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Research Article

Development and Validation of RP-HPLC Method for the estimation of vitexin in *Convolvulus arvensis* and *Neurada procumbens*

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ABSTRACT

Vitexin, a C-glycosyl flavonoid was estimated in the extracts of *Convolvulus arvensis* and *Neurada procumbens* by reversed phase high-performance liquid chromatography (RP-HPLC). C18 column was used as stationary phase and methanol-0.05 % o-phosphoric acid in the ratio of 20:80 was used to separate the compounds from the extracts by isocratic program. Vitexin was detected at 340 nm by a UV-Visible detector, which showed a sharp peak with the retention time of 6.692 minutes. The quantitative study revealed that 0.727 mg/g of vitexin in the extract of *Convolvulus arvensis* and 0.0372 mg/g of vitexin in the extract of *Neurada procumbens*. The linearity range was found to be between 100 ng to 200 µg/ml with the correlation coefficient of $r^2 \geq 0.9994$. The limit of detection and limit of quantification of vitexin were found to be 30 and 100 ng/ml. The developed method is simple, precise and accurate for the quantification of vitexin in the extracts and might be helpful in the quality control of these plants.

INTRODUCTION

Vitexin is an apigenin flavone glycoside (5, 7, 4-trihydroxyflavone-8-glucoside) found in a number of plants such as passion flower, bamboo leaves, chaste tree, hawthorn, pigeon pea, mung bean, mimosa, wheat leaves and pearl millet¹. It is a C-

glycosyl flavonoid and has gained a growing attention due to its wide ranges of biological activities². It is reported to possess antioxidant^{3,4}, anticancer⁵⁻⁷, spasmolytic⁸, antithyroid⁹, anti-aging¹⁰, cardioprotective¹¹⁻¹³, neuroprotective¹⁴⁻¹⁶, antinociceptive¹⁷, antiviral^{18,19} and antidepressant like properties²⁰ referring its actions on multiple

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systems of the body like cardio vascular, endocrine and central nervous systems. Most of the C-glycosyl flavonoids show higher antioxidant activity than their corresponding O-glycosyl flavonoids, which necessitates the researches on vitexin and plants containing C-glycosylflavonoids²¹. Qualitative detection and quantitative estimation of vitexin in medicinal plants are important to correlate the biological activities of a particular medicinal plant. Plants and plant extracts could also be standardized with the use of vitexin as marker compound which is important for the quality assurance of herbal drugs. Hence, quantization of vitexin in plants might be helpful in predicting the possible biological activities of the plants. *Convolvulus arvensis* L (Family-Convolvulaceae) and *Neurada procumbens* L (Family-Rosaceae) are reported to possess various biological activities especially on the cardiovascular system^{22, 23}. *Convolvulus arvensis* diuretic, purgative, aphrodisiac and nervine tonic²⁴. It is also useful in rheumatism, chronic ulcers and skin diseases²⁵. It was demonstrated to have tumor inhibiting and immune stimulating activities. *Neurada procumbens* a tonic for heart and also useful in diarrhea and dysentery²⁶. These plants contain flavonoid compounds, which have not been studied in detail. Thus, estimation of flavonoid compounds, especially vitexin might be useful in not only predicting the possible biological activities of the plant, but also useful in quality control of the plants or their extracts. Vitexin and related flavonoid compounds in several plants have been identified and estimated by various methods including LC-MS²⁷, HPTLC²⁸, HPLC²⁹ and UPLC³⁰. In the present study, an accurate and precise HPLC method has been developed for the estimation of vitexin *Convolvulus arvensis* and *Neurada procumbens*.

MATERIALS AND METHODS:

Sample preparation:

The leaves of *Convolvulus arvensis* and the aerial parts of *Neurada procumbens* were collected, cleaned, dried in an oven below 50 °C and powdered. Around 20 g of powder was extracted with 2x100 ml of methanol by maceration for 24 h. The extract was then filtered and the solvent was removed under vacuum using a rotary evaporator. The dried extracts were subjected for the HPLC analysis.

Chemicals and reagents:

The standard compound, vitexin and HPLC grade solvents (methanol, water and o-phosphoric acid) were purchased from Sigma Aldrich, USA. For HPLC analysis, the prepared solutions were filtered through a 0.22µm filter.

Instrument and chromatographic conditions:

The chromatographic separation was performed using Shimadzu HPLC instrument (Japan) with quaternary LC-10A VP pumps and a variable wavelength programmable UV-visible detector. The instrument was controlled by a software, Class VP 5.032. Standard and sample solutions were injected through a rheodyne injector with a 20 µl loop. C18 column (150 mm x 4.6 µm) was used for the analysis. Methanol and 0.05 % o-phosphoric acid in the ratio of 20:80 was used to elute the compounds of the extracts by isocratic program. The detection wavelength was set at 340 nm by a UV-Visible detector at ambient temperature. The flow rate was fixed as 1 ml/min. The retention time and peak area of vitexin was noted.

Standard and sample solutions:

Vitexin stock solution was prepared in methanol. Accurately weighed quantity of vitexin (10 mg)



was dissolved in 5 ml methanol by sonication in a 10 ml volumetric flask. The volume was made up to 10 ml with methanol. Various lower concentrations were prepared from the stock solution by appropriate dilution with methanol. 1 mg/ml solution of vitexin was prepared in methanol and various lower concentrations were prepared by diluting with methanol. Accurately weighed quantity of extracts (100 mg each) was dissolved separately in 5 ml of methanol in a 10 ml volumetric flask by sonication. The volume was made up to 10 ml with methanol to obtain 10 mg/ml solutions. Before injecting in to the HPLC system, the standard and sample solutions were filtered through 0.22 μ m syringe filter.

Validation:

The results of the method validation are useful to judge the reliability and consistency of analytical results. The developed method for the analysis of vitexin was validated in accordance with the guidelines of International Conference on Harmonization (ICH) for validation of analytical procedure. Linearity, precision, accuracy, robustness, limit of detection and limit of quantification were determined to validate the method³¹.

Linearity:

The various concentrations of vitexin solutions were injected into the HPLC system for the construction of calibration curves and to determine the linear range of detection. Vitexin was quantified in the extracts from calibration curve which was constructed by plotting peak areas against concentrations.

Precision:

Intra-day and inter-day precisions were determined to find out the intermediate precision.

Intra-day precision was performed by analyzing three different concentrations of standard in triplicate and the values were expressed as % RSD. Inter-day precisions were performed by analyzing three different concentrations of standard in triplicate on three different days.

Accuracy:

The accuracy was determined by standard addition method by calculating the recoveries of vitexin at three levels. Specified amounts of vitexin standard was mixed with the pre-analyzed sample solutions at three different levels (50, 100 and 150 %) and the amount of vitexin was analyzed by peak areas. The mean recoveries for vitexin at each level and the respective RSD were determined.

Robustness:

Robustness was determined by making small changes in the method deliberately. Deliberate changes were made in the mobile phase composition and column temperature of analysis to determine robustness. The standard deviation and % RSD of retention time and the peak area were determined. The low % RSD values of the analysis indicate the robustness of the method.

Limit of detection and quantification:

The limit of detection (LOD) is defined as a signal-to-noise ratio of 3:1 for this method. The limit of quantification (LOQ) is the lowest analytical concentration of a compound that could be measured with accuracy and precision. The signal to noise ratio is usually ten times. The limit of quantification of vitexin was determined by introducing three injections at a concentration near the LOQ.

RESULTS AND DISCUSSION:

Method development:



The RP-HPLC method was developed to resolve vitexin from other related flavonoid compounds in the methanol extracts of *Convolvulus arvensis* and *Neurada procumbens*. Several mobile phase compositions were investigated to get a satisfactory separation with symmetric peak shape. Based on several trials, C18 column with methanol and 0.05 % o-phosphoric acid in the ratio of 20:80 as mobile phase by isocratic program was found to be suitable for the separation of vitexin in the methanol extract. Sharp peaks obtained from the standard indicating the suitability of the column and mobile phase selection. The compounds in the extracts were detected at 340 nm using a UV-Visible detector at ambient temperature which was found to be suitable for the detection and quantification of vitexin in the extracts. The retention time of vitexin was found to be 6.692 minutes. RP-HPLC method is fast, sensitive and efficient in the separation and estimation of flavonoids³². Therefore, reversed phase column with polar mobile phase has been used in the present investigation which resulted in well resolved sharp peaks. The chromatograms of standard and samples were shown in figures 1-3.

Quantization of vitexin in extracts:

The developed method for the quantification of vitexin showed well-resolved peaks. The standard showed one symmetric peak with the retention time of 6.692 ± 0.036 minutes (Figure 1). From the chromatograms of extracts, it is evident that the compounds in the extracts were well separated with the optimized chromatographic conditions. The retention time of the vitexin in the extracts was very close to the retention time of the standard. Thus, vitexin was detected in the extracts of *Convolvulus arvensis* and *Neurada procumbens*. Peak area of vitexin was noted for standard and samples for the quantitative analysis. The analysis revealed that 0.727 mg of vitexin was found in 1 g

of the extract of *Convolvulus arvensis* whereas the extract of *Neurada procumbens* showed 0.0372 mg of vitexin per gram of extract. *Convolvulus arvensis* showed the higher quantity of vitexin than *Neurada procumbens*

Linearity:

The calibration curve of the standard, vitexin was constructed by plotting peak area against concentration. The linear regression analysis showed the r^2 values for vitexin is 0.9994. This value is indicating the linear relationship between the concentrations of the standard and area under the curve. Calibration curves were linear in relatively wide ranges of concentrations from 0.1-200 $\mu\text{g/ml}$ with high correlation coefficient values between peak area (y) and amount of each compound (x, μg). The optimized parameters of the HPLC method were given in table 1.

Precision:

The % RSD values of peak area and retention time for vitexin are not more than 2 % in inter day and intraday analysis (Table 2). The analysis of intraday precision showed that the % RSD ranges from 0.550 to 1.44 for the peak area of vitexin, while the % RSD ranges from 0.35 to 1.57 for the peak area of vitexin in the inter day precision analysis. The inter system precision study showed the % RSD from 0.70 to 1.23. Thus, there were no significant differences found in the precision analysis. From the results of intraday and interday analysis, it can be concluded that the developed method showed a good precision.

Accuracy:

The recovery of the vitexin ranged from 97.42 % to 101.83 % for *Convolvulus arvensis* and 99.27 to 101.94 % for *Neurada procumbens* in the accuracy study. The % RSD values are also in good



agreement with the acceptable ranges (Table 3). The results of the standard addition analysis indicated that the method developed is accurate and suitable for the estimation of vitexin in plant extracts.

Limit of detection and limit of quantitation:

LOD and LOQ were determined to be 30ng/ml and 100 ng/ml for vitexin. The developed method is sensitive for detecting vitexin at a nanogram level. A study on the estimation of vitexin from *Thunbergia laurifolia* by HPLC showed the LOD and LOQ as 0.69 and 2.08 $\mu\text{g/ml}$ ³³. But, the present method detects vitexin at nano gram level which indicates the developed method is more sensitive in the detection and estimation of vitexin.

Robustness:

Robustness of the method was determined by making small changes in the mobile phase composition and column temperature. The other parameters kept unchanged. The mobile phase consisting of methanol-0.05 % o-phosphoric acid in the ratio of 20:80 was changed as methanol-0.05 % o-phosphoric acid (22:78) and methanol-water (18:82) and vitexin was estimated. RSD (%) of peak area was found to be in the ranges from 0.37 to 0.57. Similarly, the RSD (%) of peak area was recorded which ranges from 0.38 to 1.38, when

analyzing the vitexin at the different column temperature such as 20, 25 and 30 °C. Based on the results, it is evident that there was no significant changes in the analysis and hence, the developed method is considered to be robust.

CONCLUSION:

A RP HPLC method was developed and validated for the estimation of vitexin in the plants, *Convolvulus arvensis* and *Neurada procumbens*. The method is simple, precise, sensitive and accurate for the estimation of vitexin in the plant extracts. The method could be conveniently used for the standardization of these plant extracts.

Table 1. The optimized parameters of the HPLC method

Parameters	Observations
Mobile phase	Methanol-0.05 % o-phosphoric acid (20:80)
Column	C18 150x4.6 mm particle size 5 μm
Detection wavelength (nm)	340 nm
Flow rate	1 ml/min
Injection volume	20 μl
Retention time (minutes)	6.692 minutes
Detection wavelength (nm)	340
Detector	UV visible detector
Method	Isocratic method

Table 2. Inter-day and intraday and precision analysis of vitexin

Concentration ($\mu\text{g/mL}$)	Inter-day precision				Intra-day precision			
	Mean area $\pm$ SD	% RSD	Mean $R_t \pm$ SD	% RSD	Mean area $\pm$ SD	% RSD	Mean $R_t \pm$ SD	% RSD
50	653951 $\pm$ 10091	1.54	6.784 $\pm$ 0.023	0.33	641768 $\pm$ 3535	0.55	6.822 $\pm$ 0.040	0.59
100	1183033 $\pm$ 4160	0.35	6.769 $\pm$ 0.041	0.60	1159997 $\pm$ 10972	0.95	6.882 $\pm$ 0.098	1.42
200	2347136 $\pm$ 3690 1	1.57	6.806 $\pm$ 0.050	0.73	2337124 $\pm$ 33679	1.44	6.658 $\pm$ 0.054	0.80



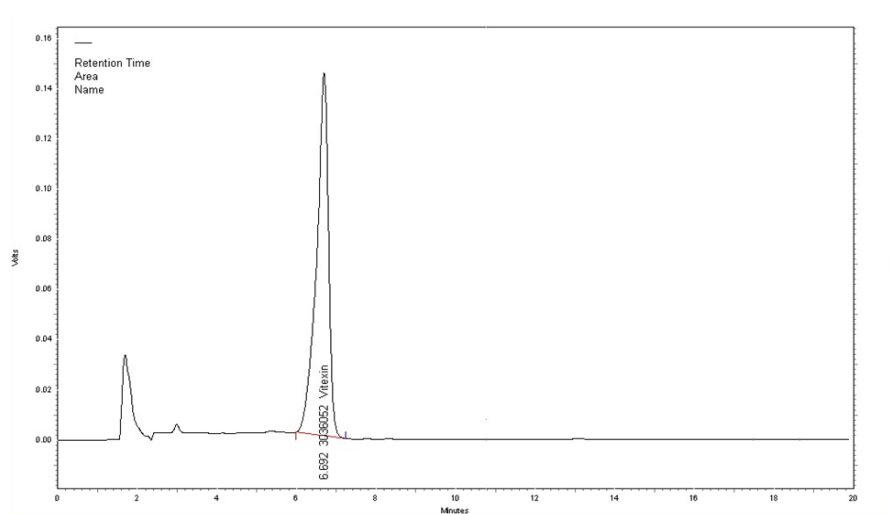
Table 3. Recovery analysis of vitexin by standard addition method

% of standard spiked to the sample		Theoretical content	Amount of drug recovered ($\mu\text{g/ml} \pm \text{SD}$)	% of drug recovered	% RSD
<i>Convolvulus arvensis</i>	0	1.817 $\mu\text{g/ml}$	1.78 $\pm$ 0.052	98.09	2.94
	50	2.725 $\mu\text{g/ml}$	2.65 $\pm$ 0.031	97.42	1.17
	100	3.634 $\mu\text{g/ml}$	3.69 $\pm$ 0.055	101.58	1.48
	150	4.542 $\mu\text{g/ml}$	4.63 $\pm$ 0.038	101.83	0.82
<i>Neurada procumbens</i>	0	93.50 ng/ml	94.13 $\pm$ 0.351	100.68	0.37
	50	140.24 ng/ml	139.30 $\pm$ 0.557	99.36	0.40
	100	187.43 ng/ml	190.63 $\pm$ 1.604	101.94	0.84
	150	233.79 ng/ml	232.00 $\pm$ 1.670	99.27	0.72

Table 4. Robustness studies of vitexin

Parameters		Vitexin	
		Retention time RSD (%)	Peak area RSD (%)
	78:22	0.85	0.37

Mobile phase composition	80:20	1.02	0.41
	82:18	0.33	0.57
Column temperature ($^{\circ}\text{C}$)	20	0.67	0.38
	25	0.56	0.25
	30	1.18	1.38

**Figure 1. HPLC Chromatogram of Vitexin**

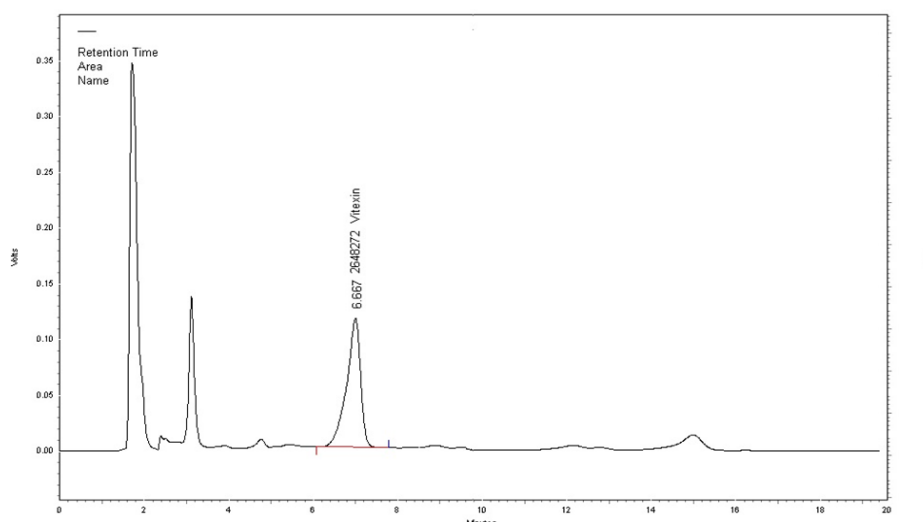


Figure 2. HPLC Chromatogram of *Convolvulus arvensis*

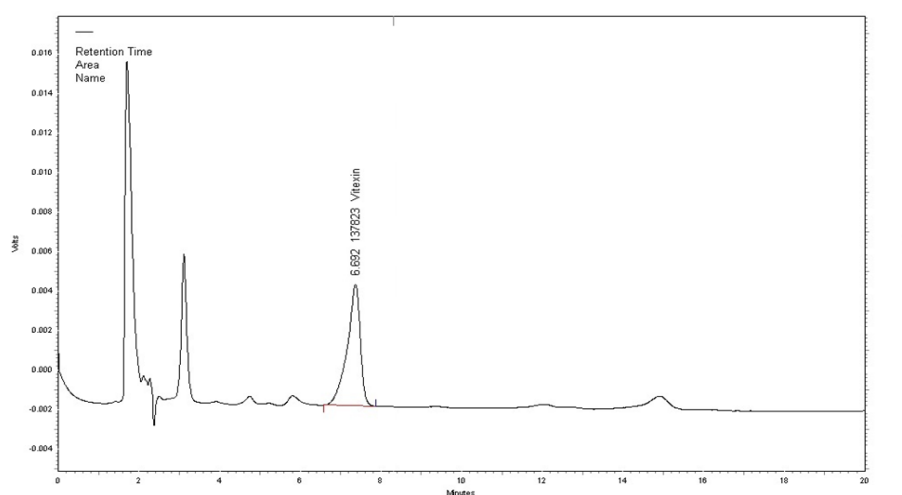


Figure 3. HPLC Chromatogram of *Neurada procumbens*

Conflict Of Interest:

The authors declare no conflict of interest

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