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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR DETERMINATION OF PICEATANNOL FROM *CYPRUS ROTUNDUS* LINN (NAGARMOTHA) EXTRACT

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ABSTRACT

Piceatannol is a stilbenoid and an important phytomolecule present in Cyperus rotundus Linn. plant. The major activity concerned is antiobesity activity of piceatannol. Compared with the analytical methods published in literatures, all methods have their drawbacks. Capillary electrophoresis needs a more expensive and less available instrument as compared with HPLC, even though only a tiny amount of sample is required for analysis. For GC analysis, stilbenoids need to be derivatized to enhance their volatility. In addition to the inherent limitations of instruments, some methods involve rather complicated extraction steps. Thus, a small quantity of Piceatannol presented in plants may be lost during the extraction, derivatization, or other sample preparation procedures. Hence, a method with fewer sample preparation steps is certainly preferred. There is no literature reported for the analysis of piceatannol in Cyperus rotundus linn. extract by HPLC, so there is need to establish a simple, sensitive, reliable, rapid and validated HPLC method for estimation and quantification of Piceatannol in C. rotundus Linn. extract. Following method provides a very reliable and accurate way for the quantification of piceatannol in Cyperus rotundus extract. The method was developed and validated on a C18 column with a gradient program using buffer and acetonitrile (ACN) as a mobile phase system. A good linear relationship between the concentrations (20–150%) and peak areas were obtained with the correlation coefficient (r^2) of 0.9991.

Keywords – Piceatannol, Cyperus rotundus Linn, Stilbenoids, HPLC, Rhizomes, Quantification.

1. INTRODUCTION

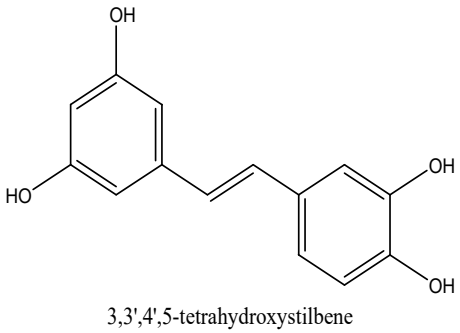
Cyperus rotundus (*C. rotundus*) Linn. (Cyperaceae) is a grass like plant well known for its pharmacological activities. The rhizomes of the plant have versatile biological activity. Phytochemical surveys revealed that the plant contained flavonoids, tannins, glycosides, furochromones, monoterpenes, sesquiterpenes, sitosterol, alkaloids saponins, terpenoids, essential oils, starch, carbohydrates, protein and amino acids[1]. Piceatannol was isolated from the rhizomes of *Cyperus rotundus* plant. It is a natural stilbenoid chemically known as (E)-4-[2-(3,5-Dihydroxyphenyl)ethenyl]-1,2 benzenediol,3,3',4,5' Tetrahydroxy-trans-stilbene having molecular formula $C_{12}H_{14}O_4$ (Molecular weight :244.2 g/mol). Piceatannol was found to possess many therapeutical and pharmacological potentials including antiobesity (antiadipogenic), antioxidant, anticancer, antidiabetic, antiasthmatic, in wound healing, in bone formation, antitumor[2]. The objective of the present work is to quantify the content of piceatannol in *Cyperus rotundus* Linn. extract followed by development of a

standard analytical protocol for the same by HPLC. HPLC is a popular method for the analysis of herbal medicines because it is easy to use and is not limited by the volatility or stability of the sample compound. HPLC is a powerful analytical technique due to its simplicity, sensitivity and reliability for the estimation of Phyto molecules. The present study aimed to quantify the content of piceatannol present in *Cyperud rotundus* formulation by HPLC. The proposed method was validated as per the ICH guidelines.

Fig. 1: *Cyperus rotundus* plant

Fig. 2: *Cyperus rotundus* rhizomes

Fig. 3: *Cyperus rotundus* Extract



3,3',4',5-tetrahydroxystilbene

Fig. 4: Chemical structure of Piceatannol

2. MATERIALS AND METHODS

2.1 Materials

HPLC grade Methanol, Acetonitrile, and Water were obtained from Merck Chemicals (Mumbai, India). Analytical grade formic acid was obtained from Merck Chemicals (Mumbai, India). *Cyperus rotundus* Linn extract was obtained from Sava Health Care Ltd, R & D Centre, Pune. A reference standard for Piceatannol was obtained from TCI Chemicals (India) Pvt. Ltd.

2.2 Chromatographic system

The HPLC system composed of Empower software certified for QA/QC. It consists of vacuum degasser, automatic front panel control, PDA detector, thermostat column compartment with C18 column [4.6mm x 250mm, pore size 5µm], high performance autosampler, thermostat for high performance autosampler. Its integrated solvent and sample management capabilities provide the flexibility and ruggedness needed to accommodate an enormous range of HPLC separation challenges.

2.3 Chromatographic condition

Two solvents were used, solvent-A containing ultra-pure water filtered through 0.22µm membrane filter [pH adjusted to 2.1 with formic acid] and solvent-B containing ACN degassed with sonication. A gradient program was made and the detection was performed at 327nm using diode array detector (Alliance 2998) using a gradient program.

2.4 Method

Solution of 10 ppm of piceatannol standard was prepared in the diluent. This solution was taken to develop a method by applying various solvent ratios for gradient program and chromatograms are recorded. Chromatograms are shown in figure 5 and 6.

Chromatographic conditions selected:

HPLC parameters consist of an Inertsil ODS column of 250 mm length and 4.6mm internal diameter with 5µ particle size of silica. A mobile phase was prepared by taking Millipore water and dissolving formic acid until pH is 2.1 (A), and Acetonitrile (B). Both mobile phases sonicated and degassed. Column temperature was kept at 45°C and sampler temperature 10° C. Flow rate was 1 ml/min and injection volume 20µl with a gradient program of 40min at 327nm wavelength.

Table-1 : Gradient Program

Time	Mobile phase A	Mobile phase B
0.01	80	20
20	80	20
22	70	30
29	65	35
34	65	35
37	80	20
40	80	20

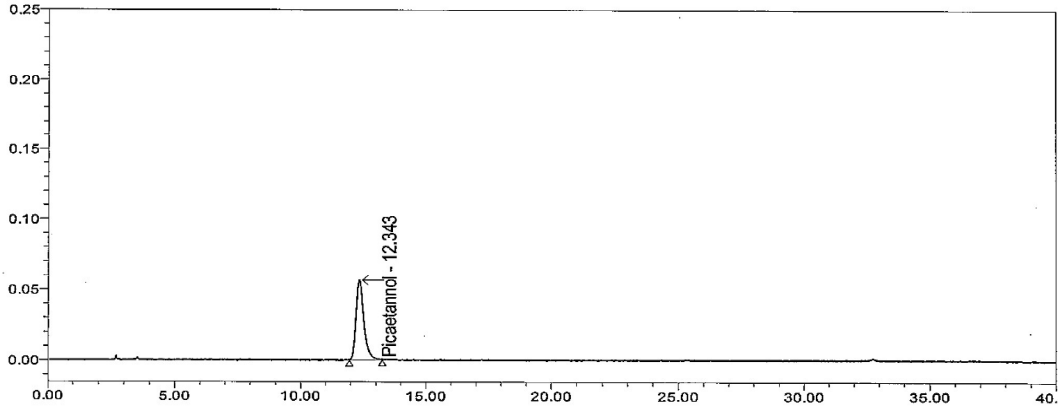


Fig. 5: Chromatogram of Piceatannol Standard Solution (RT-12.343 min)

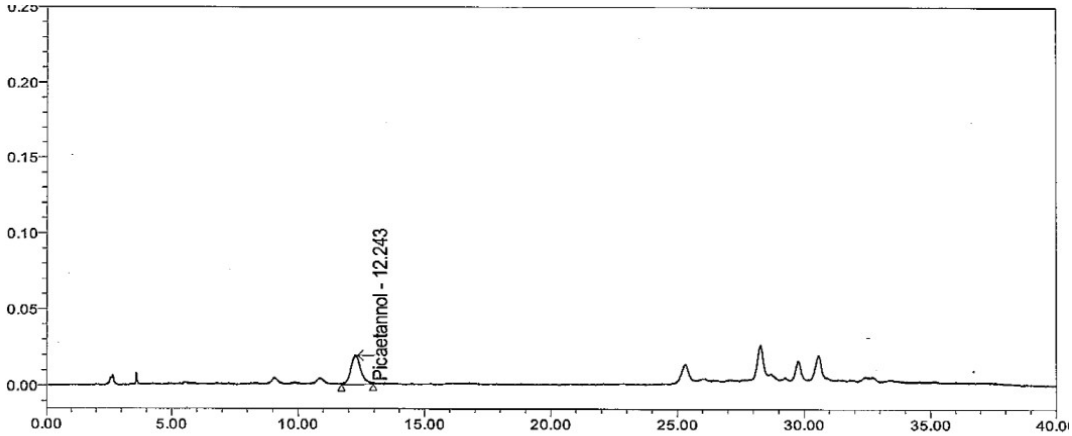


Fig. 6: Chromatogram of Picetannol Extract (Sample solution RT-12.243 min)

2.5 Method Validation

Validation of the optimized HPLC method was carried out with the following parameters.

2.5.1 Linearity

The linearity of method was demonstrated by preparing solutions of 10 ppm concentration (Stock solution) and by taking different aliquots of stock solution different dilutions were prepared over the concentration levels ranging from 20% to 150% of the standard solution of piceatannol. The solution of 200 µL was loaded in autosampler tray and 20 µL was being injected into column. All measurements were repeated three times for each concentration. The calibration curves of the area under curve versus concentration were recorded.

2.5.2 Accuracy

The accuracy of the method was determined by analysing the sample solution at three different concentration levels 50, 100 and 150% of the usual sample preparation concentration, and injected for the accuracy studies. The area under curve obtained was checked and analyzed for the recovery percentage.

2.5.3 Precision

The precision of the method was checked and verified by system precision, method precision and intermediate precision (Ruggedness) variation studies. In system precision studies six injections of standard solution of 2ppm was prepared as per the usual analytical method and injected into the system. In method precision six replicates of sample solution of the same batch were prepared and injected into the system. In intermediate precision (ruggedness) six replicates of a single batch samples were prepared and analysed by different analyst, on different day and on different instrument.

2.5.4 Robustness of the method

To determine the robustness of the developed method, minute changes were made in the flow rate and analytical wavelength of the analysis and is studied for the deviations from optimized method.

2.5.5 System suitability parameters

To perform the system suitability tests the standard solution of 2 ppm was freshly prepared and injected under the condition of optimized method to study the validation parameters.

2.5.6 Selectivity (Specificity)

To determine the peak purity selectivity studies was performed by injecting diluents, standard solution and sample solution.

Filter validation:

To determine the accuracy of filters, filter validation studies were performed. Standard and sample solution was prepared as per the analytical method. Some part of this solution was centrifuged at 4000 rpm for 10 minutes. Supernatant was decanted into another test tube. This solution was injected as centrifuged solution. The remaining half part of the solution was then filtered through 0.45 µ Nylon membrane filter and vials are filled by discarding 0mL, 2mL, and 5mL of solution. These solutions are the injected and analyzed. The absolute % difference between centrifuged and filtered sample was recorded.

3. RESULTS AND DISCUSSION

The developed and validated HPLC method was aimed to establish chromatographic conditions, capable of qualitative and quantitative determination of Piceatannol in *Cyperus rotundus* Linn. extract. Piceatannol was completely separated on C18 column by HPLC using gradient program of Buffer and ACN as a mobile phase system.

3.1 Linearity and range

The method gave a linear response to Piceatannol in *Cyperus rotundus* formulation within the concentration range of 20-150% with correlation coefficient (r^2) of 0.9991 as shown in figure 6.

Table-2: Linearity results

Linearity level	Concentration (%)	Concentration (PPM)	Area
1	20	0.401	32293
2	30	0.602	46857
3	50	1.003	84507
4	80	1.604	148507
5	100	2.005	192530
6	120	2.406	228073
7	150	3.008	288076

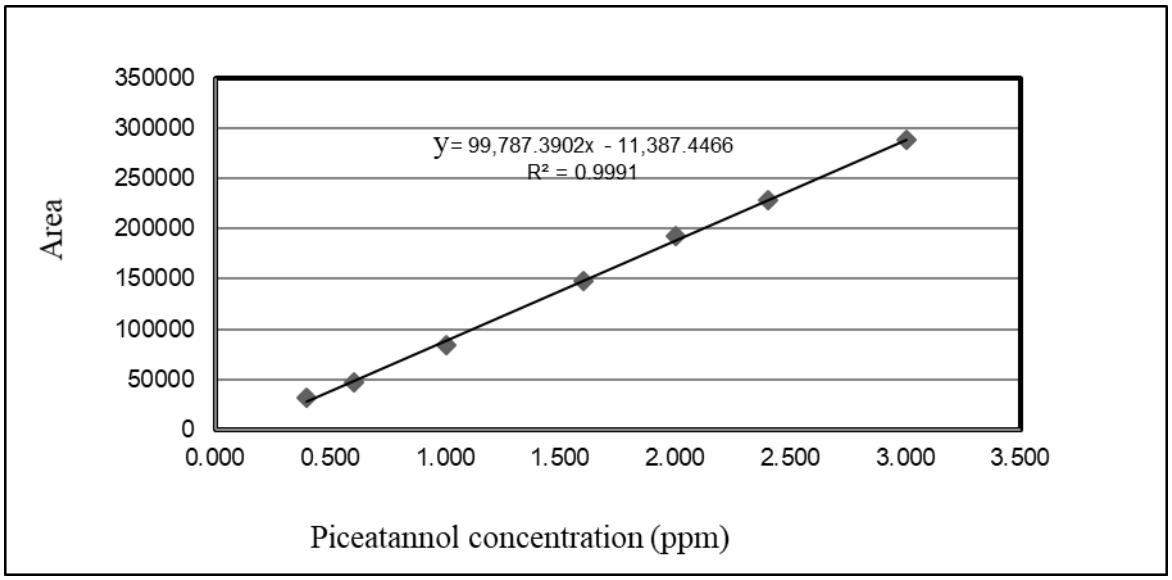


Fig. 7: Standard Calibration Curve of Piceatannol

3.2 Accuracy

Piceatannol recovered in the range of 98.0 to 102.0 % for various concentrations.

Table 3. Accuracy results

Concentration (%)	Area (Average)	Amount Recovered	Recovery %
50	158947	0.180	100.00
100	336492	0.181	100.55
150	529116	0.183	101.66

3.3 Precision

The system precision, method precision and intermediate precision (ruggedness) are calculated. The RSD values were below 2%, indicating a good precision.

Table-4: System precision results

Injection no.	Area
1	234374
2	233516
3	234186
4	234512
5	234413
6	234040
Average	234174
Standard deviation	363.9856
% RSD	0.16

Table-4: Method precision results

Preparation	Area of sample solution	% Content w/w (Piceatannol)
1	440760	0.179
2	435636	0.177
3	448369	0.181
4	459412	0.184
5	458251	0.185
6	443713	0.180
Average		0.181
Standard deviation		0.0030
% RSD		1.7

Table 5: Intermediate precision results

Preparation	Area of the sample solution	% Content w/w (Piceatannol)
1	452818	0.180
2	435476	0.175
3	445156	0.176
4	433028	0.175
5	452682	0.179
6	436606	0.175
Average		0.177
Standard deviation		0.0023
% RSD		1.3

3.4 Robustness

The robustness of the method gave the mean, standard deviation (SD) and RSD within the limits.

Table-6: Change in flow 0.9 ml/min

Injection no.	Area	Piceatannol	
		Tailing factor	Theoretical plates
1	161284	1.6	5905
2	161219	1.5	5708
Average	161252		
STDEV	45.9619		
% RSD	0.03		

Table-7: Change in flow 1.1 ml/min

Injection no.	Area	Piceatannol	
		Tailing factor	Theoretical plates
1	139175	1.8	5246
2	138905	1.8	5070
Average	139040		
STDEV	190.9188		
% RSD	0.14		

Table-8: Change in wavelength 324nm

Injection no.	Area	Piceatannol	
		Tailing factor	Theoretical plates
1	167787	1.5	6207
2	168400	1.5	6269
Average	168094		
STDEV	433.4565		
% RSD	0.26		

Table-9: Change in wavelength 330nm

Injection no	Area	Piceatannol	
		Tailing factor	Theoretical plates
1	157953	1.4	6309
2	159876	1.5	6104
Average	158915		
STDEV	1359.7663		
% RSD	0.86		

3.5 Filter validation

The absolute % difference in area between centrifuged and filtered samples was within limits.

Table-10: Filter validation results

Standard Solution	Absolute % Difference in Area
Centrifuged	NA
0 mL discarded	1.12
2 mL discarded	0.56
5 mL discarded	0.00

3.6 System suitability studies

The system suitability parameters such as retention time, theoretical plate number and peak purity of optimized method were associated with confined values.

Table 11 : System suitability parameters

Area	239785
Theoretical plates	5655
Tailing factor	1.40

3.7 Selectivity studies

Table-12: Selectivity study results

Sr. No.	Solution	Observation
1	Diluent	No interference is observed at the retention time of analyte peak.
2	Standard Solution	Peak purity passes for analyte peak
3	Sample Solution	Peak purity passes for analyte peak

3.8 Overall Validation Results

Table 13: Validation Summary

Sr. no	Parameters	%RSD	Tailing factor	Theoretical plates
1	Specificity (Selectivity)	0.94	1.4	6332
2	Linearity	0.14	1.3	5889
3	System Precision	0.35	1.4	5692
4	Method Precision	0.35	1.4	5692
5	Intermediate Precision	0.14	1.3	5889
6	Accuracy (% Recovery)	0.94	1.4	6332
7	Filter Study	0.35	1.4	5692
8	Robustness- Low Flow	0.03	1.6	5905
9	Robustness- High Flow	0.14	1.8	5246

10	Robustness- Low Wavelength	0.26	1.5	6207
11	Robustness- High Wavelength	0.86	1.4	6309

4. CONCLUSION

A new HPLC method was developed and validated for the estimation of Piceatannol from *Cyperus rotundus* Extract. The method is simple, accurate, precise and robust and can be used for routine analysis of Piceatannol in extracts as well as its marketed formulation.

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