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DEVELOPMENT AND VALIDATION OF A HPLC ANALYTICAL METHOD FOR DETERMINATION OF ELLAGIC ACID IN *EPILOBIUM ANGUSTIFOLIUM* EXTRACT

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Keywords:

Canadian willow herb extract, HPLC, Validation, Quality control

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ABSTRACT: Development of quality assessment parameters for natural products is a prominence necessity to justify their acceptability and activity. Establishment of authentic and reliable analytical methods which profile the quantitative phytochemical composition of marker constituents in multicomponent composition like extract is a challenging task. A simple, rapid, precise, and reliable HPLC method was developed for the separation and estimation of ellagic acid from *Epilobium angustifolium* (Canadian willow herb) extract. The estimation was carried out using Sunfire C18 column, 5µ (4.6 × 250 mm); mobile phase consisting of 0.1% orthophosphoric acid and acetonitrile; the flow rate of 1 mL/min and ultraviolet detection at 280 nm with a properly resolved having run time of 35 min. The method was validated as a final verification of method development concerning precision, linearity, accuracy, ruggedness, and robustness. The correlation coefficient (r^2) > 0.999, a method is considered to be linear as the correlation coefficient was found to be within acceptance criteria. The % RSD of peak area response due to ellagic acid in five replicate injections of standard solution was to be less than 2.0%, and system suitability parameters were passed. The % Average recovery of ellagic acid in Canadian willow Extract observed within the acceptance criterion of 98 - 102% indicates the accuracy of the method. The present validation proves that the HPLC-method is suitable for the determination of assay of ellagic acid from Canadian willow herb, extract at prescribed conditions.

INTRODUCTION: Plant species represent a great source of biologically active compounds whose effects on heritable material are mostly unknown. Investigation of medicinal plants is valuable on two levels: as a measure of safety for the continued use of medicinal plants and as a source of potential chemotherapeutic drugs.

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Due to widespread use in pharmacy and non-traditional medicine of extracts of medicinal plants, it could be necessary to investigate more on their safety [1]. The chromatographic or spectroscopic fingerprint profiles serve as guidelines to the phytochemical profile of the drug in ensuring the quality, while quantification of the marker compound/s would serve as an additional parameter in assessing the quality of the sample.

More recently a concept of multiple fingerprints construction and multidimensional fingerprinting have gained much attention, a large amount of chromatographic and spectroscopic signals enable more comprehensive data analysis [2].

To screen the natural samples for the presence of most active compounds fingerprinting analysis has been introduced. It was originally developed with the use of high-performance liquid chromatography. Apart from qualitative and quantitative data, it gives also the possibility to fish out the active compounds from a myriad of compounds present in herbal samples [3]. Chemical fingerprinting has been demonstrated to be a powerful technique for the quality control of herbal medicines. A chemical fingerprint is a unique pattern that indicates the presence of multiple chemical markers within a sample [4]. HPLC is a versatile, robust, and widely used technique for the isolation of natural products, HPLC is a chromatographic technique that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture.

Currently, this technique is gaining popularity among various analytical techniques as the main choice for fingerprinting study for the quality control of herbal plants [5]. Natural products are frequently isolated following the evaluation of a relatively crude extract in a biological assay to fully characterize its properties. The resolving power of HPLC is ideally suited to the rapid processing of such multi-component samples on both an analytical and preparative scale [6]. Several authors describe the use of HPLC for characterization and quantification of secondary metabolites in plant extracts, mainly phenol compounds, steroids, flavonoids, alkaloids [7]. The genus *Epilobium* is widely distributed around the world and consists of over 200 species, with the most common being *Epilobium angustifolium* L; commonly known as Canadian Willowherb Various members of the genus *Epilobium* have been used in folk medicine to treat a variety of diseases [8]. This botanical family can be commonly found in large parts of Western Europe, Siberia, China, Japan, North America and also in Ukraine [1].

Epilobium angustifolium is widely used in non-traditional medicine to treat gastrointestinal disorders, mucous membrane lesions, such as mouth ulcers, and to improve the speed of wounds healing, skin sores, swelling, and inflammations [9]. Canadian willow herb is a multifunctional active. Its primary function is as a nonsteroidal anti-irritant ingredient, which is attributed because of various Phytoconstituents like polyphenols, flavonoids, *etc*. *In-vitro* experiments have demonstrated free radical scavenging ability. Also, Canadian Willow herb shows strong antibacterial activity on *P. acnes*. This paper aims to develop and validate the HPLC method using suitable conditions for the determination of ellagic acid in *Epilobium angustifolium*.

MATERIAL AND METHODS:

Procurement of Sample: Canadian willow herb extract (5% clear) was imported as a gift sample from Lucas Meyer Cosmetics Canada (Product Code: 3378501); Ellagic acid (Potency-99.1%) purchased from Yucca Laboratories, Mumbai.

HPLC Method Development and Validation: This study aimed to validate the HPLC method for quantification of ellagic acid from Canadian willow extract as per the ICH Guidelines [10].

Standard Test Procedure:

Requisites: Ortho-phosphoric acid (AR grade), acetonitrile (HPLC grade), Water (HPLC grade), methanol (HPLC grade) all chemicals of Sigma Aldrich.

Chromatographic Condition:

- ✓ Column: Sunfire C18 column, 5µ (4.6×250 mm)
- ✓ Flow rate: 1 mL/minute.
- ✓ Column temperature: 30 °C ± 5 °C
- ✓ Sample temperature: 25 °C ± 5 °C
- ✓ Injection volume: 10 µL
- ✓ Detector: UV at 280 nm
- ✓ Run time: 35 min
- ✓ Retention time: About 16 min for Ellagic acid
- ✓ Diluent: Methanol (HPLC grade)

Mobile Phase Composition for HPLC: Solvent A – 0.1% Orthophosphoric acid solution and Solvent B – Acetonitrile

Details of Gradient Program:

TABLE 1: GRADIENT PROGRAM

Time (min)	Flow (mL/min)	% Solvent A	% Solvent B
0	1.0	99	01
20	1.0	99	01
21	1.0	05	95
30	1.0	05	95
31	1.0	99	01
35	1.0	99	01

Preparation of Standard Solution of Ellagic Acid: Weigh accurately about 50 mg of ellagic acid into 50 mL volumetric flask make up the volume with diluent. Further, dilute 20.0 mL of resulting solution into 50 mL volumetric flask and make up the volume with diluent. Use the resulting solution as a standard solution.

Preparation of Test Solution: Weigh accurately about 200 mg Canadian willow extract into 50 mL volumetric flask. Add 30 mL of diluent and then sonicate in an ultrasonic water bath for 20 min. Cool the solution and make up the volume with diluent up to the mark. Then filter through 0.45 µ syringe filter. Use the resulting solution test solution.

Analysis of Extract: Inject standard solution in five replicate and test solution in duplicate. Calculate system suitability parameters from standard chromatogram mentioned below.

Theoretical plates for analyte peak: NLT 2000
Tailing factor for analyte peak: NMT 2.0
% RSD for replicate injections of the standard: NMT 2.0

Estimation of Ellagic acid Present in Extract by HPLC: Test solution was injected, and the peak area was reported, the percentage of ellagic acid was calculated.

Validation Parameters: The validation has performed by using current ICH guidelines. The tests were performed about the Specificity, Linearity, and range, Accuracy, Precision, Robustness and Solution stability Validation parameters [10, 11].

RESULTS AND DISCUSSION:

HPLC Method Development and Validation: Validation can be defined as (ICH) Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics. The method was validated for several parameters like Specificity, Linearity, Accuracy, Precision, Robustness, Solution Stability as per ICH guidelines (Q2) R1. [10]

Specificity: The specificity of the method for Assay is demonstrated by injecting solutions into the HPLC system.

- Diluent as a Blank
- Standard Solution
- Test Solution

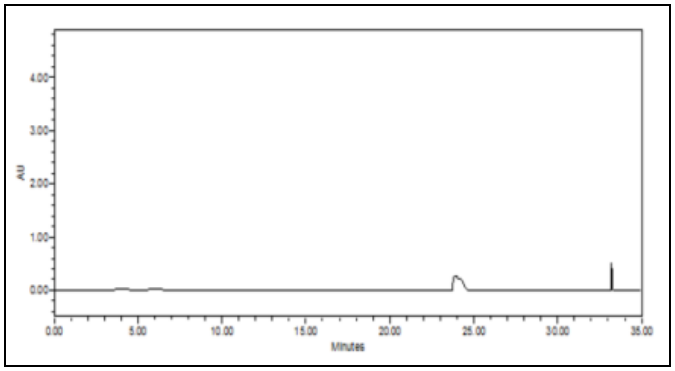

FIG. 1: HPLC CHROMATOGRAM OF BLANK SOLUTION

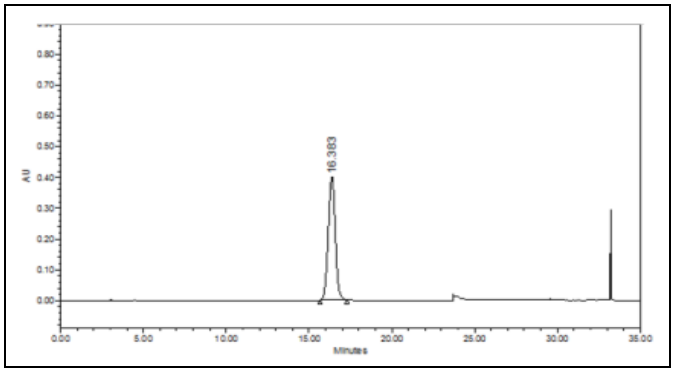

FIG. 2: HPLC CHROMATOGRAM OF STANDARD SOLUTION

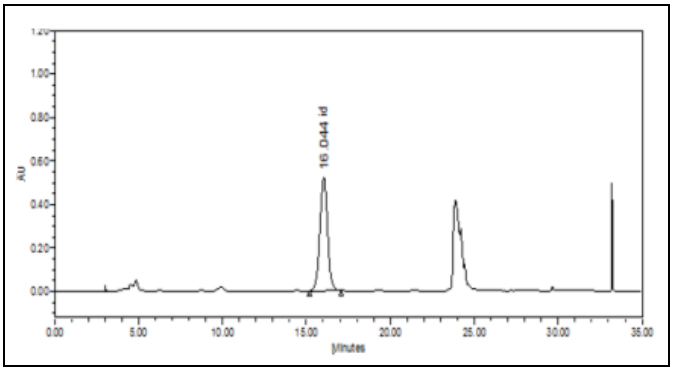

FIG. 3: HPLC CHROMATOGRAM OF SAMPLE SOLUTION

TABLE 2: SPECIFICITY OF ELLAGIC ACID

S. no.	Sample Name	Analyte Name	Purity Flag	Specificity
1	Canadian willow extract	Ellagic acid	No	Specific
2	Standard	Ellagic acid	No	Specific
3	Blank	No Peak	-	-

By comparing the chromatograms of the blank solution, Standard solution and Test solution the following evaluations were made.

- No peak was co-eluted with the analyte peak from Blank solution
- No purity flag was observed for ellagic acid from the standard solution
- No purity flag was observed for ellagic acid from the test solution.

The method is considered to be specific as per the above-mentioned observations.

Linearity: The linearity of peak area response for Ellagic acid was determined from 50% to 150% level of working concentration for ellagic acid. The stock solutions of ellagic acid were diluted in seven different known concentrations. Graphs of concentration (as x-value) versus area (as y-value) were plotted. The correlation coefficient, y-intercept, and slope of the regression were calculated and Tabulated in **Table 3**.

TABLE 3: LINEARITY OF ELLAGIC ACID

% Level	Conc. of ellagic acid (ppm)	Average peak area of ellagic acid
50	16	1088163
70	21	1374626
80	24	1652409
100	29	1956486
120	40	2811458
130	48	3423684
150	16	1088163

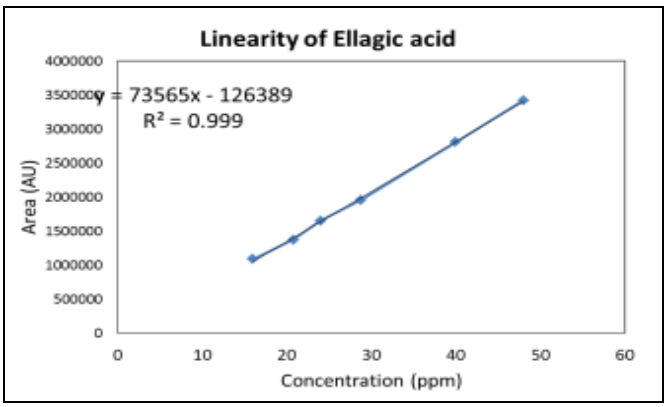

FIG. 4: HPLC CHROMATOGRAM OF SAMPLE SOLUTION

The Correlation coefficient (r^2) > 0.999, Method is considered to be linear as the Correlation coefficient was found to be within acceptance criteria.

Precision:

System Precision: System precision was evaluated from five replicate injections of standard as per the proposed method. The Peak area, average, and % RSD were calculated.

TABLE 4: SYSTEM PRECISION FOR ELLAGIC ACID

Inject. no.	Peak area of ellagic acid
1	2047982
2	2048031
3	2036917
4	2034722
5	2041344
Mean	2041799
% RSD	0.30

% RSD for Area of ellagic acid < 2.0% and the % RSD observed within acceptable limit indicates the precision of the system.

Method Precision: The six test solutions were prepared separately. Each test solution was analyzed as per the proposed procedure. The % assay, average, and % RSD were calculated.

TABLE 5: METHOD PRECISION FOR ELLAGIC ACID

Sample no.	% Assay of ellagic acid
1	1.39
2	1.41
3	1.43
4	1.45
5	1.44
6	1.42
Mean	1.42
% RSD	1.41

% RSD for Area of ellagic acid < 2.0% and the % RSD observed within acceptable limit indicates the precision of the system.

Intermediate Precision: The intermediate precision was determined by comparison of two independent analyses on 2 different days. The data of the 1st-day analysis was taken from the analysis of "Method precision."

TABLE 6: INTERMEDIATE PRECISION FOR ELLAGIC ACID

Name of Analyte	S. no.	Assay (% w/w, Analysis-1)	Assay (% w/w, Analysis-2)
Ellagic acid	1	1.39	1.47
	2	1.41	1.45
	3	1.43	1.48
	4	1.45	1.46
	5	1.44	1.47
	6	1.42	1.47
	Average	1.42	1.46
	% RSD	1.41	0.71
	Overall % RSD	1.86	

The relative standard deviation from analysis 1 and analysis 2 was to be within limits. The overall % RSD for % assay of ellagic acid from analysis 1 and analysis 2 was < 2.0%, % RSD of % assay results from 6 determinations are within acceptance criteria for day 1 analysis & day 2 analysis. Hence, the method of assay for ellagic acid from Canadian willow extract is rugged.

Range: The range of ellagic acid was determined by calculating precision at 50%, 100% and 150% level of the working standard concentration.

TABLE 7: INTERMEDIATE PRECISION FOR ELLAGIC ACID

Name of analyte	Injection no.	Peak area at 50% level	Peak area at 100% level	Peak area at 150% level
Ellagic acid	1	1086378	1952424	3428740
	2	1089948	1962712	3425347
	3	1087633	1950260	3418627
	Average	1087986	1955132	3424238
	% RSD	0.17	0.34	0.15

% RSD for area of ellagic acid < 2.0%. The % RSD of determinations for each level is within acceptance criteria shows the Range for the method.

Robustness: The influence of slightly changed parameters of the chromatographic conditions was tested according to ICH guidelines to demonstrate sufficient robustness of the method. The tests are carried out by injecting Standard solution by varying each of the parameters of chromatography.

TABLE 8: ROBUSTNESS PARAMETER OF ELLAGIC ACID

S. no.	Parameters	Working Parameter	- Changes	+ Changes
1	Flow	0.9 mL/min	1.0mL/min	1.1mL/min
2	Column temperature	28 °C	30 °C	32 °C
3	Wavelength	275 nm	280 nm	285 nm

TABLE 9: ROBUSTNESS FOR ELLAGIC ACID

Robustness Parameter		% RSD	Peak Tailing	Theoretical Plates	Remark
		Ellagic acid			
Wavelength (nm)	275	1.41	1.32	32760	Pass
	280	0.60	1.28	34338	Pass
	285	1.73	1.31	32811	Pass
Column Temperature (°C)	28	0.42	1.26	35139	Pass
	30	0.83	1.38	26276	Pass
	32	0.73	1.30	33692	Pass
Flow (mL/min)	1.0	0.38	1.25	37031	Pass
	1.0	0.52	1.21	44453	Pass
	1.1	0.45	1.27	30454	Pass

The % RSD of peak area response due to Ellagic acid in five replicate injections of standard solution was to be less than 2.0 %, and system suitability parameters were passed. The % RSD and system suitability parameters for results obtained with varied chromatographic conditions are within limits. Hence, the method is robust.

Accuracy: The accuracy was determined from recovery studies. A known but varying amount of Canadian willow extract was spiked into the pre-analyzed extract test solution at 80%, 100% and 120% recovery levels of working concentration in triplicate. The spiked test solution was analyzed according to the proposed procedure. The percentage recoveries were calculated against respective levels.

TABLE 10: RECOVERY FOR ELLAGIC ACID

Analyte	Recovery level	% Recovery	Average % Recovery
Ellagic acid	80% - 1	101.21	101.40
	80% - 2	101.24	
	80% - 3	101.77	
	100% - 1	100.29	101.29
	100% - 2	99.48	
	100% - 3	101.10	
	120% - 1	100.52	100.99
	120% - 2	101.84	
	120% - 3	100.59	

The % Average recovery of ellagic acid in Canadian Willow Extract observed within the acceptance criterion of 98-102% indicates the accuracy of the method.

Stability in Standard and Test Solution: The standard and test solutions were prepared as per the proposed method and kept at room temperature. The standard and test solutions were analyzed at initial and at different time intervals.

As the percentage relative changes of ellagic acid and extract were within the limit, Standard solution and test solution is stable up to 24 h at room temperature [10].

TABLE 11: SUMMARY OF RESULTS AND VALIDATION

Parameters	Acceptance Criteria	Result
Specificity	No Interference for Analyte Peak	Specific
Linearity	r^2 >0.999	0.999
Precision		
System Precision	% RSD <2.0	0.30
Method Precision	% RSD < 2.0	1.41
Intermediate Precision	% RSD <2.0	1.86
Accuracy		
80%	Average % Recovery should be in the range of 98% - 102%	101.40
100%	Average % Recovery should be in the range of 98% - 102%	100.29
120%	Average % Recovery should be in the range of 98% - 102%	100.99
Solution Stability	% relative change of Ellagic acid in standard and Test solution w.r.t initial <5.0%	The Standard and Test solution were stable for 24 h
Robustness	System suitability parameters should comply	Robust

CONCLUSION: The specificity of the HPLC test for assay of ellagic acid was proven by chromatographic comparison and method was found to be specific. The linearity of the proposed method was determined from the correlation coefficient and the method was found to be linear and within the range of 50% to 150% of working concentration. The accuracy of the method was calculated by recovery study & the proposed method was found to be accurate as all the parameter of the method complies as per the acceptance criteria. Standard & test solutions were found to be stable up to 24 h at Room temperature. The present validation proves that the HPLC-method is suitable for the determination of Assay of ellagic acid from Canadian willow extract at prescribed conditions.

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interest.

REFERENCES:

1. Halyna O, Oleksandra O, Samuele V, Samuela C, Elisabetta A and Michela C: *Epilobium angustifolium* L. A medicinal plant with therapeutic properties. The Euro Biotech Journal 2017; 1(2): 126-31.
2. Ostrovska H, Oleshchuk O, Vannini S, Cataldi S, Albi E, Codini M, Moulas A, Marchyshyn S, Beccari T and Ceccarini MR: *Epilobium angustifolium* L.: A medicinal plant with therapeutic properties. The Euro Biotech Journal 2017; 1(2): 126-131.
3. Ciesla L: Biological fingerprinting of herbal samples using liquid chromatography. Hindawi Publishing Corporation Chromatography Research International 2012; 1-9.
4. Feshchenko H, Oleshchuk O, Lukanyuk M and Feshchenko BM: Investigation of phenolic compounds content in *Chamerion angustifolium* L. herb freeze-dried extract. The Pharma Innovation Journal 2017; 6(3): 40-43.
5. Songlin Li, Quanbin H, Chunfeng Q, Jingzheng S, Chuen LC and Hongxi X: Chemical markers for the quality control of herbal medicines: An Overview. Chin Med 2008; 3(7): 1-23.
6. Sayik A, Yusufoglu AS, Acik L, Turker G, Aydin B and Arslan L: DNA- binding, biological activities and chemical composition of wild growing *Epilobium angustifolium* L. extract from Canakkale, Turkey. Journal of the Turkish Chemical Society 2017; 4(3): 811-840.
7. Boligon AA and Athayde ML: Importance of HPLC in the analysis of plants extracts. Austin Chromato 2014; 1(3): 2.
8. Piwowarski JP, Bobrowska-Korczak B, Stanisławska I, Bielecki W, Wrzesien R, Granica S, Krupa K and Kiss AK: Evaluation of the effect of *Epilobium angustifolium* aqueous extract on LNCaP cell proliferation in *in-vitro* and *in-vivo* models. Planta Medica 2017; 83: 1159-1168.
9. Igor AS, Andrew GR, Liliya NK, Jovanka MV, Mark AJ and Mark TQ: Therapeutic potential of polyphenols from *Epilobium angustifolium* (Fireweed). Phytotherapy Research 2016; 30(8): 1287-97.

10. ICH Guidelines, http://www.ich.org/products/guidelines.html, on 26th June 2018.
11. Kaškoniene V, Stankevicius M, Drevinskas T, Akuneca I, Kaškonas P, Surviliene KB, Ragazinskiene AMO, Kornysova O, Briedis V and Ugenskiene R: Evaluation of phytochemical composition of the fresh and dried raw material of introduced *Chamerion angustifolium* L. using chromatographic, spectrophotometric and chemometric techniques. Phytochemistry 2015; 115: 184-193.

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