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RP-HPLC Method for Identification and Quantification of Vitexin from the Plant *Justicia gendarussa*

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ABSTRACT

A reverse phase HPLC method was developed and validated for the identification and estimation of vitexin in the leaves extracts of *Justicia gendarussa*. Optimum separation was attained using acetonitrile: 0.1% ortho phosphoric acid (20:80v/v) as the mobile phase in an isocratic elution mode using Inertsil ODS-3V, 4.6mm×250mm, 5µm column at the flow rate of 1 mL/minute. The retention time is 11.05 minutes for Vitexin. The wavelength of maximum absorption selected for quantification was 335 nm. As per ICH guidelines the method was validated for system suitability, precision, linearity, solution stability, LOD and LOQ, robustness. In system precision, the % RSD obtained is less than 2% for both retention time and area response. The correlation coefficient is 0.999 for linearity. The results for robustness are not affected by small, deliberate changes. Vitexin was identified and quantified in the leaves extract of *Justicia gendarussa* using the developed HPLC method.

KEY WORDS: RP-HPLC, Validation, Vitexin, *Justicia gendarussa.*

1. INTRODUCTION

Justicia gendarussa Burm. f. (Acanthaceae) commonly known as black adusa. The plant grows in moist environment in India, Malaysia, China, Indonesia, Philippines, Sri Lanka, and Bangladesh. Usually, *J. gendarussa* has been used to treat lot of aliments. *J. gendarussa* possess anti-angiogenic effect (Periyanayagam, 2009), hepatoprotective potential and antioxidant (Krishna, 2010), antifungal activity (Sharma, 2011), antibacterial activity, analgesic activities and anti-inflammatory (Jothimanivannan, 2010), antinociceptive activity, larvicidal and adulticidal activity (Senthilkumar, 2009), these things are being reported by different researchers. *J. gendarussa* contains triterpenoids, gendarusin A and B (Prajogo, 2007), tannins, steroids, alkaloids and flavonoids. The leaves and roots are febrifuge, acrid, thermogenic, emetic, anodyne, diaphoretic, insecticidal and antipyretic (Warrier, 1995; Khare, 2007).

Flavonoids are polyphenolic compounds. By solvent extraction process greater than 4000 chemically different flavonoids have been isolated and they were identified in different plant extracts (Rodders, 1996; Bandow, 2003). The flavonoids have produced interest because of their useful effects on human health. These flavonoids have been reported to have anti-allergic, antiviral, anti-inflammatory, anti-platelet anti-tumour and antioxidant activities. Vitexin is a naturally arising flavonoid and lignin compound that is identified in various plant sources. Vitexin with is used to prevent heart diseases and the solubility is poor in water (Zu, 2012). Vitexin efficacy have been proved in numerous cardiovascular diseases and it is cardio protective (Zhang, 2008; Lu, 2013). It exhibited potent anti-inflammatory, hypertensive, anti-spasmodic and anti-metastatic potential properties. Hypertensive effect of Vitexin was endorsed to blocking properties of ganglion, antiserotonin, antibradykinin and antioxidative properties (Jin, 2005). Vitexin contains other effects such as anticonvulsant effects (Abbasi, 2012) enhancing memory retrieval, synergistically affect cell growth and apoptosis of colon cancer cells (Papi, 2013), vitexin encourages apoptosis in human leukaemia cells via a mitochondrial signalling pathway (Lee, 2012) and antiglycation activity. Our present study is to develop simple, sensitive, precise and reproducible method by HPLC for the quantification of Vitexin.

2. MATERIALS AND METHODS

Chemicals and Reagents: Standard Vitexin was purchased from Sigma-Aldrich, Bangalore, India. HPLC grade solvents acetonitrile, methanol and orthophosphoric acid were purchased from Spectrochem, Bangalore, India. HPLC water was used throughout the experiment. All the materials used were stored at recommended storage conditions.

Preparation of extract: The leaves of *Justicia gendarussa* were washed with running water and then with double distilled water. Dried for one month in shade and powdered coarsely. The leaf powder of the plant was weighed and extracted using Soxhlet method for 24 hours at 50°C with methanol. Filtered the extract through Whattman filter paper. Concentrated the solvent extract at 45°C until total dryness.

Extraction of Flavonoid: To separate the compound from methanol extract of *Justicia gendarussa*, the residue was dissolved in 500mL of distilled water. Partitioned the filtrate with petroleum ether, ethyl ether and ethyl acetate. Ethyl acetate extract was concentrated by vacuum oven. The extract from ethyl was runned through column chromatography on Sephadex LH-20 eluted with methanol-water gradient to obtain Vitexin (Methanol: Water 1:1).

Chromatographic conditions of HPLC analysis: The isolated compound in methanol was filtered and analysed in HPLC (Dionex Ultimate 3000), by injecting 10µl through the Inertsil ODS-3V, 4.6mm×250mm, 5µm with 1 mL/min

as the flow rate. The column temperature kept at column temperature 30°C. Mobile phase is prepared by mixing of acetonitrile: 0.1% orthophosphoric acid (20:80v/v), filtered with 0.45µ pore size filter and sonicated for 5 minutes and the run time was 30 minutes. Peaks are monitored at wavelength 335 nm by UV detection. Wavelength is fixed as per lambda maxima obtained from UV spectra. Methanol is used to dilute standard and sample. Chromatographic peak is identified on the basis of retention time.

Method Validation:

System suitability: To check the system is working appropriately and can give precise and accurate results, the system suitability parameters are to be set.

Specificity: Specificity is to check, if there is any interference of peaks obtained from blank with respect to main peak. Specificity parameter of the method is performed by injecting blank, standard and sample preparation. Recorded the retention times of blank, vitexin peak from standard and sample preparation.

System precision: The system precision is to confirm that the analytical system is working appropriately and is checked with standard chemical. With six determinations, the retention time and area responses should be measured and calculate for RSD. Injected blank (diluent) (1 injection), standard preparation (6 injections) into the chromatograph. Recorded the chromatographs. Calculated RSD for retention time and area of 6 determinations.

Method precision: In method precision, is to check the constant results obtained from, single homogeneous sample which is analyzed for six times. Calculated the percentage with respect to standard preparation.

Stability in analytical solution: Estimated by injecting both the standard and sample preparations at fixed interval to check the analytical solution stability.

Linearity: The linearity of method is its capacity to produce test results that are directly related to the concentration within a particular range with respect to analyte. Performed the linearity for vitexin standard in the range of 50% to 150% ie., 20 ppm, 32 ppm, 36 ppm, 40 ppm, 44 ppm, 48 ppm and 60 ppm.

Recorded the area response at each level and calculated slope, intercept, correlation and regression coefficient (R square). Tested the intercept for statistical equivalence to zero. Plotted a graph of concentration (ppm) on X-axis and area response under the curve on Y-axis.

Limit of detection and limit of quantitation: Limit of detection is the lowermost amount that can be detected in a analyte, but not quantitated, under the specified experimental situations. Limit of quantitation is the lowermost amount that can be quantitated in analyte with acceptable accuracy and precision, under the specified experimental situations. Calculated slope, correlation coefficient and the residual standard deviation from the linearity curve. From the slope and residual standard deviation calculated limit of detection and quantitation.

Limit of detection (LOD): Calculate limit of detection from linearity curve as per formulae given below.

LOD = 3.3 x Residual standard deviation / Slope

Limit of quantitation (LOQ): Calculate limit of Quantitation from linearity curve as per formulae given below.

LOQ = 10 x Residual standard deviation / Slope

Robustness: The robustness of an analytical method is a measure that should not be changed by small variations and the method should produce similar results. Robustness is performed by changing column temperature by ± 5°C, flow rate by ± 0.2 mL/min, organic phase by ± 2% and wavelength by ± 3nm.

3. RESULTS AND DISCUSSION

System suitability: The tailing factor and theoretical plates of vitexin peak in standard is 1.28 and 8402 respectively. The % RSD for 6 standard injections is 0.5. From the results, it was concluded that the system is suitable for analytical method validation.

Specificity: The blank peaks were not interfered with vitexin peak (Figure-1). The retention time of vitexin peak in standard is 11.05 and for sample is 11.07 (Figure-2 and Figure-3). The peak of Vitexin is pure and the match factor is 999 (Figure-4).

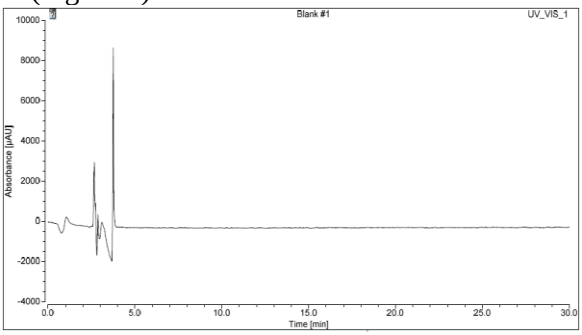

Figure.1. Chromatogram for blank solution

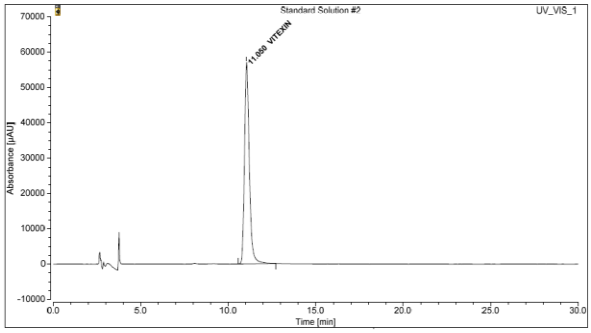

Figure.2. Chromatogram for standard solution

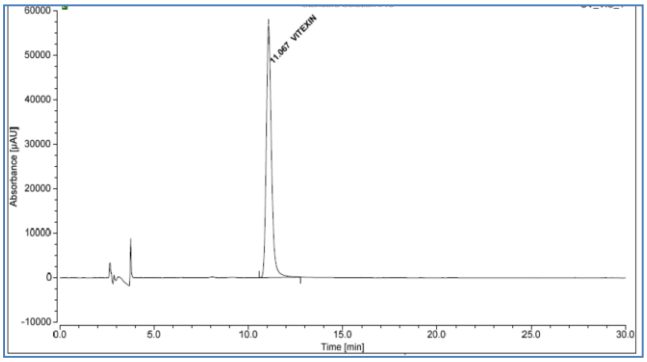

Figure.3. Chromatogram for sample solution

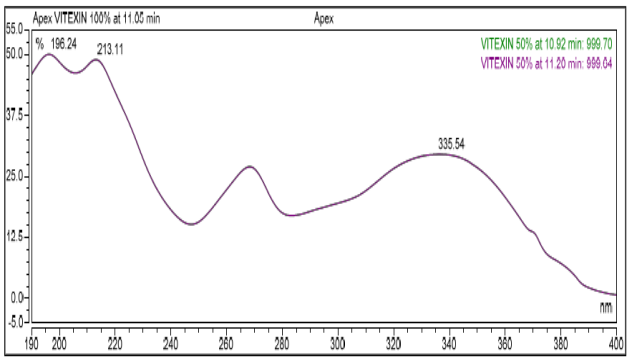

Figure.4. Peak purity spectra of vitexin in sample solution

System precision: From the Table.1, it was determined that retention times & area responses are consistent as evidenced by relative standard deviation. Hence, it was determined that the system precision parameters meet the requirement of validation.

Table.1. System precision for vitexin standard

Injection No.	Retention Time (in min)	Areas response
1	11.005	1093656.056
2	11.057	1081284.029
3	11.067	1080899.164
4	11.057	1092065.513
5	11.063	1086029.587
6	11.060	1087844.208
Mean	11.059	1086963.093
RSD	0.1%	0.5%

Method precision: From the Table.2, it was concluded that the method is precise.

Table.2. Method precision of vitexin for 6 injections

Set No.	Vitexin (in %)	Set No.	Vitexin (in %)
1	100.1	5	100.4
2	100.0	6	100.2
3	100.5	Mean	100.2
4	99.9	RSD	0.2

Stability in analytical solution: From the Table-3, it is concluded that, the vitexin standard and sample preparation is stable up to 24 hours (% difference is 0.8 % and 1.8% respectively) at room temperature (25°C).

Table.3. Solution stability of standard and sample solution at room temperature (25°C)

Time (Hrs.)	Standard solution		Sample solution	
	Area	% Difference	Area	% Difference
Initial	1096452.542	-	1086713.025	-
6	1096871.923	0.0	1090473.470	0.3
12	1097242.065	0.1	1095344.812	0.8
18	1099482.731	0.3	1105845.324	1.8
24	1105753.286	0.8	1106047.621	1.8

Linearity: From the statistical treatment of the linearity data for Vitexin, it is clear that the response of Vitexin was linear between 50 % and 150 % of the working concentration for Vitexin (Figure.5). The correlation and regression coefficients are found more than 0.998 Table.4. In addition, the values are arbitrarily scattered around zero (Figure.6), the linear model was in good fit Table-5. To calculate whether the y-intercepts were different from zero, the P–value was determined. The P value is greater than 0.05 then intercept is equal to zero. For Vitexin, P value is 0.18.

Table.4. Data for linearity

Conc. in ppm	Area Response	Conc. in ppm	Area Response
20.0	563449.167	44.0	1146665.913
32.0	832365.155	48.0	1250419.940
36.0	956371.717	60.0	1537205.124
40.0	1021473.341		
Correlation Coefficient	0.999	Slope	24563.265
Regression Coefficient	0.998	Residual standard deviation	14734.545

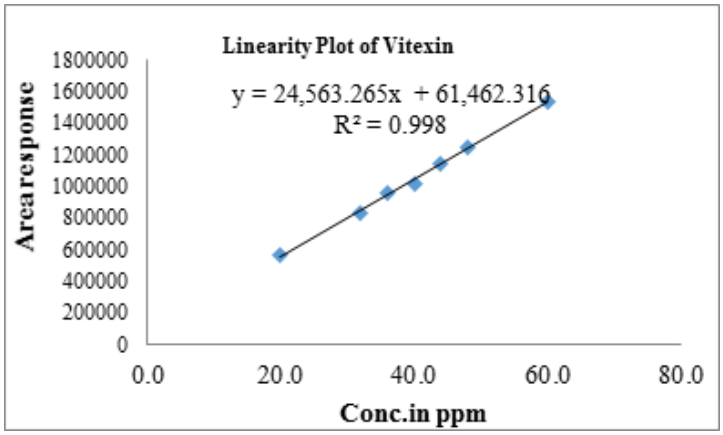

Figure.5. Linearity plot for vitexin

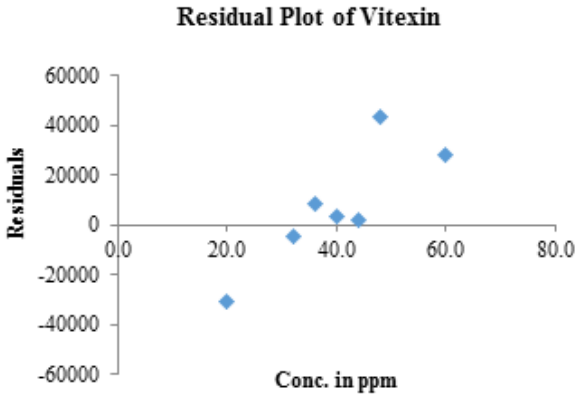

Figure.6. Residual plot for vitexin

Table.5. Data for residual output

Observation	Predicted Y	Residuals	Standard Residuals
1	1789606.73	-30913.65	-1.048
2	2136268.61	-4433.38	-0.150
3	2829592.36	8234.77	0.279
4	3176254.24	3322.49	0.113
5	3522916.12	1650.74	0.056
6	3869577.99	43164.52	1.464
7	4129574.40	27860.54	0.945
8	5256225.50	-48886.04	-1.658

LOD and LOQ: Distinct visible peak was observed at LOD level concentration. LOD and LOQ were found to be 1.98ppm, 5.99ppm respectively. These values indicate the method is sensitive.

Robustness: From the Table.6 results, it was concluded that, the method is robust.

Table.6. Theoretical plates, tailing factor and % RSD results obtained by changing in flow rate, temperature, organic phase and wavelength

Changes	Theoretical plates for vitexin peak in standard solution	Tailing factor for vitexin peak in standard solution	The % RSD for vitexin peak from five replicate injections of standard solution
Decrease in Flow (-0.2ml/min)	9168	1.27	0.7
Increase in Flow (+0.2ml/min)	7510	1.24	0.2
Decrease in Temperature (-5°C)	8140	1.26	0.4
Increase in Temperature (+5°C)	8699	1.25	0.2
Decrease in Organic Phase (-2%)	8412	1.25	0.6
Increase in Organic Phase (+2%)	8567	1.24	0.7
Decrease in nm (-3nm)	8402	1.23	0.7
Increase in nm (+3nm)	8434	1.26	0.2

4. CONCLUSION

A simple and efficient RP-HPLC method was developed for identification and quantification of Vitexin from the leaves extract of Justicia Gendarussa. As per ICH guidelines the HPLC method was developed validated. The developed method is simple, rapid, specific, precise and reproducible. The developed method can be used for the routine identification and quantification in quality control of herbal formulation containing Vitexin and also for pharmacokinetic studies of related extracts and drugs.

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