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Rapid validated HPTLC method for estimation of betulinic acid in *Madhucalongifolia bark* extract

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ABSTRACT

Betulinic acid (pentacyclictriterpenoid) is an important marker component present in *Madhucalongifolia* bark. *M. longifolia* bark has several medicinal uses including as epilepsy, inflammation, diabetes mellitus, analgesic, anthelmintic, pneumonia, piles and skin diseases. To establish a simple, sensitive, reliable, rapid and validated high-performance thin-layer chromatography method for estimation of betulinic acid in hydro-alcoholic extract of *M. longifolia* bark. The separation was carried out on a thin-layer chromatography aluminium plate pre-coated with silica gel $60F_{254}$, eluted with toluene, methanol and formic acid (8:1:1 v/v). Post chromatographic derivatisation was done with anisaldehyde–sulphuric acid reagent and densitometric scanning was performed using a Camag TLC scanner III, at 640 nm. The system was found to produce a spot for betulinic acid (R_f = 0.49). A good linear relationship between the concentrations (0.2–1.2 µg) and peak areas were obtained with the correlation coefficient (r) of 0.931. The limit of detection and limit of quantification of betulinic acid were detected to be 0.028 and 0.062 µg per spot. The percentage of recovery was found to be 97.42%. The percentage relative standard deviations of intra-day and inter-day precisions were 0.37-1.51 and 0.31-1.27, respectively. This validated HPTLC method provides a new and powerful approach to estimate betulinic acid as phytomarker in the extract.

Keywords: *Madhucalongifolia*; Bark; betulinic acid; HPTLC; quantitative analysis; method validation

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INTRODUCTION

*Madhucalongifolia*J.F. Macbr. (Sapotaceae; mahwa or mahua), is an Indian tropical tree found largely in the central and north Indian plains and forests. The different parts of the plant are used for wide variety of ailments, such as epilepsy [1], inflammation, diabetes mellitus, analgesic, anthelmintic, pneumonia, piles and skin diseases. [2] Anti-inflammatory activity, [3] antiulcer activity, [4] and analgesic activity [5] of *Madhucalongifolia*have also been reported. The fruit consists a number of triterpenoids (including α- and β-amyrin acetate); *n*-hexacosanol, β-D-glucoside of β-sitosterol and free sitosterol. The carollas consist rich source of sugars, vitamins, minerals. The seeds yielded saponins 2,3-*di*-*O*-glucopyranoside of bassic acid (saponin A and saponin B), β-sitosterolglucoside, quercetin and dihydroquercetin. Trunk bark comprise lupeol acetate, α-amyrin acetate, α-spinasterol, erythrodiolmonocaprylate, betulinic acid and betulinic acid caprylates. [2]

Lack of an appropriate simple TLC method for the quantification of betulinic acid in *M.longifolia* bark extract, a densitometric HPTLC method is proposed to develop in the present work for quantification of betulinic acid from the methanolic extract of bark of *M.longifolica* that may be used in pharmaceutical industry for the standardization and quantification of betulinic acid in herbal dosage from. The proposed method was validated by evaluating different parameters as per ICH guidelines [6, 7].

MATERIALS AND METHODS

Plant material

Bark of *Madhucalongifolia*J.F. Macbr. (Sapotaceae), was collected (October, 2015) from the Anand (Gujarat, India) and identified by a taxonomist, Department of Bio-science, Sardar Patel University. Voucher specimens of plant have been preserved in Department of Pharmacognosy, Anand Pharmacy College, Anand, Gujarat (India).

Chemicals and reagents

Betulinic acid reference standard (98%) was obtained from Sigma Aldrich, India. Analytical-grade solvents were procured from E-Merck, Mumbai, India. Pre-coated silica gel $60F_{254}$ TLC plates (Merck, Darmstadt, Germany) were used for the analysis.

M. longifolia bark extraction procedure:

Freshly collected bark of *M. longifolia* was dried under shade and coarsely powdered. The 10 g of powder materials were extracted with methanol (70:30, 50 ml) and after standing for 48 h at room temperature, the hydro-lcoholic extract was drained off. This process of extraction was repeated till exhaustive extraction was done. The hydro-alcoholic extract was combined, filtered and concentrated under reduced pressure in a rotary evaporator at 45 °C and proceed for drying under high vacuum to produce the final extract.

Preparation of sample solutions:

1 mg of *M.longifolia* bark extract was placed in 10 ml volumetric flask. It was dissolved in adequate quantity of methanol and volume was adjusted to 10 ml with methanol to get (100 µg/ml) concentration which was further diluted with methanol to get 10 µg/µl.

Standard solutions:

Standard solutions of betulinic acid were prepared by dissolving 1.0 mg of betulinic acid compound in 10 mL of methanol (final concentration: 100 µg mL^{-1}).

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HPTLC condition:

A Camag TLC system is used for the analysis having CamagLinomat V an automatic TLC sample spotter, Camag glass twin trough chamber (20 cm × 10 cm), Camag scanner 3 along-with integrated win CATS 4 Software. Preliminary TLC was performed on a pre-coated TLC plate as 8-mm wide bands with an automatic TLC sampler (Linomat V) under a flow of N_2 gas, 10 mm from the bottom and 10 mm from the side, and the space between two spots was 15 mm of the plate. The linear ascending development was carried out in a Camag twin trough chamber (20 cm × 10 cm) which was pre-saturated with 20 ml mobile phase Toluene: Ethyl acetate: Formic acid (10:9:1) for 20 min at room temperature (25 ± 2°C and 40% relative humidity). The length of the chromatogram run was 8 cm. Subsequent to the development, TLC plates were dried under a stream of hot air and then subjected to treatment with freshly prepared anisaldehyde- sulphuric acid solution for the derivatization and performed densitometric scanning using a Camag TLC scanner III (Camag, Switzerland) with win CATS software in the absorbance-reflectance scan mode. Quantitative analysis of the plate was made in the absorption-reflection mode at 640 nm. Quantification of betulinic acid in the methanolic extract of bark of *M.longifolia* was performed.

Sample application:

Different concentrations of standard betulinic acid and extract were applied in different tracks by Linomat IV applicator. Standard solutions of betulinic acid of six different concentrations 2, 4, 6, 8, 10 and 12 µL (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µg $spot^{-1}$) were applied from stock solution of the drug. The plate was developed and derivatized which was further processed for the detector response. The plate was kept in the above mentioned solvent system and allowed to run up to a distance of 8 cm. After drying, it was scanned densitometrically at 640 nm.

Method validation

The method was validated by determining linearity, peak purity, limit of detection, repeatability (Table 1), percentage recovery (Table 2), intra-day and inter-day precision (Table 3) of betulinic acid from bark *Madhucalongifolia*. Each of the standard solutions of betulinicacid (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µg per band) was applied in triplicate. The calibration plot was prepared by plotting peak area against the amount of betulinic acid and linearity range was determined. Instrument precision was checked by scanning the same betulinic acid band (600 µg) six times. The mean, standard deviation and coefficient of variation [%] were calculated for peak area and R_F. Repeatability was tested by analyzing the betulinic acid band after application of standard solution to the plate (n=6) and calculating %CV. The accuracy of the method was tested by determination of recovery at three levels, after addition of 50, 100 and 150% betulinic acid to the sample. Recovery was calculated for each of the three levels (Table 2). Precision was studied by analyzing three bands of sample solution per plate on three plates (intra-day precision) and by analyzing three bands of sample solution per plate on second day (intermediate precision) and calculating % CV (Table 3). The specificity of the method was determined by absorbance spectrum of betulinic acid standard and the corresponding peak in the test samples in the range 200-800 nm. Different dilutions of the standard solutions were applied with methanol as blank and the Limits of Detection (LOD) and Quantification (LOQ) were determined.

Table 1: Method performance parameters for quantification of betulinic acid by proposed TLC densitometric method

Parameters	Method (Betulinic acid)	Acceptance Criteria
Selectivity	Selective	
Specificity	Specific	No interference observed
Linear Range (µg/spot)	0.2-1.2	Linearity, accuracy and precision over the range
Correlation Coefficient	0.931 ± 0.716	Within 0.9-1.1
Linear regression equation	y = 14.836x + 3703.4	
LOD (ng/spot)	20.56	
LOQ (ng/spot)	62.34	
Recovery (%)	97.42	Within 90-110%

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Repeatability (n=6)	0.37	% RSD ≤2
Intraday (n=3)	0.37-1.51	
Interday (n=3)	0.31-1.27	

Table 2: Recovery studies of betulinic acid at 50%, 100%, and 150% addition by the proposed TLC densitometric method

Concentration of standards (µg/spot)		Area Spotted		Total area (Sample + Standard)	Total area obtained	% Recovery
Sample amount	Spiked amount	Sample area	Spiked Area			
0.4	0.2	8811.2	5171.01	13982.21	14191.1	98.52802
0.4	0.4	8811.2	8312.27	17123.47	17521.1	97.73056
0.4	0.6	8811.2	9151.15	17962.35	18708.4	96.01222

Table 3: Intra and inter day precision of HPTLC method (n=3).

Concentration of standards (µg/spot)	Inter-day Precision		Intra-day Precision	
	Peak area (mean ± SD)	% RSD	Peak area (mean ± SD)	% RSD
200	5472.18 ± 82.77	1.51	5495.98 ± 42.22	0.768258
400	14171.01 ± 89.12	0.62	14151.83 ± 180.15	1.273074
600	18693.35 ± 69.75	0.37	18684.86 ± 59.51	0.318499

RESULTS AND DISCUSSION

Validation

The proposed HPTLC method was validated by evaluating different parameters such as precision, accuracy and repeatability (Table 1). The method is specific for betulinic acid as it resolved the compound (R_f = 0.47) well in the presence of other components of *Madhucalongifolia* (Fig. 1). A linear relationship was obtained in the range 0.2-1.2 µg per band and the correlation coefficient (R^2) was 0.93 (Fig.2) (Table 2). The optimized solvent system was found suitable for the estimation of the betulinic acid in *M.longifolia* extract. There was no interfering from other components present in extract. The resolution was good and components were observed at different Rf value. The total betulinic acid present in extract was found to be 0.02 % w/w.

Fig 1: HPTLC chromatoplate of betulinic acid and hydroalcoholic extract of powdered bark of *Madhucalongifolia*) after derivatization under 560 nm at different concentration 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µg/spot standard Betulinic acid and Hydroalcoholic extract of bark of *M.longifolia.*

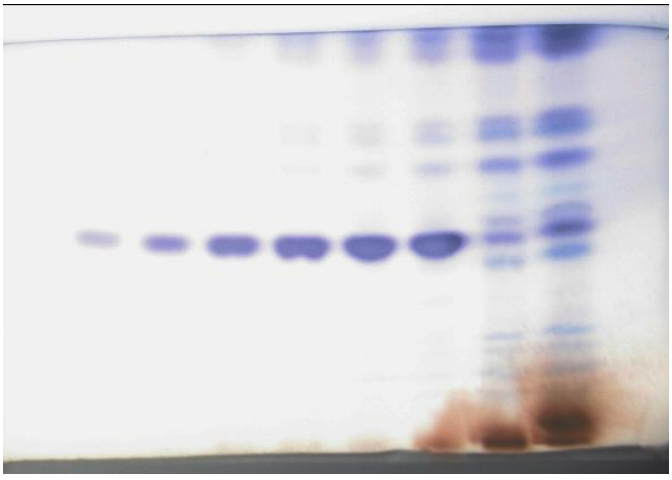

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Fig 2: Calibration curve for standard Betulinic acid (n= 6).

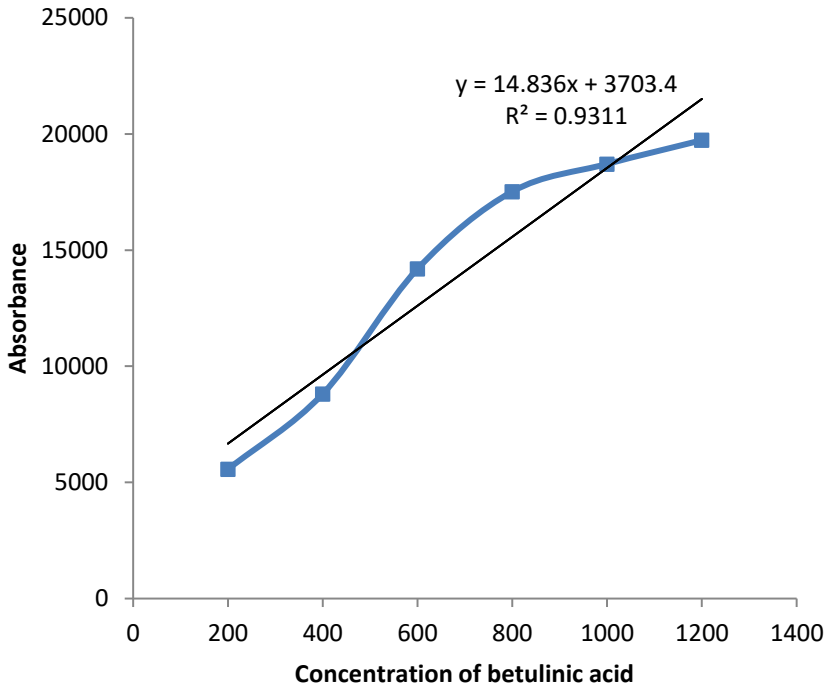

Fig 3: Densitometric chromatogram of betulinic acid and hydroalcoholic extract of *M.longifolia* at 640 nm (3D View).

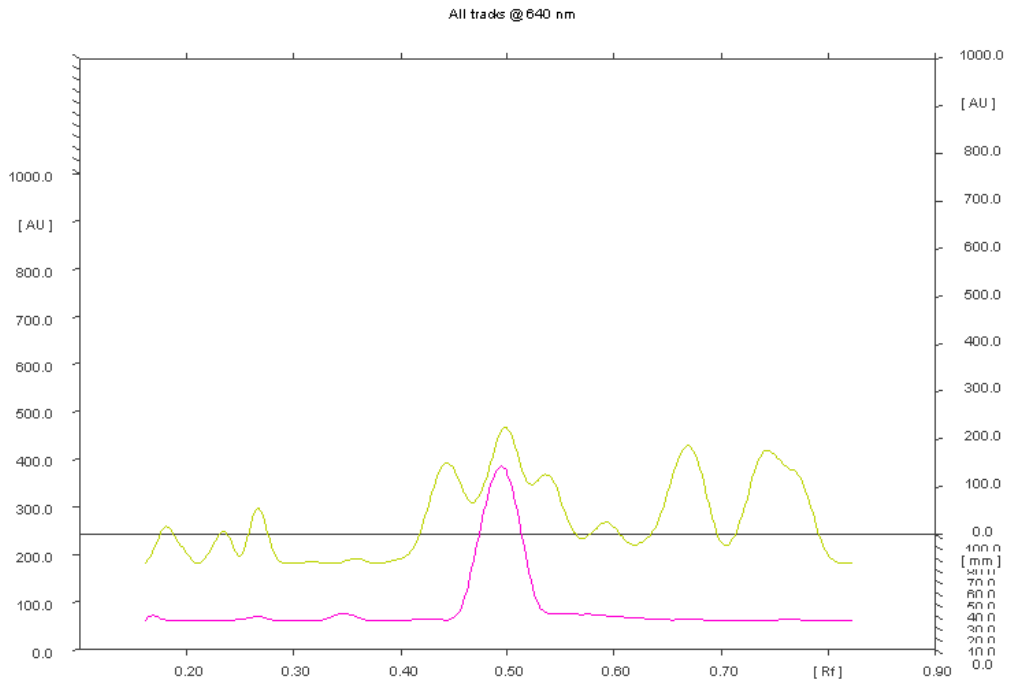

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Fig 4: Densitometric chromatogram of hydroalcoholic extract of powdered bark of *Madhucalongifolia* after derivatization at 640 nm.

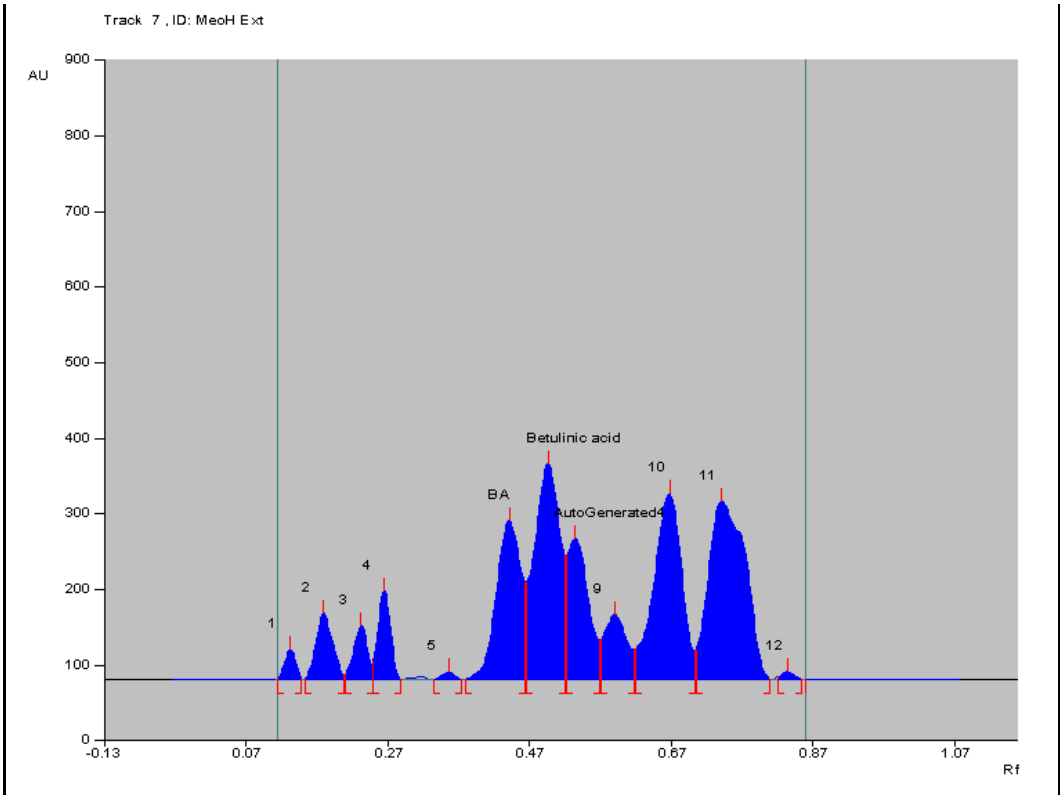
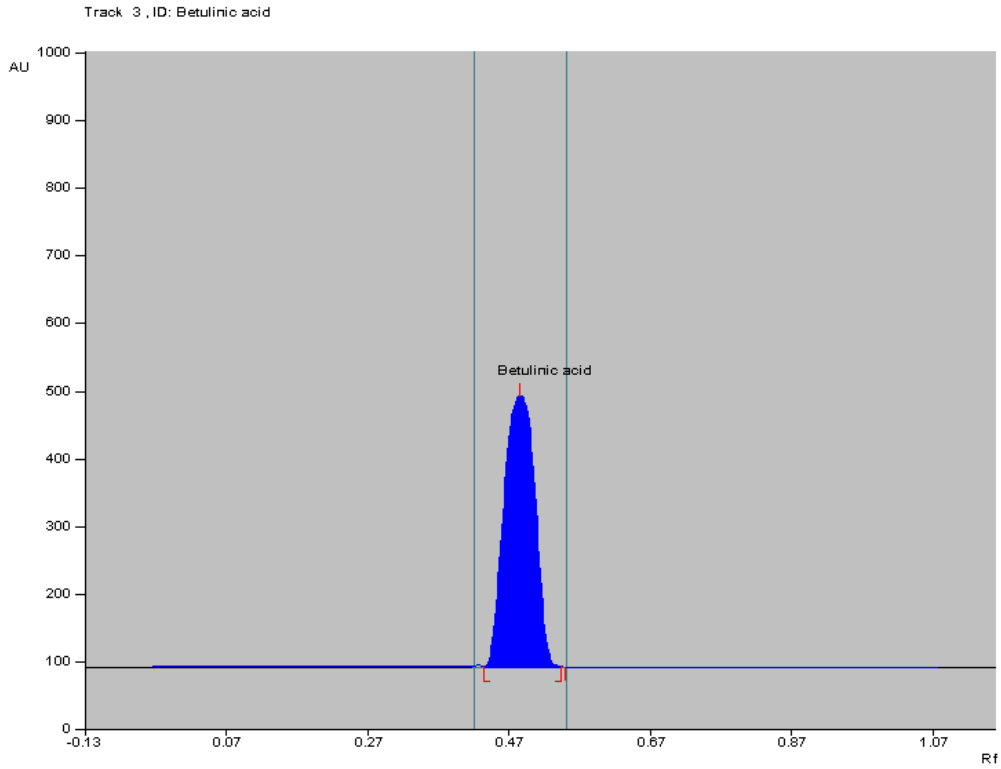


Figure 5: HPTLC Chromatogram of betulinic acid



In case of betulinic acid, the Limit of Detection (LOD) and Limit of Quantification (LOQ) were found to be 28.56 and 62.34 ng, respectively and show linearity in the range 0.2-1.2 µg/spot. Further the recovery values for betulinic acid were found to be 97-98%, which shows the reliability and suitability of the method. The peak purity test was done by comparing the spectra of the standards and its corresponding peaks in test samples. The correlation coefficients were found to be 0.931 for betulinic acid, which indicates its purity. The percentage of the bio-active marker was determined by calculation mode using peak area parameter and is found to be 0.48% w/w. The present HPTLC method is rapid, simple and accurate for quantitative monitoring of *Madhucalongifolia* plant with respect to betulinic acid.

CONCLUSION

The present investigation describes a simple, cost-effective and easily adaptable HPTLC method for simultaneous screening and quantitative determination of betulinic acid from different varieties of *Madhucalongifolia*. The method has been validated and found to be selective, linear, repeatable and accurate within established ranges.

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