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Original Article

A VALIDATED RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF BETULIN, LUPEOL AND STIGMASTEROL IN *ASTERACANTHA LONGIFOLIA* NEES

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ABSTRACT

Objective: The aim of the present study was to develop a reversed phase high-performance liquid chromatography (RP-HPLC) method for simultaneous estimation of betulin, lupeol and stigmasterol in *Asteracantha longifolia* extract.

Methods: The chromatographic separation was achieved using Luna C18 column (5 µm, 250 x 4.6 mm) under isocratic elution of acetonitrile and 0.1% acetic acid in water (94: 6, v/v) with a flow rate of 1.0 mL min^{-1} and the detection wavelength was set at 215 nm. The column temperature was maintained at 25°C and the run time was set at 30 min. The method was validated for linearity, specificity, system suitability, accuracy, limit of detection, limit of quantification, precision and ruggedness.

Results: Calibration curves showed a good linearity relationship in the concentration range of 5 - 200 µg mL^{-1} for betulin (r^2 = 0.9994) and 10 - 800 µg mL^{-1} for lupeol (r^2 = 0.9995) and 10 - 800 µg mL^{-1} for stigmasterol (r^2 = 0.9986). Limits of detection for betulin, lupeol and stigmasterol were found to be 0.33, 1.0 and 3.0 µg mL^{-1} and limits of quantification were 2.0, 5.0 and 8.0 µg mL^{-1}, respectively. Mean recovery values were in the range of 98.92 - 100.11%. The %RSD values of intra- and inter-day precision analysis were lower than 2%. System suitability parameters were also found to be satisfactory.

Conclusion: The developed method allows rapid and reliable simultaneous determination of betulin, lupeol and stigmasterol in *A. longifolia* extract.

Keywords: A. Longifolia; RP-HPLC, Betulin, Lupeol, Stigmasterol

INTRODUCTION

Asteracantha longifolia Nees. Syn. *Hygrophila spinosa* T. Anders., is an ethnomedicinal herb (family: Acanthaceae) widely distributed throughout India, Sri Lanka, Myanmar, Malaysia, and Nepal[1]. Pharmacological studies of the plant were reported to possess antitumor, anti-inflammatory, analgesic, antioxidant, antidiabetic, haematopoietic, hepatoprotective and diuretic activities[1]. Betulin, lupeol and stigmasterol are the main bioactive compounds of *A. longifolia* associated with various pharmacological activities. These have been reported to possess anti-inflammatory, antioxidant, anticancer, hepatoprotective, and hypoglycaemic activities[2-4]. The quantities of active compounds in herbal medicines greatly depend on several factors such as intraspecies variability, seasonal and geographical variations, environmental conditions, harvest period, storage time, and extraction methods[5]. Therefore, the development of chemo-profiling and standardization of the medicinal plants are important to maintain quality control as well as to get the most favourable concentrations of known active constituents present therein[6].

Several analytical methods have been employed for analyzing and quantifying betulin, lupeol and stigmasterol separately or in combination with other constituents in herbal medicines. Zhao et al. used an isocratic elution for simultaneous determination of betulin and betulinic acid in white birch bark (Betula platyphylla suk)[7]. Simultaneous estimation of betulin, betulinic acid and lupeol from Alnus glutinosa bark extract has been reported[8]. The whole plant extract of *A. longifolia* was analyzed for simultaneous quantitation of lupeol and β-sitosterol using RP-HPLC method[9]. A HPLC-UV–MS method for separation and identification of lupeol, lupeol acetate, lupenon, ursolic acid, oleanolic acid, cycloartenol, stigmasterol, β-sitosterol and cycloartenol acetate have been reported[10]. Determination of lupeol, stigmasterol and β-sitosterol in various parts of Carissa carandas has been cited[11]. All the existing methods separately demonstrated either betulin and lupeol or stigmasterol and lupeol or any one of these compounds in plant extracts. None of these methods was found to be suitable for the simultaneous determination of betulin, lupeol and stigmasterol from their mixtures. A thorough literature survey revealed that there is no report available for simultaneous estimation of betulin, lupeol and stigmasterol from complex plant extract. Thus, it is necessary to develop an analytical method for simultaneous estimation of these marker compounds by convenient RP-HPLC method for the quality evaluation of herbal medicines. Therefore, the present work was aimed to develop a simple RP-HPLC method and validate it for the simultaneous determination of betulin, lupeol and stigmasterol in *A. longifolia* extract.

MATERIALS AND METHODS

Reference standards and reagents

The reference standards betulin (purity > 98%), lupeol (purity > 94%) and stigmasterol (purity > 95%) were purchased from Sigma-Aldrich, USA. Distilled water was prepared with a Milli-Q academic water purification system (Millipore, Bedford, MA, USA). Methanol, acetonitrile and acetic acid (HPLC grade) were purchased from Merck Ltd, Mumbai, India. Before use, all the solvents were filtered through membrane filters of 0.45 mm pore size (Millipore).

Plant materials collection and extraction

Aerial parts of *A. longifolia* were collected from the local fields of East Midnapur, West Bengal, India and the sample was authenticated (voucher specimen no: VU/BOT/DB/24/11) from the Department of Botany and Forestry, Vidyasagar University, India. Dried plant material (250 g) was powdered and extracted with 70% ethanol by cold maceration process (37 ± 2°C) for 15 days and the solvent was evaporated to dryness in a rotary evaporator, yielding 2.36% (w/w) crud extract.

Instrumentation and analytical conditions

The RP-HPLC system (Waters Corporation, Milford, MA, USA) consisted of a 600 controller pump, a multiple-wavelength ultraviolet-visible (UV-Vis) detector, an in-line AF 2489 series degasser, a rheodyne 7725i injector with a 20 µL loop with

integrated Empower2 integration software (Waters Corporation, Milford, MA, USA) was used for the analysis. The separation was carried out using Luna C18 (2) 100 Å, 250 x 4.6 mm filled with 5 µm particles (Phenomenex, Torrance, CA, USA) column. Assays were performed using the externally standardized isocratic conditions. Mobile phase composition (pH 6.8) was optimized to acetonitrile and 0.1% acetic acid in water (94:6, v/v) and aliquot was degassed and filtered using a membrane filter before run the column. Analysis was carried out at a flow rate of 1.0 mL min^{-1} with the detection wavelength set at 215 nm and the injection volume was set at 20 µL. Column temperature was maintained at 25°C and run time was set at 30 min.

Standard solution preparation

Stock solutions of reference standards, betulin (1 mg mL^{-1}), lupeol (2 mg mL^{-1}) and stigmasterol (2 mg mL^{-1}) were prepared in methanol. Appropriate amount of each standard stock was mixed separately to prepare working standard solutions containing six different concentrations of betulin (5, 10, 20, 50, 100 and 200 µg mL^{-1}), lupeol and stigmasterol (10, 50, 100, 200, 400 and 800 µg mL^{-1}) for establish the calibration curves. Standard solutions contain betulin (50, 100 and 200 µg mL^{-1}), lupeol and stigmasterol (100, 200 and 400 µg mL^{-1}) were subjected for method validation. All solutions were stored at 4°C prior to analysis.

Sample preparation

Accurately weighted 10 mg solvent free dried extract was dissolved in 5 mL methanol to prepare concentration of 2 mg mL^{-1}. The aliquot was then filtered through 0.45 µm membrane filter prior to injection.

Calibration curve

The calibration curves were established by analysing (n = 6) the six different concentrations of each reference standard at concentrations ranged from 5 - 200 µg mL^{-1} for betulin and from 10 - 800 µg mL^{-1} for lupeol and stigmasterol, respectively. Calibration curves were constructed by plotting the peak areas versus the concentrations of each standard by means of linear regression.

Method validation

The developed RP-HPLC method for simultaneous quantitative analysis of betulin, lupeol and stigmasterol was validated in term of linearity, specificity, system suitability, limits of detection (LOD) and quantification (LOQ), accuracy, precision, robustness and ruggedness. Validation of the method was performed as recommended by the International Conference on Harmonization (ICH) guidelines[12].

Statistical analysis

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

RESULTS

Optimization of chromatographic conditions

Chromatographic conditions such as column, mobile phase composition, flow rate and detection wavelength were optimized to provide sufficient selectivity and sensitivity. In order to optimize the mobile phase various mixtures of acetonitrile and water were tested as an ideal mobile phase to elute three investigated compounds. When the percentage of acetonitrile was increased from 80%, the peaks were sharp pointed and well separated. The addition of 0.1% acetic acid in water increased the resolution of the peaks, whereas the distortion of peaks occurred, when the percentage of acetic acid was increased. Considering the overall resolution and separation of the peaks and the retention time, the mobile phase compositions of acetonitrile and 0.1% acetic acid in water (94: 6, v/v) having pH adjusted to 6.8 with phosphoric acid was selected for the separation. Further, Waters XTerra RP18 (4.6 x 250 mm), Hypersil ODS C18 (4.6 x 100 mm), Phenomenex Luna C8 (150 x 4.6 mm), Phenomenex Luna C18 (250 x 4.6 mm) columns were used with optimized mobile phase. Good chromatography (resolution, peak symmetry and reproducibility) was obtained using Luna C18 column. Flow rate was increased from 0.5 - 1.50 mL min^{-1}, the retention times and peak tailing were decreased for lupeol and stigmasterol. However, a flow rate of 1.0 mL min^{-1} was ideal. UV absorbance was recorded within 200 - 800 nm range. It was observed that all analytes showed best peak resolution at 215 nm. The effect of column temperature was studied at the temperature range of 20 - 35°C and best peak resolutions were observed at 25°C. Using the optimized conditions, all marker compounds were successfully resolved and eluted within 30 min.

Method validation

Linearity and range

Linearity and range of the proposed method was carried out by analyzing twelve different intermediate concentrations (n = 6) of the mixed standard solutions containing 0.5 - 800 µg mL^{-1} of betulin and 0.5 - 1000 µg mL^{-1} of lupeol and stigmasterol using the optimized chromatographic conditions. A calibration curve for each standard was prepared separately by plotting peak area versus concentration. Calibration curve was found to be linear over the concentration range 5 - 200 µg mL^{-1} for betulin ($r^2 > 0.999$) and 10 - 800 µg mL^{-1} for lupeol ($r^2 > 0.999$) and stigmasterol ($r^2 > 0.998$). The data were analyzed by linear regression analysis and the results were summarized in Table 1.

Specificity

The specificity of the proposed method was determined by analyzing the standards and test sample. The method specificity was demonstrated by good separation i.e. purity of the peak with adequate resolution of the separation of betulin, lupeol and stigmasterol (Figure 1A and 1B). The purity of the peaks was evaluated using multivariate analysis by comparison of retention time (RT) and peak area of standard compounds and extract.

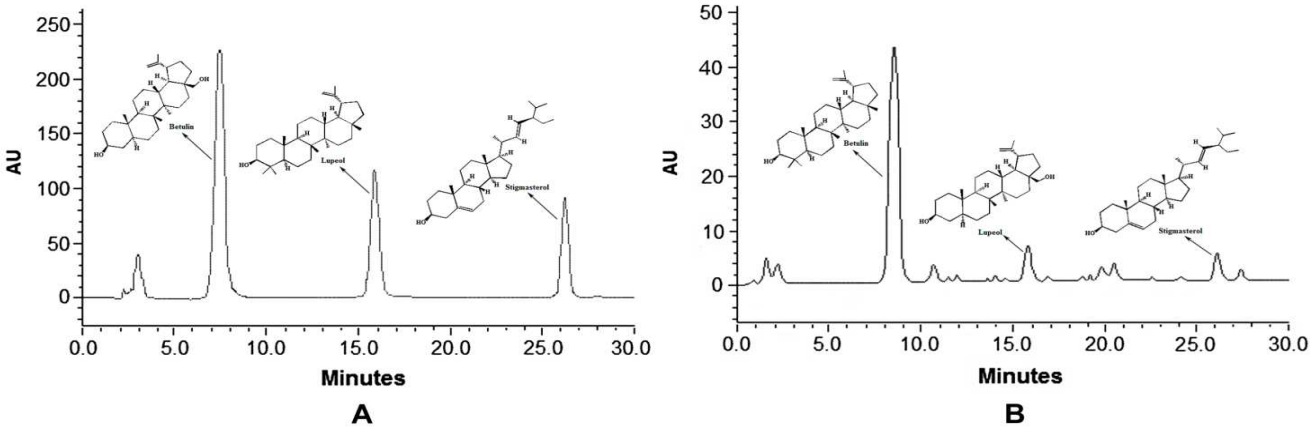

Fig. 1: (A) RP-HPLC chromatogram of the standard betulin, lupeol, stigmasterol and (B) chromatogram of *A. longifolia* extract

Table 1: Results of the linear regression analysis in the solvent and in the matrix extracts, differences between angular coefficients and curve intervals (n = 6)

Analyte	Solvent		Matrix extract		Difference in angular coefficients (%)	Concentration range (µg mL^{-1})
	Regression equation	r^2	Regression equation	r^2		
Betulin	y = 15384.39x + 11816.55	0.9994	y = 15141.82x - 10133.47	0.9987	1.58	5 - 200
Lupeol	y = 1736.71x + 11951.28	0.9995	y = 1662.17x + 13865.53	0.9986	4.29	10 - 800
Stigmasterol	y = 1369.71x + 15576.78	0.9986	y = 1278.89x + 14308.61	0.9998	6.63	10 - 800

Table 2: Intra-day and inter-day precision of the method (n = 6)

Reference standards	Concentration (µg mL^{-1})	Intra-day				Inter-day			
		RT		Response (AU)		RT		Response (AU)	
		Mean	%RSD	Mean area	%RSD	Mean	%RSD	Mean area	%RSD
Betulin	50	8.55	0.39	741981.54	0.55	8.55	0.25	738702.87	0.87
	100	8.58	0.37	1586269.58	0.60	8.57	0.41	1588647.34	0.60
	200	8.59	0.44	3081270.23	0.52	8.57	0.37	3086729.53	0.56
Lupeol	100	15.82	0.37	205836.35	0.92	15.83	0.44	201741.51	0.62
	400	15.82	0.48	698497.75	0.89	15.82	0.47	694695.66	0.84
	800	15.84	0.43	1409594.78	0.66	15.83	0.43	1400152.54	0.77
Stigmasterol	100	26.21	0.39	163871.06	0.77	26.24	0.41	158417.26	0.69
	400	26.22	0.28	583589.30	0.64	26.20	0.38	580754.61	0.74
	800	26.24	0.33	1104188.27	0.54	26.21	0.57	1107558.25	0.66

LOD and LOQ

The LOD and LOQ values were calculated based on the ICH guidelines[12], by determining the SD of the response and the slope of the linear equation. LOD and LOQ under proposed chromatographic conditions were calculated using the formula: LOD = 3.3σ/S and LOQ = 10σ/S. Where, σ is the standard deviation of the response from a number of blank run and S is the slope of the calibration plot. The LOD values of betulin, lupeol and stigmasterol were 0.33, 1.0 and 3.0 µg mL^{-1}, respectively and their respective LOQ values were found to be 2.0, 5.0 and 8.0 µg mL^{-1}.

Precision

The intra- and inter-day precisions of the method were evaluated by analysing the six replicates (n = 6) of standard solutions containing three different concentrations of each standard. The analysis was performed on the same day for intra-day precision and over a period of 6 consecutive days for inter-day precision. Respective intra- and inter-day precisions for area of responses were ranges from 0.52 - 0.60% and 0.56 - 0.87% for betulin, 0.66 - 0.92% and 0.62 - 0.84% for lupeol, and 0.54 - 0.77% and 0.66 - 0.74% for stigmasterol (Table 2). No significant differences were observed between %RSD values for inter- and intra-day analysis.

Accuracy

The accuracy of the method was evaluated by recovery study. The recovery study was performed by addition of known amounts of each standard to the pre-analysed sample (n = 3) followed by the re-analysis of the contents using the developed method. The recovery data revealed that the mean recovery values of three different concentrations of betulin, lupeol and stigmasterol were 99.52, 98.92 and 100.11%, respectively (Table 3).

Table 3: Recovery study of betulin, lupeol and stigmasterol from *A. longifolia* extract (n = 3)

Reference standards	Amount added (µg)	Total content (µg)	Amount found (µg)	Recovery (%)	Mean recovery (%)
Betulin	50	58.71	58.47	99.59	99.52
	100	108.71	107.83	99.19	
	200	208.71	208.25	99.78	
Lupeol	100	100.78	99.26	98.49	98.92
	400	400.78	399.40	99.66	
	800	800.78	789.58	98.60	
Stigmasterol	100	101.16	101.56	101.40	100.11
	400	400.16	400.23	100.02	
	800	800.16	791.41	98.91	

System suitability

System suitability was analyzed in terms of peak area, RT, tailing factor (must be < 2), theoretical plate count (should be > 20000) etc. For system suitability, six replicates (n = 6) of standard solution containing betulin (100 µg mL^{-1}), lupeol (400 µg mL^{-1}) and stigmasterol (400 µg mL^{-1}) were analysed to establish %RSD of RT, peak area, tailing factor and theoretical plate count.

The results of the system suitability parameters are given in Table 4.

Table 4: System suitability data of the proposed method (n = 6)

Reference standards	RT	%RSD	Response (AU)	%RSD	No. of theoretical plates	%RSD	Tailing factor	%RSD
Betulin	8.58	0.49	1587506.13	0.51	2309.08	1.07	0.90	1.38
Lupeol	15.83	0.31	700216.13	0.76	5521.24	0.59	1.17	1.13
Stigmasterol	26.25	0.28	582947.13	0.83	17670.60	0.81	0.96	1.08

Robustness

The robustness of the proposed method was determined by analyzing (n = 6) the standard solutions of betulin (100 µg mL^{-1}), lupeol (400 µg mL^{-1}), and stigmasterol (400 µg mL^{-1}) under small changes (± 2) in the optimum conditions set for this method such as flow rate, detection wavelength, pH and column temperature. Under the modification of such critical parameters, no significant changes were observed in the RT, peak area response and recovery of the standard compounds with %RSD values of less than 2%.

Ruggedness

Method ruggedness was evaluated by analysing (n = 6) the standard solutions containing three different concentrations of betulin (50, 100 and 200 µg mL^{-1}), lupeol (100, 400 and 800 µg mL^{-1}) and stigmasterol (100, 400 and 800 µg mL^{-1}) using the developed method in another RP-HPLC system (Prominance LC-20AD, Shimadzu Corporation, Tokyo, Japan). The RP-HPLC system consisted of a binary reciprocating pump with a SPD-M20A photo diode array detector and a rheodyne 7725i injector. The analysis was performed using Xterra RP 18 column (5 µm, 4.6 x 250 mm) (Waters Corporation, Milford, MA, USA). The %RSD obtained for RTs and peak areas of the standards were always found to be lower than 2% and the recoveries of the standards were found to be in the range of 99.03 - 100.34%.

Matrix effect

To evaluate the existence of matrix effects, post extraction standard addition technique was employed. Known amounts of each standard were mixed with the extract before injection. Percentage difference between the slopes of the standard curves prepared in solvent and plant matrix was calculated. A matrix effect greater than 10% can be considered as a significant effect on the results of quantitative analysis[13]. Calibration curves in solvent ware prepared using six different concentrations of betulin (5 - 200 µg mL^{-1}), lupeol (10 - 800 µg mL^{-1}) and stigmasterol (10 - 800 µg mL^{-1}). Results showed that the mean matrix effect values of betulin, lupeol and stigmasterol at the specified analytical ranges were 1.58, 4.29 and 6.63%, respectively (Table 1).

Determination of betulin, lupeol and stigmasterol in *A. longifolia* extract

Quantity of betulin, lupeol and stigmasterol in *A. longifolia* extract was simultaneously estimated using the developed method. Chromatographic peaks were identified by comprising the RT of individual standards with the extract. RP-HPLC chromatogram (Figure 1B) of the extract showed sharp peaks for betulin, lupeol and stigmasterol with RT of 8.56, 15.84 and 26.24 min, respectively. Quantity of each marker compound was determined from the corresponding calibration curve of the standard. The contents of betulin, lupeol and stigmasterol in the extract were found to be 8.71, 0.78 and 0.16%, respectively.

DISCUSSION

Development of new analytical methods for qualitative and quantitative estimation of active compounds in a crud plant extract is essential for pharmacokinetic, biological and toxicological studies and for standardizing them to maintain their quality and batch-to-batch reproducibility[14]. To develop a simple, rapid and sensitive RP-HPLC method for simultaneous determination of betulin, lupeol and stigmasterol in *A. longifolia* extract, the analytical column, column temperature, mobile phase composition, run time, pH, flow rate, and detection wavelength were optimized. Optimization of these chromatographic conditions enabled an efficient simultaneous separation of betulin, lupeol and stigmasterol within 30 min from their mixture as well as from the extract of *A. longifolia* (Fig 1A and 1B). Calibration curves were found to be linear over the concentration range 5 - 200 µg mL^{-1} for betulin and 10 - 800 µg mL^{-1} for lupeol and stigmasterol (Table 1). All marker compounds had a correlation coefficient (r^2) value over 0.998, indicating that there was excellent correlation between peak areas and concentrations of the marker compounds. Specificity of the method was evidenced in the identical RTs of betulin, lupeol and stigmasterol in standard solution and extract. All the standard compounds were clearly resolved with sufficient resolution and no interfering peaks of endogenous compounds were observed. Results indicated that the developed method was selective and specific to distinguish and quantify betulin, lupeol and stigmasterol from a complex plant extract. The low LOD and LOQ values of standards indicated that the developed method was very sensitive and can be used for the detection and quantification of betulin, lupeol and stigmasterol over a wide range of concentrations. Considering the LOD and LOQ values for lupeol and stigmasterol, the present method was comparable with the previously reported methods[11]. Zhao et al. reported only LOD value for betulin[7]. In the present study, LOD and LOQ values of betulin were found to be 0.33 and 2.0 µg mL^{-1}, respectively. Average recovery values (≥ 98.92%) of the standards were very close to the actual theoretically calculated values (Table 3). Mean percent recovery of three standard compounds were higher than previously available reports[1,11]. Good recovery values were obtained for each standard suggested that the accuracy of the proposed method was acceptable. Matrix effects can be influenced by the co-elution of any other endogenous compounds with the analytes which may causes significant errors in the accuracy and precision of analytical methods[15]. In the present study, matrix effects were found to be insignificant as the matrix effects of the three analytes were less than 10% with correlation coefficients (r^2) over 0.998 (Table 1). None of the methods studied earlier reported the matrix effects on the qualification of betulin, lupeol and stigmasterol simultaneously in herbal matrix. Results of inter- and intra-day precession showed that %RSD values of peak area and RT were lower than 2% with high repeatability (Table 2). There was not much variation in the inter- and intra-day precision, thus the method is extremely adaptable because of the good precision and excellent repeatability of the method. The results of inter- and intra-day precisions of the present study were highly precise in comparison with the previously published methods[7,11]. System suitability test was performed to verify that the proposed method has acceptable peak resolution with high reproducibility. Results of system suitability parameters reflected that the %RSD values for RT, area of response, tailing factor and theoretical plate count of all standards were less than 2%, indicating the suitability of the proposed method (Table 4). It was observed that the RT, peak area and recovery of the standard compounds were not significantly affected by small changes in the parameters as evident from the low values of %RSD (< 2%) indicating robustness of the method.

This rapid and simple RP-HPLC method was developed to determine the contents of betulin, lupeol and stigmasterol in ethanol extract of A. Longifolia. The chromatogram of the extract (Figure 1B) showed clear separation of three standards with high repeatability in their RT with that of internal standards. Therefore, the proposed present method can be useful for routine analysis of betulin, lupeol and stigmasterol in *A. longifolia* extract reliably and accurately.

CONCLUSION

In this study, a RP-HPLC method was developed for simultaneous separation and quantification of three active compounds viz. betulin, lupeol and stigmasterol in ethanol extract of A. longifolia. The proposed method was found to be very simple with excellent sensitivity, and accuracy with relatively short analysis time. This method could be useful as a reliable analytical tool for quality evaluation of *A. longifolia* and formulations containing betulin, lupeol and stigmasterol as chemical markers.

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