple and sensitive LC–MS/MS method for the quantitation of apigenin, luteolin and quercetin in *Achillea millefolium* extract has been developed and validated. The method has several advantages, good chromatographic resolution, specific and sensitive mass spectrometric conditions and the broad concentration range of 1.25–5000 ng/ml.

MATERIALS AND METHODS

Chemicals and reagents

Reference Standards apigenin (97.8 % HPLC purity), luteolin (98.1 % HPLC purity) and quercetin (99.0 % HPLC purity) were procured from Natural Remedies Pvt. Ltd. Banglore, India. Methanol of HPLC Grade was purchased from S. D. Fine chemical, while formic acid, Hydrochloric acid where of analytical grade.

Chromatography conditions

Chromatographic separation was carried out on a Gemini C18 column, (50 mm × 2.0 mm, 3 µm); using a Shimadzu CBM-20A, SPD-M20. A system equipped with a LC-20ADvp pump, a Vacuum degasser and an autosampler (Shimadzu Corporation). The column oven was maintained at 40 ± 0.3°C. The Isocratic mobile phase consisting of water and 0.1% formic acid, Acetonitrile: Methanol (50: 50 v/v) and 0.1% formic acid at the flow rate of 0.400 ml/min. The samples were kept at 10 ± 3 °C in the autosampler.

Calibration standard samples preparation

Apigenin, luteolin and quercetin standards were prepared by weighing accurately 0.4 mg of standards and appropriate volume of Methanol was added to make final concentration of it equivalent to 0.4 mg/ml accounting for its potency and the actual amount weighed. Solution was stored in refrigerator at 5 ± 3 °C and was used within 7 days from date of preparation. Both the calibration standard was prepared from the same stock solution.

Sample preparation

10 g of accurately weighed dry powder of aerial parts of *A. millefolium* was extracted using Methanol and Water. The hydro-alcoholic of *A. millefolium* was hydrolysed by refluxing with 2N HCl: toluene (1:1 v/v) for three hours. The hydrosylate after neutralizing with 5-10% Na_2CO_3 was allowed to separate. The aqueous phase was further extracted using ethyl acetate (3 × 25 ml). Collect ethyl acetate extract and evaporate it. Stock solution was prepared by weighing 100 mg of the sample then quantitatively transferred to 100 ml volumetric flask and volume was adjusted with methanol, kept in the refrigerator and tightly closed.

Method validation

Linearity

To evaluate linearity of this method, calibration standards were prepared and analyzed in triplicate on three consecutive days. Calibration curves were constructed by plotting the peak area ratio versus the spiked concentrations of apigenin, luteolin and quercetin by least square linear regression analysis.

Precision and accuracy

Precision of developed method were assessed by determining three replicates of concentration 1250, 125, 1.25 ng/ml on three consecutive days. The precision was expressed as the % relative standard deviation (RSD). Accuracy of developed method was determined at three levels (80%, 100% and 120%) of concentration 1250 ng/ml.

Limit of detection and limit of quantification

The LOD with S/N of 3:1 and the LOQ with S/N of 10:1 were calculated for both drugs using the following equations according to International Conference on Harmonization (ICH) guidelines:

$LOD = 3.3 \times \sigma/S$

$LOQ = 10 \times \sigma/S$

Where σ the standard deviation of the response and S is the standard deviation of the y-intercept of the regression line**.**

RESULTS AND DISCUSSION

Chromatographic conditions

Multiple chromatographic conditions were explored in order to have appropriate retention time, and better resolution and sensitivity. Gemini C18 column (50 mm × 2.0 mm, 3 µm) was evaluated to attain better separation and was finally chosen for the chromatographic separation. The Isocratic mobile phase consisting of water and 0.1% formic acid, Acetonitrile: Methanol (50: 50 v/v) and 0.1% formic acid was found to be optimal for this study (Table 1). In addition, under the optimized conditions, no significant endogenous interference was found.

Table 1: Chromatographic conditions.

<table>
<tr><th>Parameters</th><th colspan="3">Detail</th></tr>
<tr><td>Column</td><td colspan="3">Gemini C18, (50 mm × 2.0 mm, 3 µm)</td></tr>
<tr><td rowspan="9">Mobile Phase</td><td colspan="3">A: Water + 0.1% formic acid
B: ACN (50): Methanol (50) + 0.1% formic acid</td></tr>
<tr><td>Time(min)</td><td>A%</td><td>B%</td></tr>
<tr><td>0.00</td><td>80</td><td>20</td></tr>
<tr><td>1.50</td><td>50</td><td>50</td></tr>
<tr><td>2.50</td><td>0</td><td>100</td></tr>
<tr><td>4.00</td><td>0</td><td>100</td></tr>
<tr><td>4.50</td><td>50</td><td>50</td></tr>
<tr><td>5.50</td><td>80</td><td>20</td></tr>
<tr><td>6.00</td><td>80</td><td>20</td></tr>
<tr><td>Flow rate</td><td colspan="3">0.400 ml/min</td></tr>
<tr><td>Column oven temperature</td><td colspan="3">40 ± 0.3°C</td></tr>
<tr><td>Autosampler temperature</td><td colspan="3">10 ± 3°C</td></tr>
<tr><td>Volume of injection</td><td colspan="3">5.0 µl</td></tr>
<tr><td>Detector</td><td colspan="3">Mass detector (MS/MS)</td></tr>
<tr><td>Retention time</td><td colspan="3">Apigenin at about 3.22 minutes.</td></tr>
<tr><td></td><td colspan="3">Luteolin at about 3.18 minutes</td></tr>
<tr><td></td><td colspan="3">Quercetin at about 3.16 minutes</td></tr>
<tr><td>Run time</td><td colspan="3">5.0 minutes</td></tr>
</table>

Mass spectrometric conditions

Electron spray ionization (ESI) is the most commonly used soft ionization sources in mass spectrometry. By investigating the full scan mass spectra of apigenin, luteolin and quercetin (Figures 1-3), it was found that the signal intensity in the positive mode was much higher than that in the negative ion mode. Thus, all detections were carried out using the predominantly positive ion The most suitable mass spectrometric conditions were determined by optimizing all the parameters of the mass spectrometer such as collision energy, nebulizer gas, DL temperature, heat block temperature and drying gas flow to obtain much higher and more stable response (Tables 2 and 3).

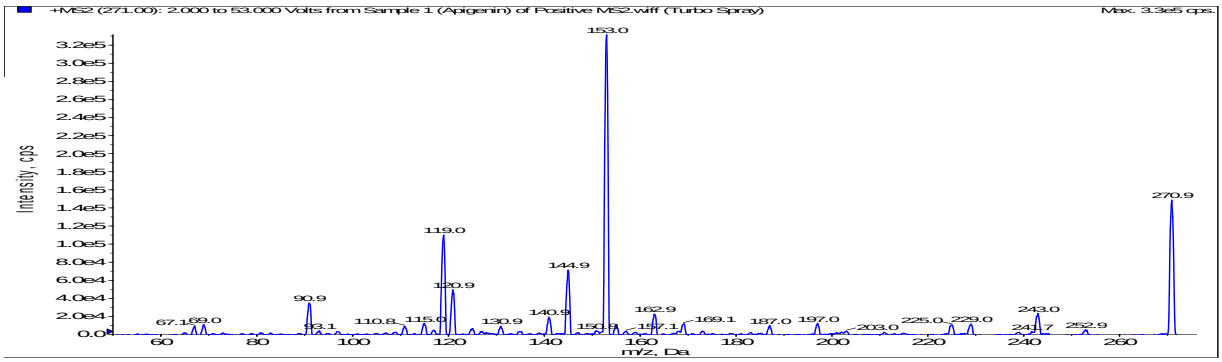


Figure 1: MS-MS spectra of Apigenin

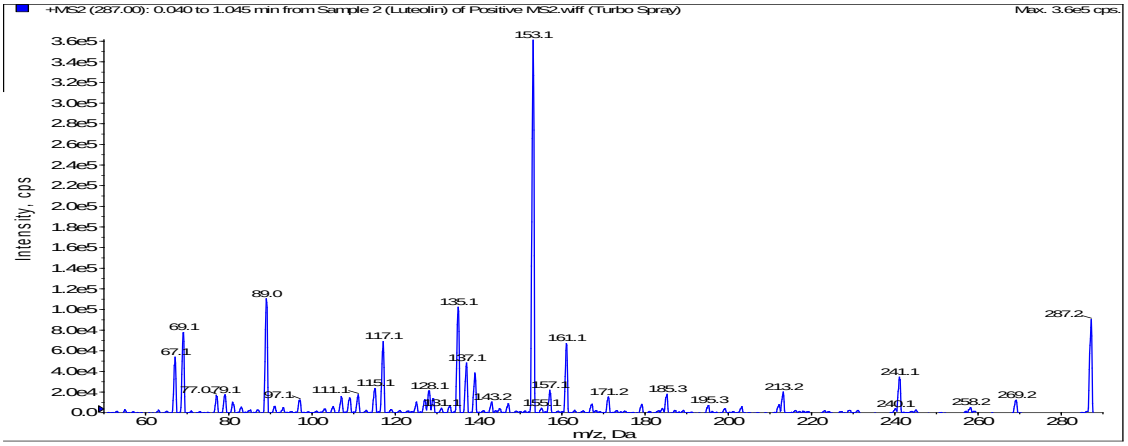


Figure 2: MS-MS spectra of Luteolin

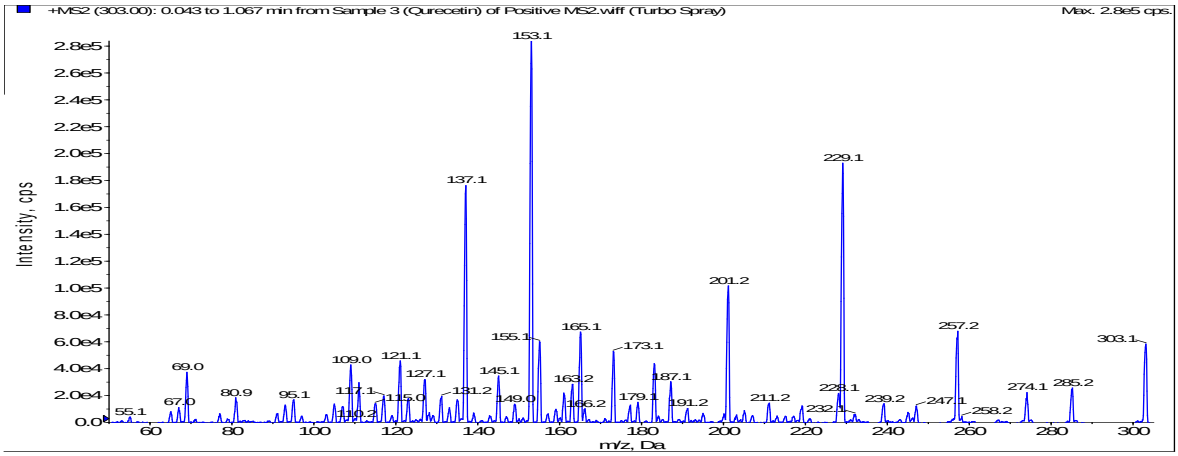


Figure 3: MS-MS spectra of quercetin.

Table 2: Mass spectrometric conditions.

Parameters	**Apigenin** **Drug 1**	**Luteolin**	**Quercetin**
Ion Source	Electro Spray ionization		
Polarity	Positive		
Parent Ion	271.0	287.0	303
Daughter Ion	121	153.1	229
Dwell Time (msec)	100	100	100
Collision Energy (CE)	42	44	37

Table 3: Mass spectrometric source dependent parameters

Parameters	**Used**
DL Temperature	250 °C
Nebulizing Gas Flow	3.0 l/min
Heat Block Temperature	450 °C
Drying Gas Flow	15.0 l/min

Method validation

Selectivity and specificity

The selectivity of the method was investigated by comparing chromatograms of apigenin, luteolin and quercetin, retention time was 3.22, 3.18 and 3.16 min, respectively. There were no significant endogenous peaks that could interfere with the analyte (Figures 4-6). The results indicated that the method exhibited good specificity and selectivity.

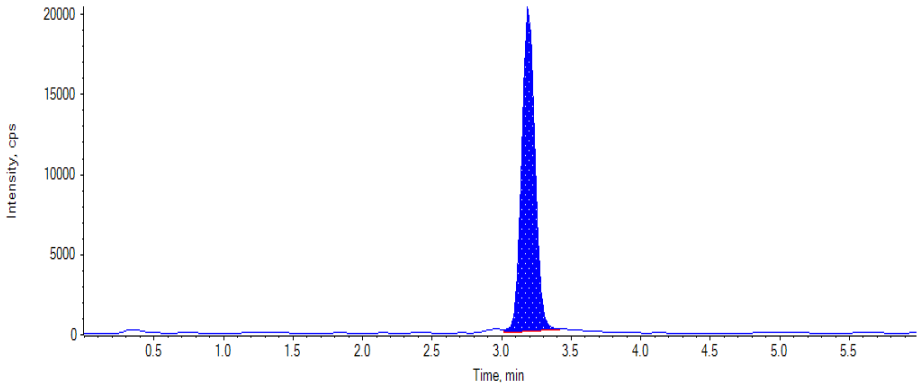


Figure 4: Chromatograms of apigenin

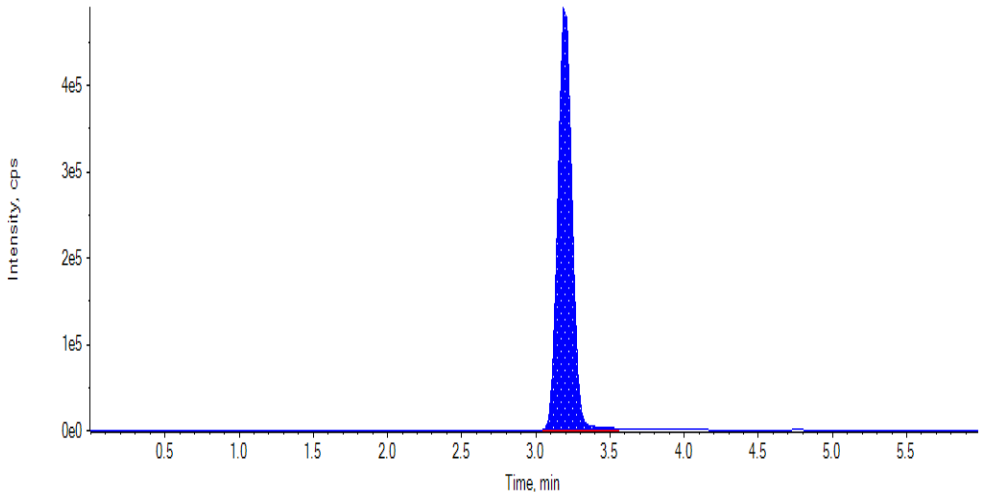


Figure 5: Chromatograms of luteolin

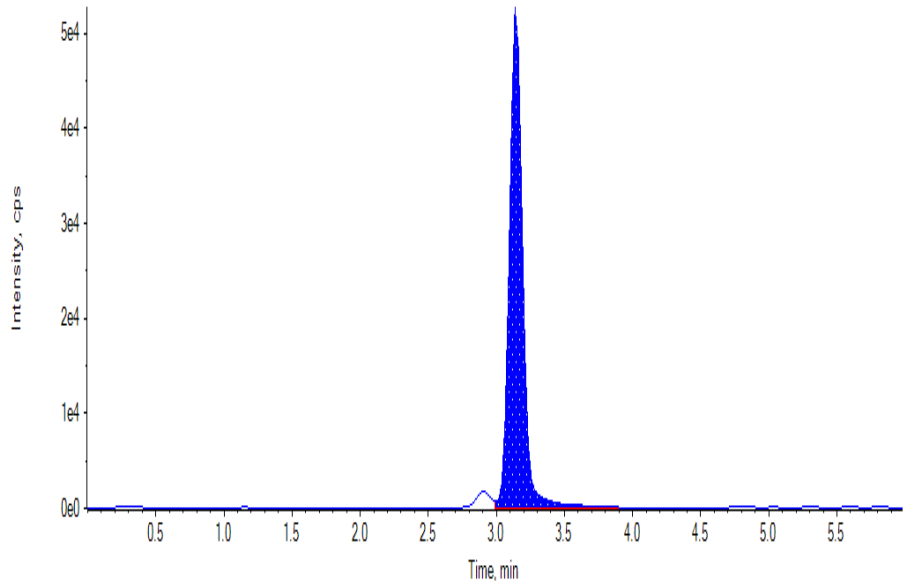


Figure 6: Chromatograms of quercetin

Linearity

The linearity was evaluated by analyzing a series of different concentrations of the standards apigenin, luteolin and quercetin where each concentration was applied triplicate. Linear regression data for the calibration curves of standard apigenin showed a good linear relationship over the concentration range of 1.25-5000 ng/ml with respect to the area (Table 4), correlation coefficient (R^2) was 0.995 and linear regression equation was found to be y = 66.81x + 8762, where y is the spot area and x is the concentration of the analyte (Figure 7), luteolin showed a good linear relationship over the concentration range of 1.25-5000 ng/ml with respect to the area (Table 5), correlation coefficient (R^2) was 0.995 and linear regression equation was found to be y = 72.89x + 8031 (Figure 8), and quercetin showed a good linear relationship over the concentration range of 1.25-5000 ng/ml with respect to the area (Table 6), correlation coefficient (R^2) was 0.991 and linear regression equation was found to be y = 86.18x + 19246 (Figure 9).

Limit of detection of developed method was found to be for apigenin 24.04 ng/ml, for luteolin 17.03 ng/ml and for quercetin 27.22 ng/ml and Limit of quantitation was found to be for apigenin 84.98 ng/ml, for luteolin 51.61 ng/ml and for quercetin 82.48 ng/ml indicating acceptable sensitivity of the method.

Table 4: Linearity study for apigenin (1.25-5000 ng/ml)

Conc. (ng/ml)	Peak area 1	Peak area 2	Peak area 3	Avg. peak area[a]	SD	%RSD
5000	329000	335000	340090	334696.7	5551.2	1.65
2500	184000	187900	191200	187700	3604.1	1.92
1250	104000	99950	99700	101216.7	2413.6	2.38
125	16500	17000	16890	16796.67	262.7	1.56
12.5	4830	5020	4950	4933.333	96.0	1.94
1.25	1165	1150	1170	1161.667	10.4	0.89
Note: a n=3 replicates, % RSD= Relative Standard Deviation						

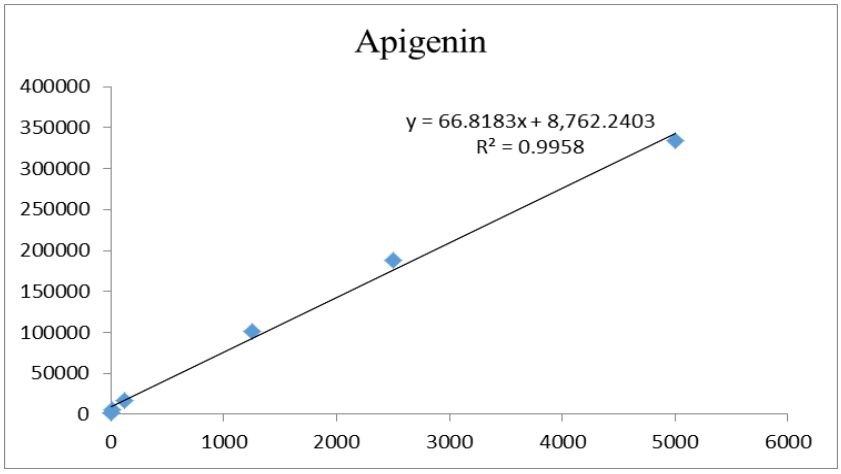


Figure 7: Calibration curve of apigenin standard (1.25-5000 ng/ml)

Table 5: Linearity study for luteolin (1.25-5000 ng/ml)

Conc. (ng/ml)	Peak area 1	Peak area 2	Peak area 3	Avg. peak area[a]	SD	%RSD
5000	365000	362940	359900	362613.3	2565.64	0.70
2500	206000	200800	210000	205600	4613.02	2.24
1250	108000	110000	107800	108600	1216.55	1.12
125	13300	13450	13400	13383.33	76.37	0.57
12.5	4290	4320	4330	4313.33	20.81	0.48
1.25	1640	1690	1660	1663.33	25.16	1.51
Note: a n=3 replicates, % RSD= relative standard deviation						

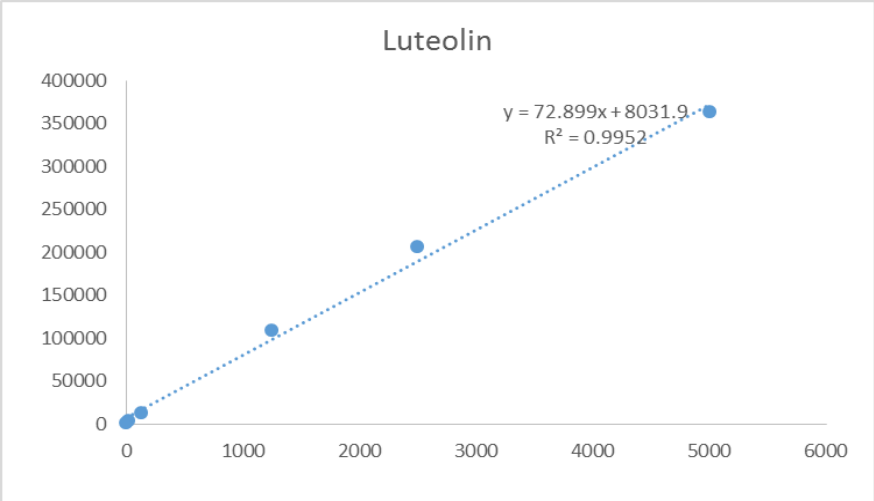
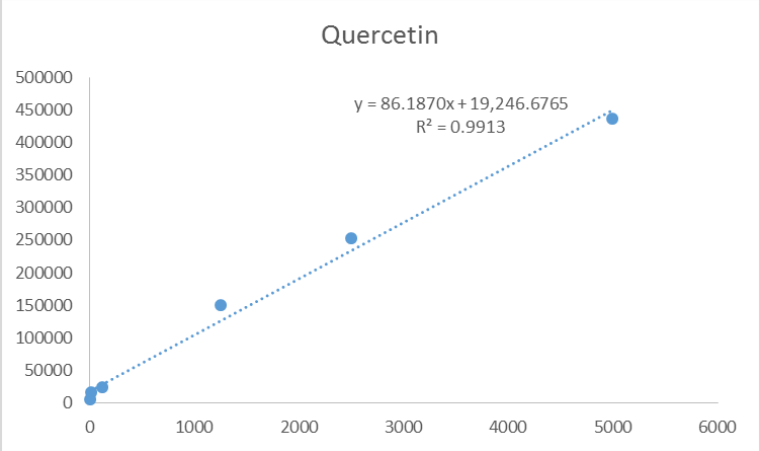


Figure 8: Calibration curve of luteolin standard (1.25-5000 ng/ml)

Table 6: Linearity study for quercetin (1.25-5000 ng/ml)

Conc. (ng/ml)	Peak area 1	Peak area 2	Peak area 3	Avg. peak area[a]	SD	%RSD
5000	426000	438100	443900	436000	9132.90	2.09
2500	249000	255000	253000	252333.3	3055.05	1.21
1250	148000	151500	147900	149133.3	2050.20	1.37
125	23800	23750	24350	23966.6	332.91	1.38
12.5	16100	15900	16000	16000	100	0.62
1.25	4150	4050	4225	4141.6	87.79	2.11
Note: a n=3 replicates, % RSD= Relative Standard Deviation						

Figure 9: Calibration curve of quercetin standard (1.25-5000 ng/ml)



Analysis of sample

Extract when analysed in triplicate using the developed method in present study was quantify for apigenin, luteolin and quercetin (Figure 10) indicating that the method can be applicable in routine quality control testing of extract. The % RSD value was found to be less than 2 (Table 7).

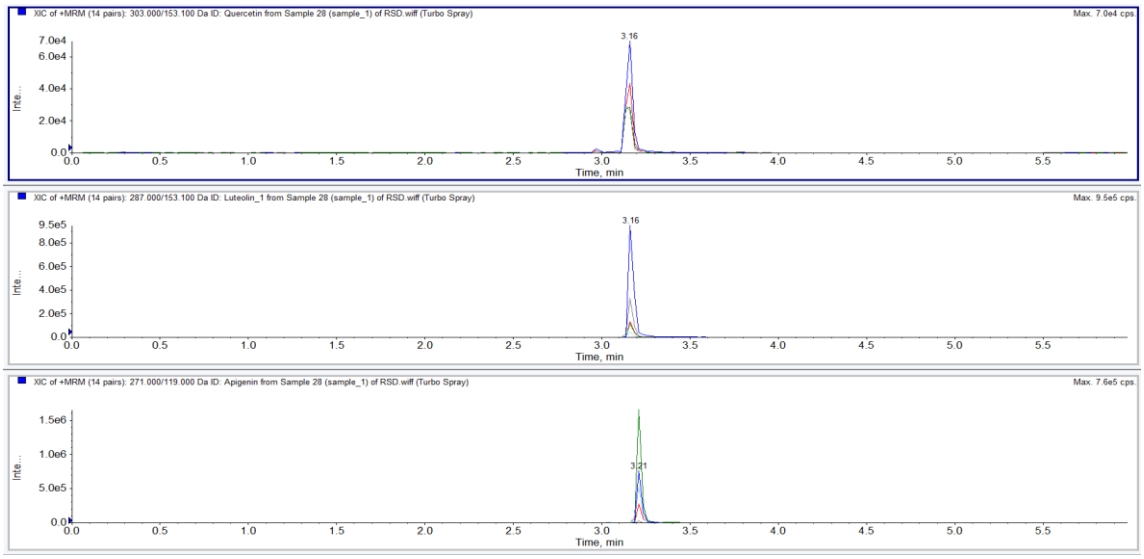


Figure 10: Chromatogram of apigenin, luteolin and quercetin in extract

Table 7: Analysis of sample

Drug	amount of drug found [a] (mg/gm) ± SD	% RSD
Apigenin	18.2 ± 0.18	1.01
Luteolin	16.3 ± 0.28	1.20
Quercetin	27.46 ± 0.22	0.91
Note: a n=3 replicates, % RSD= relative standard deviation		

Precision and accuracy

Apigenin, luteolin and quercetin samples at three concentrations were analyzed in triplicates in order to determine the intra-and interday precisions and accuracy of developed method was determined at three level (80%, 100% and 120%) of concentration 1250 ng/ml (Tables 8 and 9).

Table 8: Precision study

Amount (ng/ml)	Repeatability[a]		Interday precision[a]	
	Mean amount of drug found[a] ± SD (ng/ml)	%RSD	Mean amount of drug found[a] ± SD (ng/ml)	%RSD
Apigenin				
1250	101102 ± 18.65	1.85	101386 ± 22.33	2.13
125	16888 ± 33.52	1.36	16888 ± 54.12	1.36
12.5	5010 ± 17.42	1.40	5010 ± 12.32	1.40
Luteolin				
1250	108160 ± 12.26	1.60	107400 ± 14.25	1.44
125	13320 ± 32.78	1.62	13520 ± 17.85	1.71
12.5	4363.8 ± 10.20	1.62	4558 ± 2.32	1.90
Quercetin				
1250	14984 ± 32.96	1.33	15006 ± 54.12	1.36
125	2380 ± 22.38	1.38	2391 ± 36.78	1.56
12.5	1617 ± 14.12	1.51	1563 ± 18.95	1.56

Note: a n=3 replicates, % RSD= relative standard deviation

Table 9: Recovery of apigenin, luteolin and quercetin from *Achillea millefolium* extract.

Drug name	Recovery Level (%)	Amount added (ng/ml)	Initial amount (ng/ml)	Mean amount found	% Recovery[a]	SD	%RSD
Apigenin	80	1250	1000	207800	132.40	2.19	1.66
	100	1250	1250	187700	107.13	2.15	2.01
	120	1250	1500	226666	118.60	0.95	0.80
Luteolin	80	1250	1000	185666	108.31	2.16	1.99
	100	1250	1250	208000	109.73	1.09	1.00
	120	1250	1500	252166	121.79	1.62	1.33
Quercetin	80	1250	1000	225100	106.162	0.65	0.62
	100	1250	1250	252333	108.186	1.41	1.31
	120	1250	1500	274000	107.493	0.08	0.07

Note: [a] n=3 replicates**,** % RSD= relative standard deviation

CONCLUSION

In summary, we have developed and validated a highly sensitive, simple, efficient and reliable method for determining apigenin, luteolin and quercetin concentration in *Achillea millefolium* plant extract. This method produced excellent reproducibility, rapid sample preparation and accurate quantification for this study. The assay was shown to be accurate and precise over the concentration range of 1.25–5000 ng/ml for apigenin, luteolin and quercetin respectively.

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