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Isolation, Characterization & Phytochemical Screening, Analytical Method Development and Validation for the Determination of Catechins in *B.ciliata* by RP HPLC Method

NDVR Saradhi*[1], N Madan Gopal[1], C Madhusudhna Chetty[1], K Sushmitha[1]

[1]Santhiram college of Pharmacy, NH – 40, Nerawada, Kurnool Dist, Andhra Pradesh, India

Abstract : Ayurveda, an ancient system of medicines detailing a number of medicinal plants and their activities in human or animals. The present research work was aimed to develop an analytical procedure for the determination of catechins in the selected plant - B.Ciliata. It is famously known as stone flower/ stone breaker having various biological activities like anti urolithiatic, antiviral, antidiabetic antitumor and cardio protective activity. The methanolic extract of the plant is isolated and a method is developed by using RP HPLC for the determination of catechins in the crude plant extract using a C_{18} column (200*4.6mm, 5µ) and detected at 241 nm. The method is validated for its system suitability, Linearity, Accuracy, Precision, Robustness and sensitivity as per the ICH guidelines Q2(R1) to meet the analytical procedure in academic and industrial usage[19].

Key words : *B.Ciliata*, RP HPLC, Catechins, phytochemical Screening etc.

Introduction:

The B.Ciliata is one of the medicinal Plants discussed in the Ayurveda system of medicine[1,2]. The plant even though old but having many activities of it now days the usage of the plant leaves and roots is more. B.Ciliata is belongs to Saxifragaceae is a perennial herb. The Catechins are the phytochemicals having antioxidant, antimicrobial activity and used in kidney ailments. The earlier literatures revelas that there are only few methods which have been enlisted in the method development of catechins in various plants. There was no study conducted in the estimation of catechins in B.Ciliata with the selected stationary and Mobile Phases. The fresh leaves and rhizomes were collected from the Nandyal and its surroundings which were authenticated from the Senior Botanist Dr V J Sailaja Rani. The plant parts were shade dried and extracted with methanol, from the pure extract the RP HPLC method is developed for the estimation of catechins[3-7]. The plant leaves and rhizomes are shown in figure – 1.

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(a) Leaves of B.Ciliata (b) dried roots of B.Ciliata

Experimental:

Materials and Methods:

The chemicals used in the method were HPLC grade and Analytical Grade and their percentage of purity between 99.98 – 100.02%. chloroform, methanol, ethanol, Acetone, Distilled water. The pure form of the catechin was a gifted sample from the Hi Q laboratory, Hyd. The glass ware like sohxlet apparatus, volumetric flasks, beakers, round botton flaks used for extraction and other procedural activities is Borosil grade. The equipments are calibrated before the experiment electronic balance – Shimazdu (ATY240), Ultrasonicator, Double Beam spectrophotometer – Shimazdu (UV- 1800), HPLC – Analytical technologies (2230) powered with N 2000 software, column – waters (B.No: 080606) – 200*4.6mm, 5µ particle size stationary Phase were used for the experiment.

Plant Extraction:

The shad dried leaves were grounded to a fine size and sieved through No: 44, powder was packed in a muslin cloth and adjusted to a position of ¾ th level of sohxlet apparatus. In a 250 ml of round bottom flask, was added about 200 ml of methanol and made hot percolation was continued for 3 Hours, cooled the flask and make the crude extract was concentrated into solid on a rotary evaporator. The flakes of the crude drug was collected and stored in a SS bottle[6,7].

Mobile Phase:

An equal portion of the HPLC Grade methanol and Chloroform (50:50 v/v) into a flask, filtered through a 0.25 µ membrane filter, subjected for the vaccume filtration, the filtered diluent is collected and sonicated for 10 minutes to remove any air traps in the mobile phase. This is stored in a Borosilcate glass bottle for further use. This is termed as diluent[8, 9].

Standard solution preparation:

Pure catechin is weighed about 10 mg into clean volumetric flaks and make upto the mark with diluent. The further dilutions were made to get 3 ppm of catechin[10,11],.

Sample solution preparation:

An equivalent amount about 10 mg is taken into 10 ml volumetric flask and diluted upto the mark, further dilutions were made to get approximately 3 ppm of sample solution[12-15].

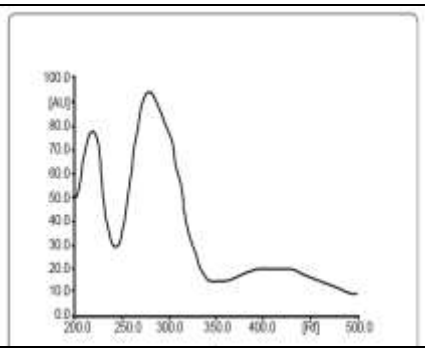
Phytochemical Screening:

General tests were performed for both standard and sample solutions for the identification of various phytochemicals. The test performed for carbohydrates, fats, proteins, alkaloids, glycosides, resins and tannins. Both show a positive results for tannins and further identification test conducted for identification of tannins by bromine water test and match stick test which showed and confirmed the presence of catechins in the plant extract[14].

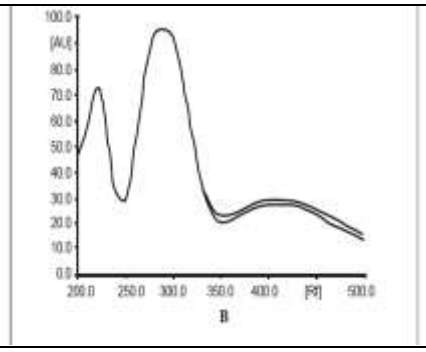
A TLC method is used for the separation of catechins from the crude plant extract. A pre coated TLC silica gel 60 F254 aluminium sheet is used as a stationary phase and methanol: water: chloroform (35:10:65 v/v) is a mobile phase. The sample and standard applied on the plate and eluted for 20 minutes. The plate was dried and sprayed with 0.55% vanillin in a 4% Hydrochloric acid. The spots were scrapped and dissolved in the methanol. Now this solution is measured for the absorbance to quantify the catechin present in the sample solution.

Measurement of Lambda Max:

A spectrum plotted using diluent as blank to the sample and standard solutions both are shown their lambda max at 241 nm. The figure 2 represented as:



Standard Catechin



Extract of B.Ciliata over standard Catechin

Results and Discussion:

Various trails have been conducted by changing the column conditions, mobile phase concentrations and UV detection wavelengths. The final optimized method developed by using Hyper chrome ODS- BP (200*4.6mm, 5µ) column with methanol: chloroform in 1:1 ratios, the flow rate maintained at1 ml/ min[16-18]. The chromatograms of the placebo, sample and standard were shown in figure – 3, 4, 5.

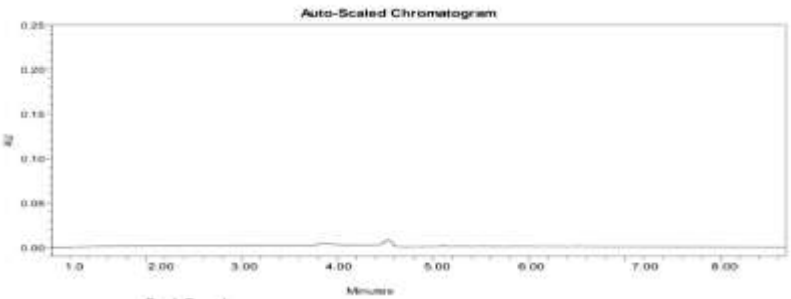


Figure : 3 – blank/ Placebu at 241 nm

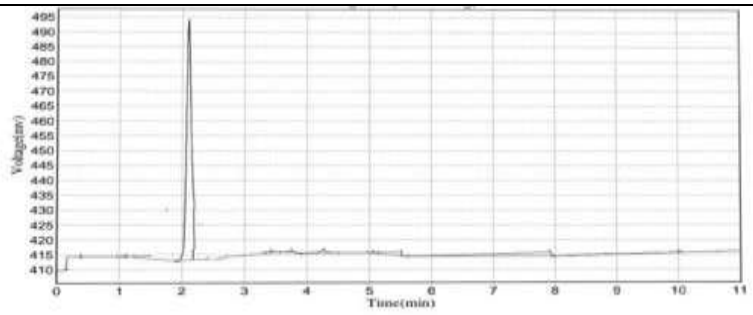


Figure :4 – Standard catechin showing peak at 241 nm

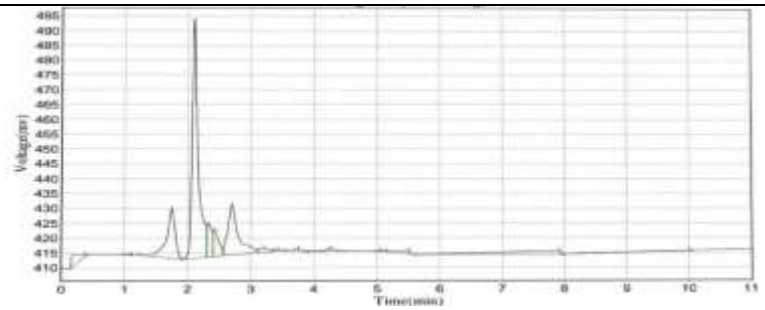


Figure :5 – Plant Extract showing peak at 241 nm

Validation of the method:

The method was validated various parameters per ICH guidelines Q2(R1)[19].

(a) **System suitability:** This procedure validates that the selected procedure for the estimation of the catechin in B.Ciliata by using the HPLC conditions. A replicate injection of same concentration of the catechin standard is injected into the system and measure the peak area, mean, SD, %RSD was calculated. The results were shown in table – 1.

(b) **Linearity:** Linearity or range is a measure of analytical procedure that the selected method is suitable for the least to maximum concentrations. The selected procedure is followed for beer lambart law. From the standard primary stock solution 1-5 ml was taken into individual volumetric flask and diluted with diluent to get 1 – 5 ppm. The peak response was measured for the individual concentration. The correlation between the individual concentrations was shown as in figure – 6.

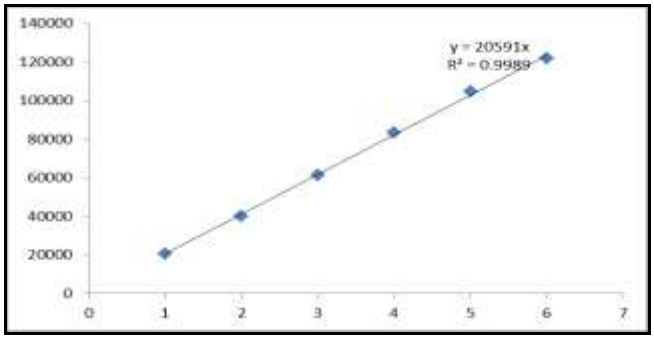


Figure 6: Linearity graph of catechin

(c) **Precision:** It is the agreement between individual test results with the procedure applied. The precision is completed in 3 steps. Repeatability, intermediate precision, reproducibility. The procedure involves the replicate injection of the same concentration of the sample injected to the HPLC, the peak area was noted between the days, analysts, instruments, and laboratories etc. the results were shown in the table – 1.

Table 1: System suitability & precision data for catechin

Injection Number	Concentration in PPM	System Suitability	Precision			
			Repeatability	Intermediate Precision	Analyst variation	Instrument variation
1	3	61587	61587	61597	61483	60823
2	3	61498	67570	61591	61532	59878
3	3	61481	61581	61599	61345	61233
4	3	61594	61594	61590	61563	60384
5	3	61595	61595	61596	61423	61482
6	3	61497	61597	61587	61082	62383
Mean		61542	61587	61595	61404	61030
Standard Deviation		55.172	10.36	3.76	176.31	879.0
% RSD		0.089	0.01	0.006	0.287	1.440
ICH Criteria		>2	>2	>2	>2	>2
Test Result		Passes	Passes	Passes	Passes	Passes

(d) **Accuracy:** It is a validated protocol for the measure the exactness of the experimental procedure. There are two ways for determining the accuracy comparison to a reference method, recovery of the analyte spiked into a blank matrix and standard addition of the analyte. This can be done by taking 3 different concentrations of the sample and injected to the HPLC system, measure the peak area of the each concentration and calculate the percentage of recovery from each level of concentration. The results were shown in table – 2.

Table No 2: Accuracy data of Catechin

S. No	Accuracy Level	Area	Spike added in mg	Recovered in mg	% Recovery
1	50%	31216	1.5	1.52	101.35
2		30682	1.5	1.49	99.62
3		30429	1.5	1.48	98.80
4	100%	60394	3.0	2.93	98.04
5		60297	3.0	2.96	97.88
6		62199	3.0	3.09	100.97
7	150%	91899	4.5	4.01	99.46
8		92385	4.5	4.52	99.99
9		92899	4.5	4.51	99.99

(e) **Robustness:** It is a measure of the capacity of the developed method. The procedure involves the change in the system conditions like column flow rate, temperature, detection wavelength etc. the results were tabulated in -3.

Table 3: Robustness data of Catechin

Parameter	Mean retention time	Mean area	% RSD
Change in detection wave length (nm)			
237	2.09	61488.6	0.0095
241	2.09	61586.6	0.0080
245	2.09	61738.6	0.0195
Change in Flow rate (ml/Min)			
0.9	2.0736	55435	0.0075
1.0	2.0946	61594	0.0048
1.1	2.1254	67755	0.0055

(f) **Sensitivity:** The sensitivity of the experiment can be calculated by LOD/LOQ of the validation parameters. The LOD is the lowest limit of quantity to be determined using developed procedure. LOQ is the highest minimum limit of quantity to be determined by using the procedure[20]. They were theoretically calculated using linearity graph for its signal to noise ratio and the results shown in the table - 4.

Table 4: Sensitivity data of Catechin

Name of the Drug	Slope	SD	LOD	LOQ
Catechins	20591	55.172	0.0088 µg/ml	0.0267 µg/ml
Acceptability			< 3.0	< 10

Conclusion:

The method initiated with methanol extract, isolation and phytochemical screening and there by HPLC method development. The instruments and chemicals used for the purpose is calibrated and fine graded suitable for the analysis through HPLC. The method developed is validated as per the ICH guidelines of Q2R1 for its system suitability, Linearity, Precision, Accuracy, Sensitivity and robustness to meet the academic and industrial needs.

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