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Short communication

Validated RP-HPLC-UV method for the determination of betulin in *Asteracantha longifolia* (L.) Nees. extract.

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Abstract

Asteracantha longifolia (L.) Nees. (Acanthaceae) is a well known medicinal plant of Indian traditional medicines. The aim of this work was to develop a validated reversed phase-high performance liquid chromatography (RP-HPLC) method for the quantitative determination of betulin in *A. longifolia* extract. The analysis was performed by RP-HPLC on Luna C18 (2) 100 Å, 250 x 4.6 mm column under isocratic elution of acetonitrile and water (80:20, v/v) with a flow rate of 1.0 ml/min and the total run time was 20 min. The column temperature was adjusted at 25 C and the detection wavelength was set at 210 nm. The method was validated for suitability, specificity, accuracy, precision, limits of detection and quantification (LOD and LOQ), robustness and ruggedness. The betulin content in *A. longifolia* extract was found to be 15.96 ± 0.34% (w/w). The calibration curve was linear over a concentration range of 10-125 μg/ml (r^2 = 0.997) and the recovery range was 98.29-99.59%. The LOD and LOQ were 1.11 and 7.35 μg/ml, respectively. The intra- and inter-day assay precisions were satisfactory and the relative standard deviations were found to be always less than 2%. The developed method was found to be simple, sensitive, accurate, robust and rugged for the quantification of betulin. This validated method can be useful for the routine quality control analysis of betulin content in *A. longifolia* extract and its formulations.

Keywords: Asteracantha longifolia; betulin, RP-HPLC

Introduction

Asteracantha longifolia (L.) Nees. (family: Acanthaceae) is a well known medicinal herbs in Indian traditional medicines specifically in Ayurveda and Unani medicines [1]. Traditionally it is used in inflammation, anaemia, urinary discharges, hepatic obstruction, diarrhea, joint pains and abdominal disorders [2]. The quantification of known bioactive compounds is essential for the quality evaluation of herbal medicines [3]. In line of therapeutic uses, plant was reported to show antitumor, anti-inflammatory, analgesic, antipyretic, hepatoprotective, hematopoetic, diuretic, antidiabetic and antioxidant activities [1,2]. The plant was reported to contain betulin, β-sitosterol, stigmasterol, lupeol, 25-oxo-hentriacontanyl acetate and methyl8-n-hexyltetracosanoate [2]. The betulin is one of the most bioactive compound can be used for the standardization of this plant because of its wide range of bioactivities like anticancer [4], antiviral [5], analgesic and anti-inflammatory activities [6]. Some methods like HPTLC [7], HPLC [8], FT-IR and FT-Raman spectroscopy [9] have been suggested for the quantitative determination of betulin. To best of our knowledge, no reliable analytical method is available for the quantitative determination of betulin for the standardization of *A. longifolia*. Keeping in view of biological importance of betulin, the aim of the present work was to develop a validated RP-HPLC method for the quantitative determination of betulin in *A. longifolia* extract according to ICH recommended guidelines.

Material and methods

Chemicals and reagents

The standard betulin (purity 98.0%) was procured from Sigma-Aldrich, USA. HPLC grade acetonitrile and methanol were purchased from Merck Ltd, Mumbai, India.

Instrumentation and chromatographic conditions

The HPLC system (Waters Corporation, Milford, MA, USA) was consisted of a 600 controller pump, a multiple-wavelength ultraviolet-visible (UV-Vis) detector, a rheodyne 7725i injector with a 20 ml loop. The separation was performed using Luna C18 (2) 100 Å, 250 x 4.6 mm filled with 5 μm particles (Phenomenex, Torrance, CA, USA) column. The separation was carried out using the isocratic elution of acetonitrile and water (80:20, v/v) and the wavelength was set at 210 nm. The column temperature was maintained at 25 C and each injection volume was 20 μl. The run time was set at 20 min with a flow rate of 1 ml/min. The peak identification was performed by comparison of the retention time (RT) of the reference standard with the extract.

Plant material and extraction

A. longifolia whole plant was collected from Midnapore, West Bengal, India and the sample was authenticated from the

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Department of Botany and Forestry, Vidyasagar University, India. The voucher specimen (specimen no: VU/BOT/DB/19/11) has been deposited at the herbarium of the Department of Botany and Forestry, Vidyasagar University, India. The air dried (20-25 C) plant material (500 g) was powdered and extracted with 70% ethanol by cold maceration process for 15 days at 25ºC. The extract was concentrated and dried through rotary evaporation followed by lyophilization. The yield of extract was found to be 2.78% (w/w).

Sample and standard preparation

The extract (100 mg) was dissolved in 100 ml of mobile phase solution to prepare 1 mg/ml extract solution. The standard, betulin (5 mg) was dissolved in mobile phase solution (20 ml) to get standard stock solution of 250 µg/ml and it was diluted to prepare different concentrations (10-125 µg/ml) of standard solutions. All aliquots were filtered through Whatman's syringe filters (NYL 0.45 µm) prior to the analysis.

Calibration curve

The calibration plot was prepared by analyzing the five different concentrations of standard betulin solution ranging from 10-125 µg/ml. The calibration curve was constructed by linear regression analysis of the peak area against the respective concentration of betulin. The quantification of betulin in the extract was quantified in reference to this calibration curve.

Method validation

The HPLC method was validated as per the recommended guidelines of International Conference on Harmonization [10]. The method was validated for system suitability, specificity, limits of detection and quantification, precision, accuracy, robustness and ruggedness.

System suitability

The system suitability test was performed to establish the percentage relative standard deviations (% RSD) of RTs, peak area responses, tailing factors and theoretical plates. The test was performed by analysing six replicates (n = 6) of standard solution (75 µg/ml) and % RSD was calculated.

Specificity

The method specificity was confirmed by analyzing the chromatogram of standard betulin and extract for peak purity. The peak purity of betulin was determined using multivariate analysis by comparison of RT and peak area.

Limits of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were assessed by determining standard deviation ( ) of the response and the slope (S) of the linear equation using the formula: LOD = 3.3 /S and LOQ = 10 /S, where = standard deviation of the response from the number of blank run and S = slope of the calibration curve.

Accuracy

The accuracy of the method was determined from recovery study of betulin in the extract. The method was based on the addition of six different amounts (0-125 µg) of betulin with the pre-analyzed extract sample (n = 3) followed the by re-analysis of the contents. The mean amount of the standard achieved was taken as 'real value' to calculate the spike recovery.

Precision

Inter-day and intra-day precision were evaluated by analysing the six replicates (n = 6) of extract and different concentrations (10-125 µg/ml) of standard betulin on the same day and on different (n = 6) days. The mean and % RSD was calculated for intra-day and inter-day runs.

Robustness

The robustness of the method was based on the analysis (n = 3) of samples under slight variation of optimum conditions set for this method. The samples were analyzed with small changes in the mobile phase ratio, flow rate, detection wavelength, pH and column temperature to determine their effect on the RT, peak area response and recovery. The % RSD of RTs and peak area responses and percentage of mean recovery were calculated.

Ruggedness

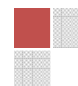
Ruggedness of the method was performed by the analysis (n = 3) of different concentrations of standard and extract using the developed method in another HPLC system. The HPLC system (Prominance LC-20AD, Shimadzu Corporation, Tokyo, Japan) was consisted of a binary reciprocating pump with a SPD-M20A Photo diode array detector (Shimadzu Corporation, Tokyo, Japan) and a rheodyne 7725i injector with a 20 ml. The separation was achieved using Xterra, RP 18, 5 µm, 4.6 x 250 mm column (Waters Corporation, Milford, MA, USA). The % RSD of RTs and peak area responses were calculated.

Statistical analysis

The results were statistically analyzed using GraphPad Prism version 5.0. The results are calculated as the mean ± SD/SEM.

Results and discussion

The present work was designed in order to develop and validate an RP-HPLC method for quantitative determination of betulin in *A. longifolia*. The present study is the first that report a validated RP-HPLC method for the quantification of betulin in *A. longifolia*. The chromatogram of the extract (Figure 1)



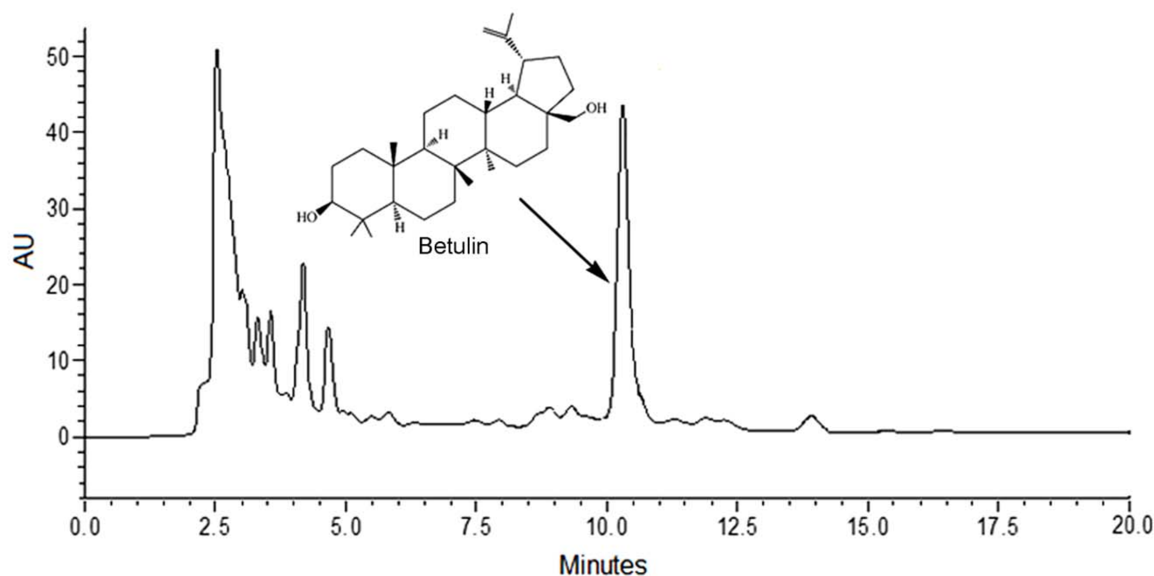


Figure 1: HPLC chromatogram of ethanolic extract of *A. longifolia*

showed sharp peak for betulin with RT at 10.29 ± 0.03 min which was comparable with the RT of standard betulin (RT, 10.30 ± 0.02 min). The calibration curve showed a linear relationship between peak area and concentration in the range of 10-125 μg/ml with a correlation coefficient (r^2) of 0.997. The quantity of betulin in extract was found to be 15.96 ± 0.34% (w/w). The chromatograms of the extract illustrated the clear separation of betulin with adequate peak resolution and there was no peaks at RT range of 10.29 min which indicates the method is selective for betulin. The % RSD for RTs, peak area responses, tailing factors and theoretical plates were found to be less than 2% indicating the system suitability of the method (Table 1).

Table 1: System suitability parameters (n =6)

Injection number	Retention time (RT)	Response	No. of theoretical plates	Tailing factor
1	10.28	1259882.22	3795.46	1.2
2	10.30	1261432.17	3699.56	1.19
3	10.19	1265611.73	3695.16	1.22
4	10.33	1262246.33	3680.67	1.19
5	10.23	1256877.28	3721.56	1.21
6	10.41	1265987.06	3791.64	1.19
Mean	10.29	1262006.13	3730.67	1.20
% RSD	0.77	0.27	1.35	1.05

The developed method was found to be suitable for detection and quantification of betulin. The LOD and LOQ for betulin quantification were found to be 1.11 and 7.35 μg/ml, respectively. The recovery data (Table 2) revealed, the mean recovery values of six different concentrations was ranged from 98.29 to 99.59% (average = 99.01%) which was very close to the actual theoretical values. The good recovery values suggest the accuracy of the proposed method was acceptable. The result of inter-day and intra-day precision is given in Table 3.

Table 2: Recovery experiment of betulin (n = 3)

Betulin added to extract (μg)	Total content of betulin (μg)	Actual content of betulin found (μg)	Recovery (%)	Mean recovery (%)
0.0	15.96	15.69	98.29	99.01
10.0	25.96	25.71	99.04	
18.80	34.76	34.35	98.82	
37.50	53.46	53.24	99.59	
75.0	90.96	90.36	99.34	
125.0	140.96	139.55	99.00	

Table 3: Intra-day and inter-day precision (n = 6)

Amount (µg/ml)	Intra-day precision				Inter-day precision			
	Retention time (RT)		Response (AU)		Retention time (RT)		Response (AU)	
	Mean	% RSD	Mean area	% RSD	Mean	% RSD	Mean area	% RSD
10.0	10.32	0.22	10382.80	0.97	10.31	0.28	10383.20	0.75
18.8	10.32	0.27	169241.00	0.89	10.31	0.15	169277.00	0.81
37.5	10.29	0.49	1135447.83	0.35	10.30	0.29	1141021.00	0.69
75.0	10.33	0.46	1263299.16	0.17	10.33	0.52	1265383.50	0.24
125.0	10.30	0.19	1470193.16	0.10	10.29	0.16	1470900.33	0.17
Extract	10.29	0.55	1613784.83	0.20	10.31	0.46	1614316.83	0.22

No significant differences were observed in the inter- and intra-day analysis. The % RSD of standard and extract were found to be lower than 0.97% with a high repeatability in the RTs indicates the proposed method is very suitable for analysis of betulin. No significant changes in the RT, peak area response and recovery of betulin were observed under the modification of critical parameters developed for this method and the % RSD was less than 2% which indicates the proposed method is robust (Table 4).

Table 4: Robustness of method (n= 3)

Parameter	Proposed	Variation	Retention time (RT)	% RSD	Response (AU)	% RSD	Recovery (%)
Mobile phase (v/v)	80:20	82:18	10.31	0.37	1270095.66	0.30	98.97
		78:22	10.24	0.93	1258045.16	0.70	99.19
Flow rate (ml/min)	1	1.2	10.27	0.36	1270243.16	0.18	98.93
		0.8	10.26	0.62	1259324.22	0.28	99.04
Wavelength (nm)	210	212	10.30	0.27	1266012.66	0.25	99.13
		208	10.27	0.42	1258147.16	0.58	99.37
Column temperature (C)	25	27	10.31	0.46	1271061.50	0.47	99.24
		23	10.29	0.32	1256600.16	0.84	99.18
pH	7.0	7.2	10.27	0.36	1267267.83	0.50	99.20
		6.8	10.30	0.40	1262615.16	0.53	99.26

Table 5: Ruggedness of the method (n= 3)

Amount (µg/ml)	Retention time (RT)	% RSD	Response (AU)	% RSD	Recovery (%)
10.0	10.31	0.18	10321.20	0.38	98.27
18.8	10.32	0.43	170085.33	0.44	98.82
37.5	10.29	0.20	1141238	0.39	99.32
75.0	10.30	0.44	1272614.16	0.19	99.07

Method ruggedness was determined by comparing the results of RTs, peak area responses and the assay of betulin obtain from another HPLC system. The % RSD was found to be always less than 2% (Table 5) which indicates the method is rugged.

Conclusions

The present work deals with a new RP-HPLC method for the quantitative determination of betulin in *A. longifolia* extract. The method shows a good linearity, specificity, accuracy and precision which might be useful for the routine quality control analysis of *A. longifolia* extract and its formulations.

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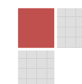

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