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A VALIDATED HPLC METHOD FOR THE DETERMINATION OF BETULIN IN THE STEM BARK OF *TECTONA GRANDIS* LINN.

Preet Amol Singh [*1], N. B. Brindavanam [2], G. P. Kimothi [2], Rajesh Verma [2] and Vidhu Aeri [1]

Department of Pharmacognosy and Phytochemistry [1], Jamia Hamdard University, New-Delhi, India.
Bio-resource Development Department [2], DRDC, Ghaziabad, India.

Keywords:

Tectona grandis Linn. ; betulin; HPLC; validation; ICH guidelines.

Correspondence to Author:
Dr. Vidhu Aeri

Ph. D,
Associate Professor,
Department of Pharmacognosy and Phytochemistry, Jamia Hamdard University, Hamdard Nagar, New Delhi, 110062, India.

Email: vidhuaeri@yahoo.com

ABSTRACT: Betulin, a known pentacyclic tri-terpenoid, possessing diverse pharmacological properties was determined using HPLC in stem bark methanol extract of *Tectona grandis* Linn. distributed in greater part of India. Furthermore, *Tectona grandis* Linn. is used traditionally in treatment of bronchitis, dysentery, headache, leukoderma, leprosy and constipation. The present study was aimed to validate HPLC method for the determination of betulin in *Tectona grandis* Linn. (bark). The chromatographic separation was achieved using Waters HPLC instrument, reverse phase Hypersil C18 column (250 mm) under isocratic elution of acetonitrile-water 85:15 (v/v) with a flow rate of 1.0mL min $^{-1}$ and the run time was set at 15 min. The detection was done at 210 nm and the column temperature was maintained at 25^0 C. The method was linear for betulin over the concentration range of 28.42- 53.99 ppm (R^2 = 0.9974) in *Tectona grandis* Linn. (bark). The quantification of betulin in methanol extract of *Tectona grandis* Linn. was validated in terms of system precision, method precision, linearity, recovery, robustness, ruggedness, LOD and LOQ according to ICH guidelines. The % RSD values were found to be in an acceptable range as per ICH guidelines.

INTRODUCTION: *Tectona grandis* Linn. (Verbenaceae), is known as teak, distributed in south and southeast Asia and is found in greater part of India. It is popularly cultivated for its commercial wood due to its beautiful surface and its resistance to termite and fungal damage [1]. It is a tall, branched, deciduous tree with large leaves, possessing good pharmacological properties. Traditionally, the bark of this plant is used to treat bronchitis, dysentery, headache, leukoderma, leprosy and constipation [2].

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Betulin, lup-20(29)-ene-3β,28-diol, is a pentacyclic triterpene alcohol with a lupane skeleton, is abundantly found in its free form in the outer bark of white birch [3]. The preparations based on lupane series are used in the treatment of many diseases and this is linked to betulin which is used as such or it is transformed to betulinic acid, its biologically more active derivative. Betulin and betulinic acid was also simultaneously determined in white birch bark using RP-HPLC [4].

Betulin which is the main component of the extract of birch bark exhibit choleretic, liver-protecting, wound healing, cholesterol lowering action and anti-inflammatory activity. Plants extract containing lupeol, betulin and betulinic acid is known to possess anti-tumour property [5]. Several analytical methods have been developed for the

determination of betulin in various plants. Banerjee et al., simultaneously determined betulin, lupeol, stigmasterol in *Asteracanthalongifolianees*. According to the literature surveyed, it is revealed that there is no systematic documentation available for the validation of betulin in the bark of *Tectona grandis* Linn. Therefore, the present study was aimed to develop a novel method for the determination of betulin using validated HPLC technique in *Tectona grandis* Linn. (bark).

MATERIALS AND METHODS:

Apparatus and chromatographic conditions:

The HPLC (Waters, Alliance 2695) consisting of a 100 µl injector (Alliance), waters reciprocating pump, 4 line in-line alliance degasser and 2998 PDA detector with Empower2 integration software (Waters Corporation) was used for the analysis. The chromatographic separation was achieved using reverse phase Hypersil C18 column (250 mm) under isocratic elution of acetonitrile-water 85:15 (v/v) with a flow rate of 1.0mL min $^{-1}$ and the run time was set at 30 min. The detection wavelength was set at 210 nm and the injection volume was set at 20µl. All chromatographic operations were carried out at ambient temperature. Column temperature was maintained at 25^{0}C. The chromatographic peaks of betulin in solvent extracts were confirmed by comparing their retention time and UV spectra with betulin standard. The experiment was performed in the month of September 2015 in the analytical lab of Dabur, DRDC, Ghaziabad (U.P), India.

Standard solutions and reagents:

The reference standard betulin was obtained from Sigma-Aldrich Chemical Corporation (USA). Distilled water was prepared with a Milli-Q academic water purification system (Millipore, Bedford, MA, USA). HPLC grade acetonitrile (Merk Ltd, Mumbai, India) were used for the HPLC analysis. All the solvents were membrane filtered through 0.45 mm pore size (Millipore).

Plant material:

Tectona grandis Linn. (bark) was collected from the environmental park of Patiala, Punjab, India in the month of September 2015, and the sample was authenticated (voucher specimen no: PUN-59201) from the Department of Botany, Punjabi University, Patiala (Punjab), India.

Preparation of standard solution:

Stock solution of reference standard betulin was prepared by dissolving appropriate weights of standard betulin in methanol at concentration of 41.216 ppm and stored in refrigerator. Working solutions were freshly prepared every day at same concentration of betulin in methanol.

Preparation of sample solution:

Accurately weighed equivalent to 4 g of solvent free coarse powder of Plant material, refluxed with 30 ml of methanol for 1 hour and repeated for twice. The filtrates were combined and dried under reduced pressure. The dried extract was dissolved in methanol and volume was made up to 25 ml using volumetric flask. The concentration of betulin in *Tectona grandis* (bark) extract was 40.276 ppm. The volumetric flasks were tightly capped and stored at ambient temperature.

Method Validation:

The HPLC method developed for the quantitative analysis of betulin in *Tectona grandis* Linn. (bark) was validated in terms of system precision, method precision, linearity, recovery, robustness, ruggedness, limits of detection (LOD) and limits of quantification (LOQ) according to International Conference on Harmonization (ICH) guidelines. Quantification of betulin was computed using external standard method.

RESULTS AND DISCUSSION:

Optimization of chromatographic conditions:

Zhao et al., determined betulin and betulinic acid simultaneously in white birch bark using RP-HPLC using reverse phase C18 column with acetonitrile-water 86:14 (v/v) at 210 nm. According to Zhao et al., acetonitrile was selected as a solvent system in order to minimize background noise and to improve detection limit. In our study, different ratios of acetonitrile-water were tried for the separation of betulin but acetonitrile-water 85:15 (v/v) was found to be the best for the separation of betulin in *Tectona grandis* Linn. (bark) using reverse phase Hypersil C18 (250mm) column at a flow rate of 1.0 ml/min. It was observed that betulin showed best peak resolutions at 210 nm

using PDA detector. It can be seen from **Fig.1(a)** and **(b)** that good separation can be achieved within 15 min using the described conditions as retention time of betulin in standard and sample solution was 10.780 and 10.768 mins respectively.

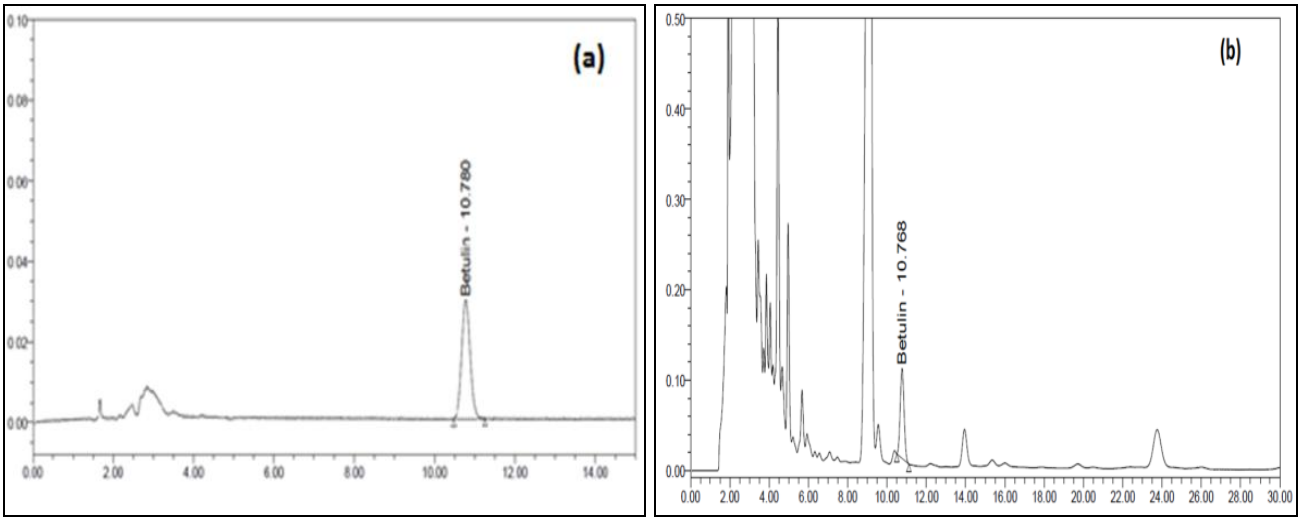

FIG.1: REPRESENTATIVE CHROMATOGRAMS OF BETULIN DETECTED AT 210 nm: (A) BETULIN IN STANDARD SOLUTION AND (B) BETULIN IN METHANOL EXTRACT OF *TECTONA GRANDIS* LINN. (BARK)

Method validation:

System precision:

Repeatability of sample application and measurement of peak area were analyzed using six replicates of standard and expressed in terms of % Relative Standard Deviation (%RSD). The %RSD in system precision was 0.88 which indicated an acceptable level of precision for analytical system.

Method precision:

Six replicates of a single batch of raw material were prepared and analyzed using proposed method. Average area of the peak of betulin in each replicate was noted, content of the betulin (%w/w) in each sample was noted when compared to betulin standard. The average area of standard betulin was 386591.7. The %RSD of betulin in *Tectona grandis* samples was 1.77% indicating that the method has an acceptable level of precision.

Linearity: Linearity response of betulin indicated that the response was linear over the specified range. The stock solution was prepared by dissolving 17.76 mg of betulin in methanol and making the volume up to 25 ml. Dilutions were prepared by adding 1ml to 1.9 ml of stock solution to volumetric flask and making volume up to 25 ml using methanol as shown in **Table 1**. The y-value (8747.8x+578.89) and the values of the regression coefficients (R^2=0.9974) confirmed the linearity of the method as shown in **Fig. 2**. The quantification of betulin undertaken was proved to be simple, sensitive and reproducible.

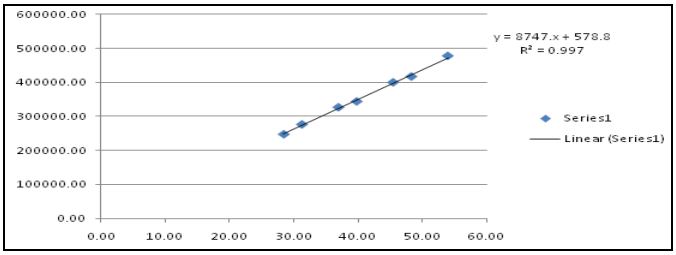

FIG.2: REPRESENTS THE LINEARITY PLOT OF BETULIN

TABLE 1: DILUTIONS FOR THE LINEARITY PLOT IN *TECTONA GRANDIS* (BARK)

Dilutions	Conc. (ppm)	Area 1	Area 2	Av. Area
1 ml /25ml	28.42	246062.00	249707.00	247884.50
1.1 ml /25ml	31.26	275061.00	278589.00	276825.00
1.3 ml /25ml	36.94	329637.00	324369.00	327003.00
1.4 ml /25ml	39.78	345774.00	342408.00	344091.00
1.6 ml /25ml	45.47	396999.00	402567.00	399783.00
1.7 ml /25ml	48.31	414742.00	418944.00	416843.00
1.9 ml /25ml	53.99	478298.00	476499.00	477398.50

Recovery of betulin in *Tectona grandis* (bark):
The result displayed in Table 2 indicate that the used method allowed a recovery of betulin between 94.23-96.31%, confirming that both accuracy and recovery were satisfactory.

TABLE 2: RECOVERY STUDIES OF BETULIN IN *TECTONA GRANDIS* (BARK):

S.No	Content of betulin (%w/w)	Stocked soln. spiked (17.76 mg/25 ml)	Betulin recovered (ppm)	Theoretical (ppm)	Recovery (%)	Average recovery (%)
REC. 80% A	0.041	1.1 ml /25ml	67.08	71.53	93.78	94.23
REC. 80% B	0.041	1.1 ml /25ml	67.01	71.53	93.67	
REC. 80% C	0.042	1.1 ml /25ml	68.12	71.53	95.22	
REC. 100% A	0.047	1.4 ml /25ml	76.59	80.06	95.67	95.19
REC. 100% B	0.047	1.4 ml /25ml	75.41	80.06	94.19	
REC. 100% C	0.048	1.4 ml /25ml	76.62	80.06	95.70	
REC. 120% A	0.052	1.7 ml /25ml	84.95	88.58	95.90	96.31
REC. 120% B	0.053	1.7 ml /25ml	84.90	88.58	95.84	
REC. 120% C	0.054	1.7 ml /25ml	86.09	88.58	97.18	

Robustness:
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters, proving its reliability during normal usage. For determination of method robustness, a number of chromatographic parameters, for e.g., flow rate, column temperature, detection wavelength and mobile phase composition were varied within a realistic range and quantitative influence of the variables were determined in % RSD as shown in **Table 3**. %RSD of parameters after variations were found to be acceptable range.

TABLE 3: The %RSD OF RESPECTIVE PARAMETERS IN ROBUSTNESS STUDIES

Chromatographic parameters	Variations done	%RSD
Flow rate	1.1 ml/min	2.90
	0.9 ml/min	5.02
Column temperature	30^0 C	0.62
	20^0 C	1.16
Wavelength	215 nm	3.02
	205 nm	4.35
Mobile phase	Acetonitrile +2%	9.68
	Acetonitrile -2%	1.50

Ruggedness:
To have an idea about the ruggedness, it is recommended to introduce deliberate variations to the method, such as different days, analysts, instruments, reagents, variations in sample preparation or sample material used. Changes were made separately and the effect of each set of experimental conditions were evaluated with respect to precision and trueness. The samples were subjected to different instrument, analyst and column of different company was used. The %RSD of betulin in for ruggedness was 5.12.

LOD and LOQ:
The limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected but not necessarily quantified. The samples were diluted accordingly to reach the accurate limit of detection. In order to determine the detection and quantification limit, the betulin concentration in the lower part of the linear range of the calibration curve was used. The limit of quantification (LOQ) is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated and are represented in **Table 4**. The limits of quantification and detection were determined based on the technique of signal-to-noise ratio using the equations (1) and (2) below:

$$LOQ ¼ 10σ=S \quad (1)$$

$$LOD ¼ 3:3σ=S \quad (2)$$

where σ is the standard deviation of the intercept of the calibration plot and S is the slope of the calibration curve.

TABLE 4: THE LOD AND LOQ STUDIES OF BETULIN IN *TECTONA GRANDIS*LINN. (BARK):

Betulin	LOD (ppm)	LOQ (ppm)
	8.24	24.73

CONCLUSION: In this study, the HPLC method was validated for the determination of betulin in *Tectona grandis* Linn. (bark). To the best of our Knowledge and literature surveyed, it was revealed that no systematic work has been carried out for the determination of betulin in *Tectona grandis* Linn. (bark) using validated HPLC technique. The HPLC method described in this paper is simple, accurate, sensitive, reproducible and falls best for the determination of betulin with good peak resolution and short analysis time (15 min). Easy availability of *Tectona grandis* due to its acceptable geographical conditions in India, it can be used as a natural source of betulin and can find its way in pharmaceutical industries.

DECLARATION OF INTEREST: The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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