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ESTIMATION OF BETULINIC ACID IN EXTRACTS AND FORMULATIONS CONTAINING *ALBIZIA LEBBECK* BY HPLC

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ABSTRACT

A triterpene acid, betulinic acid has been studied extensively because of its wide range of biological properties specially its anti-tumor activity. The present method discusses a new, simple and sensitive reversed phase HPLC-DAD method for quantitative estimation of betulinic acid in stem bark extract and formulations of *Albizia lebbeck*. Betulinic acid was analysed using MZ-Analytical C18 column (250 mm X 4.6 mm, 5 µm) as stationary phase. Elution was carried out using acetonitrile: water (92:08, v/v) at flow rate of 1.0 ml/min. Detection was carried at 205 nm. Column oven temperature and injection volume were kept at 25 ^{0}C and 20 µl, respectively. Linearity of the proposed method was found to be over the range 0.3-60 µg/ml. LOD and LOQ were found to be 0.1 µg/ml and 0.3 µg/ml, respectively. The method was found to be accurate with % recovery of 92.40 - 96.54 %.

Key Words: *Albizia lebbeck*; Betulinic acid; Fabaceae; Shirish; Standardization.

INTRODUCTION

Betulinic acid, chemically (3β)-3-Hydroxy-lup-20(29)-en-28-oic acid, a naturally occurring pentacyclic triterpenoid has been known to possess various biological activities such as anti-inflammatory, antinociceptive, antimalarial and antiretroviral properties. Recently it has been discovered that this molecule has got potential anticancer activity. [1]

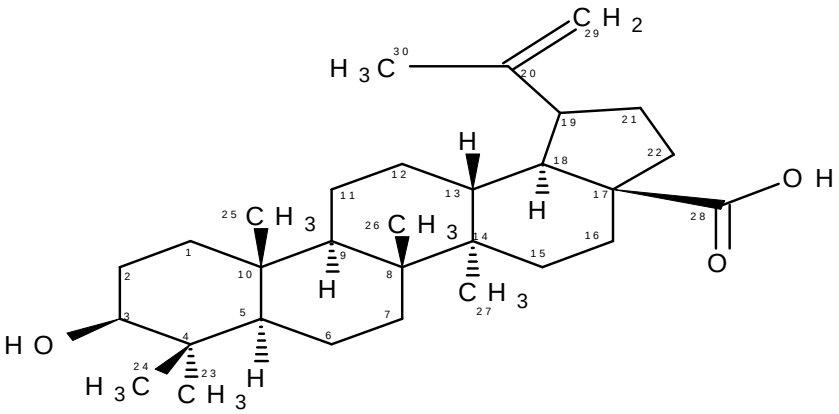

Fig. 1 Structure of betulinic acid

Betulinic acid is found in several species of plants, mainly *Betula pubescens*.[2] It is also found to be present in *Uapaca* species [3], *Berlina grandiflora* [4], *Triphyophyllum peltatum*, *Ancistrocladus heyneanus*, *Diospyros leucomelas* and *Tetracera boiviniana*, *Syzygium formosanum*.[5] The molecule is also found in stem bark of *Albizia lebbeck*.[6] Many HPLC methods have been reported for quantitative determination of betulinic acid alone or in combination in different medicinal plants.[7-13] But no HPLC method has been developed and validated for betulinic acid in stem bark extract of *Albizia lebbeck*. The present work discusses development and validation of a new, simple and rapid reversed phase HPLC-DAD method for quantitative determination of betulinic acid in ethyl acetate extract of stem bark of *Albizia lebbeck* and its successive application to standardize extracts/herbal formulations containing *Albizia lebbeck*.

MATERIALS AND METHODS

Reagents and standards

Solvents and purified water used were of HPLC grade from S. D. Fine chemicals, Mumbai, India. All the solutions were filtered using 0.2 µ PTFE filter (Goettingen, Germany) before injecting into HPLC chromatograph. Standard of betulinc acid (purity 98% w/w) was procured from Extrasynthese, France.

Instrument

Analysis was carried out using Dionex- UltiMate 3000 HPLC chromatograph (Germany), consisting of quaternary pumps, auto-sampler, column oven and diode array detector (DAD). Data were analysed using Chromeleon software version 6.80.

Procurement of plant material

Dried stem barks of *Albizia lebbeck* were procured from three different geographical sources namely Valsad district of Gujarat, Tirunelvelli district of Tamilnadu and Mumbai, Maharashtra in February 2012. Different herbaria of these stem barks were prepared and deposited at Botanical Survey of India (BSI), Pune under names ALGSOD8, ALTSOD9 and ALMSOD7, respectively and authenticated.

Preparation of bark extract

Collected barks were dried and pulverized. Around 10 g of bark powder was extracted with 100 ml of ethyl acetate. The method was repeated thrice to ensure complete extraction. Ethyl acetate extracts thus obtained were combined together. The combined ethyl acetate extract was dried under reduced pressure using rotary evaporator.

Preparation of standard solution

A stock solution of 1000 µg/ml was prepared by dissolving 50 mg of standard betulinic acid in 50 ml of methanol by sonication for 10 min. From this stock solution, working standard of 500 µg/ml was prepared by diluting 25 ml of stock solution upto 50 ml with methanol. From this, various aliquots were taken and diluted with appropriate volume of methanol to produce different concentrations which were used to validate the method.

Preparation of sample solution

Ethyl acetate extract of stem bark of *Albizia lebbeck* (5 mg) was dissolved in 5 ml of methanol, sonicated for 10 min and filtered through 0.2 µ PTFE filter.

HPLC method development

Solvents such as methanol or acetonitrile were tried in various combinations with water or 0.1 % TFA in isocratic mode and gradient mode as mobile phase. Effects of these combinations on retention time, number of plates, resolution and peak symmetry were observed. In all cases, no separation of components present in extract was achieved. Thus the following chromatographic conditions which gave optimum retention time and peak symmetry were selected: Column: MZ-Analytical C18 column (250 mm X 4.6 mm, 5 µm) Mobile Phase: Acetonitrile: water (92:08, v/v) Flow rate: 1.0 ml/min

Detection Wavelength: 205 nm

Temperature: 25 ^{0}C

Injection volume: 20 µl

Method Validation [14]

The developed method was validated for various parameters such as linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, robustness and system suitability as per ICH guidelines.

Specificity

To assess specificity, two injections of blank (methanol), six individual injections of standard solution (40 µg/ml) and two injections of sample solution (5 mg of ethyl acetate extract in 5 ml of methanol) were applied before all measurements and any interference from blank or sample was checked.

System suitability

To assess system suitability, six individual injections of standard solution (100 µg/ml) and two injections of sample solution were applied before all measurements and system suitability parameters such as resolution, theoretical plates, asymmetry and repeatability of the peak area were evaluated.

LOD and LOQ

The LOD and LOQ were calculated based on signal to noise ratio method. Betulinic acid solutions in increasing concentrations were injected until signal to noise ratio of 3.0 and 10.0 were obtained for determination of LOD and LOQ, respectively.

Linearity and range

To determine the linear relationship, duplicate injections of different concentrations 0.3, 10, 20, 30, 40, 50 and 60 µg/ml of standard betulinic acid solution were applied using 20 µl loop and area were recorded at 205 nm. A plot of average peak area and concentrations was obtained and correlation coefficient (r^2) was determined.

Accuracy

The accuracy of the developed method was evaluated through the analyte recovery test at three concentrations levels. Known amount of sample was spiked with 32, 40 and 48 µg/ml of the standard solutions of betulinic acid and % recovery was calculated.

Repeatability and intermediate precision

Repeatability (intraday) and intermediate precision (interday) were determined through analysis of the samples in triplicate at three concentrations level that is 80 %, 100 % and 120 % for two different days and % relative standard deviation (% RSD) was calculated.

Robustness

The robustness of the method, related to the variation in retention time, area, and % w/w of betulinic acid in the sample was evaluated by deliberately changing the detection wavelength (204, 205 and 206 nm), mobile phase flow rate (0.9, 1.0 and 1.1 ml/ min), column oven temperature (24, 25 and 26 ^{0}C) and mobile phase composition (acetonitrile: water, 91.5: 8.5, 92:08 and 92.5: 7.5, v/v). For each altered condition, six injections of the standard solution (40 µg/ml) and two injections of sample were applied.

Estimation of betulinic acid in extracts and formulations

The developed and validated method was applied for quantitative determination of betulinic acid in three extracts and different marketed herbal formulations such as granules, herbal tea, *Vati,* and syrup containing *Albizia lebbeck.* Different extracts were prepared from bark of *Albizia lebbeck* procured from three different geographical regions of India. To estimate % w/w of betulinic acid present in herbal formulations, samples were prepared as given below

Preparation of sample solutions for analysis of marketed formulations

Granules

Accurately weighed 10.560 g of granules were extracted with 100 ml of ethyl acetate. The resulting solution was filtered and dried. Accurately weighed 0.067 g of ethyl acetate extract thus obtained was dissolved in 5 ml of methanol and sonicated for 10 min. Accurately measured 1.0 ml of above solution was diluted to 10 ml with methanol. The solution was filtered and injected in HPLC.

Herbal tea

Accurately weighed 10.029 g of herbal tea was extracted with 100 ml of ethyl acetate. The resulting solution was filtered and dried. Accurately weighed 0.0514 g of ethyl acetate extract thus obtained was dissolved in 5 ml of methanol and sonicated for 10 min. Accurately measured 1.0 ml of above solution was diluted to 10 ml with methanol. The solution was filtered and injected in HPLC.

Vati

Twenty *Vatis* were individually weighed and their mean weight was determined. The *Vatis* were triturated and accurately weighed 10.018 g of *Vati* power was extracted with 100 ml of ethyl acetate. The resulting ethyl acetate solution was filtered and evaporated to dryness. Accurately weighed 0.0505 g of ethyl acetate extract thus obtained was dissolved in 5 ml of methanol and sonicated for 10 min. Accurately measured 1.0 ml of above solution was diluted to 10 ml with methanol. The solution was filtered and injected in HPLC.

Syrup

Accurately measured 50 ml of syrup was extracted 3 X 100 ml of ethyl acetate. The ethyl acetate extract was concentrated to dryness. Accurately weighed 0.05 g of ethyl acetate extract thus obtained was dissolved in 5 ml of methanol and sonicated for 10 min. The solution was filtered and injected in HPLC.

RESULTS AND DISCUSSION

Betulinic acid was found to be soluble in methanol hence reversed phase HPLC was the method of choice. Detection wavelength was selected based on λ_{max} of the compound. To determine λ_{max} of betulinic acid, UV spectrum of 10 µg/ml of standard betulinic acid was obtained. It showed absorbance maximum at 191.8 nm. Based on literature survey and because of the interference at lower wavelength, the analysis was carried out at 205 nm. Initially, column selected was Eclipse XBD C18 column (250 mm X 4.6 mm I.D., 5 µm). The injection volume was kept at 20 µl. Effect of various mobile phases on system suitability parameters was observed. In all cases, no separation of components present in extract was achieved. Therefore, another column C18 MZ-Analytical column (250 mm X 4.6 mm I.D., 5 µm) was tried. Acetonitrile in combination with water in isocratic mode gave proper peak shape and desired resolution as compared to other mobile phases. As concentration of acetonitrile was increased from 95 to 99 %, though there was decrease in retention time of betulinic acid, however marked decrease in resolution between the components of extract and number of theoretical plates were observed. Acetonitrile: water in ratio of 92:08, v/v produced desired peak shape, resolution and number of theoretical plates. Analysis was carried out at column oven temperature of 25 ^{0}C. The flow rate of mobile phase was kept at 1.0 ml/min which gave less retention time of betulinic acid and was optimum for resolution of peaks in extract. The developed method was found to be specific as no interference from blank or other components from sample was observed (Fig. 2 and Fig. 3).

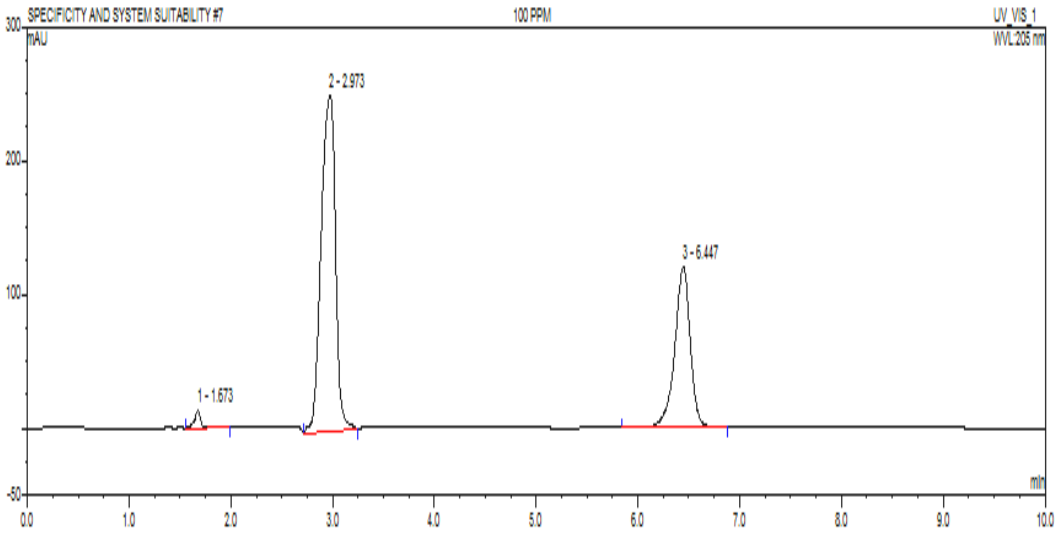

Fig. 2: HPLC chromatogram of standard betulinic acid

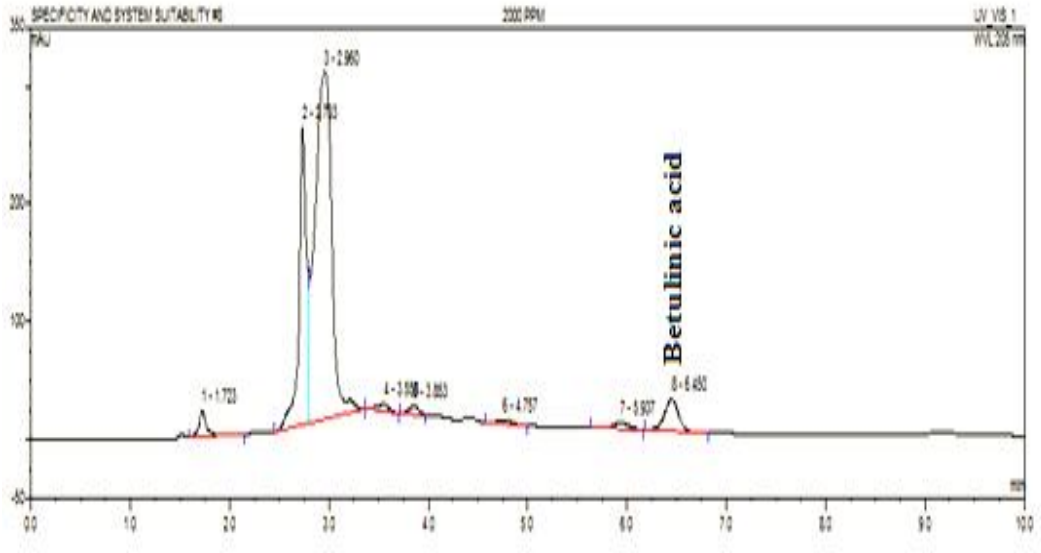

Fig. 3: HPLC chromatogram of ethyl acetate extract of *Albizia lebbeck*

Peak with mean retention time of 6.447 min represents betulinic acid. Total run time was 10 min. System suitability parameters such as number of theoretical plates (N) were more than 9000, resolution (Rs) was 1.86, peak asymmetry was less than 2.0 and % relative standard deviation (% RSD) obtained for six injections of standard solution was less than 1.0 representing that the method and instrument were appropriate for carrying out analysis. The proposed method was found to be linear over the range 0.3-60 µg/ml with correlation coefficient (r^2) of 0.9932. LOD and LOQ for the developed method were found to be 0.1 µg/ml and 0.3 µg/ml, respectively. The recoveries obtained during accuracy studies were found to be between 92.40-96.54 % which is within the 90-110 % range that is considered acceptable (Table 1). So the developed method was found to be accurate.

Table 1: Results of accuracy studies for betulinic acid

Component	Amount Present[a] (µg/ml)±SD	Amount Found[a] (µg/ml)± SD	% Recovery[a]± SD
Betulinic acid	30.6706±0.07	29.2444±0.11	95.3497± 0.15 %
	40.5608±0.02	37.4806±0.06	92.4060±0.12 %
	49.0125±0.06	47.3174±0.03	96.5413± 0.07 %

[a] n=3, triplicate injections

During precision studies, values of % RSD obtained at all level were less than 2.0 indicating that the proposed method was precise (Table 2).

Table 2: Results of intraday and interday precision studies for betulinic acid

Component	Amount level (mg/5 ml)	Intraday (% RSD)[a]		Interday (% RSD)[a]
		Day 1	Day 1	Day 2
Betulinic acid	4	0.9978	0.1506	0.0451
	5	0.7177	0.2106	0.0624
	6	0.2366	0.2787	0.9851

[a] n=3, triplicate injections

The robustness was evaluated by calculating the overall mean, standard deviation and % RSD for each variable. % RSD was less than 2.0 for the variables such as detection wavelength, flow rate, column oven temperature and mobile phase composition. The present method was found to be robust for the % w/w of betulinic acid present in the sample (Table 3). In the analysis of extracts, % w/w of betulinic acid in extract-II (sample procured from Tamilnadu) was found to be higher than the other two extracts. In the analysis of the formulations, granules showed higher % w/w of betulinic acid than other formulations (Table 4).

Table 3: Results of robustness study for betulinic acid

Parameters	Mean Retention time	Mean area	% RSD of area	% w/w of betulinic acid
Detection wavelength (nm)				
204	6.632	9.1480	1.1882	2.5136
205	6.632	8.4238	0.8262	2.5131
206	6.632	7.7205	0.0971	2.5418
Flow rate (ml/min)				
0.9	7.349	9.3503	0.1058	2.5435
1.0	6.632	8.4238	0.8262	2.5131
1.1	6.012	7.7248	0.4051	2.5473
Column oven temperature (^{0} C)				
24	6.686	8.5648	0.8196	2.5275
25	6.632	8.4238	0.8262	2.5131
26	6.508	8.5835	0.2446	2.5036
Mobile phase composition (v/v)				
Acetonitrile: water (91.5: 8.5)	6.635	8.6484	0.5607	2.4286
Acetonitrile: water (92: 08)	6.632	8.4238	0.8262	2.5131
Acetonitrile: water (92.5: 7.5)	6.861	8.4252	0.3857	2.5176

[a] n=6, six injections

Table 4: % w/w of betulinic acid in extracts and formulations containing *Albizia lebbeck*

Extracts/Formulations	% w/w of betulinic acid [a] ± SD	mg of betulinic acid [a] ± SD
Extract-I (Gujarat)	2.5330±0.01	-
Extract-II (Tamilnadu)	3.4142±0.01	-
Extract-III (Maharashtra)	1.2698± 0.00	-
Granules	2.7028±0.04	0.8162±0.01/Teaspoon
Herbal Tea	1.1450±0.01	0.5978±0.00/ Teaspoon
Vati	0.8396±0.00	0.0758± 0.00/*Vati*
Syrup	0.0272±0.00	0.0083±0.00/ Teaspoon

[a] n=3, triplicate injections

CONCLUSION

Thus, a simple, rapid, accurate and convenient HPLC method was developed using betulinic acid as a marker compound and validated as per ICH guidelines. This developed and validated method was successfully applied for estimation of betulinic acid in three ethyl acetate extracts and marketed herbal formulations containing *Albizia lebbeck*. This sensitive, accurate and precise HPLC-DAD method can be very well utilized to determine batch to batch variations and routine analysis of herbal formulations containing *Albizia lebbeck* by herbal manufacturer.

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REFERENCES

1. Yogeeswari P, Sriram D. Betulinic Acid and Its Derivatives: A Review on their Biological Properties. Curr Med Chem, 2005; 12(6): 657-66.
2. Kovac-Besovic EE, Duric K, Kalodera Z, Sofic E. Identification and isolation of pharmacologically active triterpenes in *Betuale cortex*, *Betula pendula* Roth., Betulaceae. Bosn J Basic Med Sci, 2009; 9 (1): 31-8.
3. Nyasse B, Nono J-J, Nganso Y, Ngantchou I, Schneider B. *Uapaca* genus (Euphorbiaceae), a good source of betulinic acid. Fitoterapia, 2009; 80(1): 32-4.
4. Enwerem NM, Okogun JI, Wambebe CO, Okorie DA, Akah PA. Anthelmintic activity of the stem bark extracts of *Berlina grandiflora* and one of its active principles, Betulinic acid. Phytomedicine, 2001; 8(2): 112-14.
5. Shyur L-F, Lau ASY. Recent Trends in Medicinal Plants Research, UK; Academic Press; 2012: pp.84

6. Desai S, Tatke P, Gabhe SY. Isolation of catechin from stem bark of *Albizia lebbeck*. International Journal of Analytical, Pharmaceutical and Biomedical sciences, 2014; 3(2): 31-5.
7. Zhao G, Yan W, Cao D. Simultaneous determination of betulin and betulinic acid in white birch bark using RP-HPLC. J Pharm Biomed Anal, 2007; 43(3): 959-62.
8. Guo S, Duan J, Tang Y, Su S, Shang E, Ni S, Qian D. High performance liquid chromatography-two wavelength detection of triterpenoid acids from the fruits of *Ziziphus jujuba* containing various cultivars in different regions and classification using chemometric analysis. J Pharm Biomed Anal, 2009; 49(5): 1296-02.
9. Kumar D, Mallick S, Vedasiromoni JR, Pal BC. Anti-leukemic activity of *Dillenia indica* L. fruit extract and quantification of betulinic acid by HPLC. Phytomedicine, 2010; 17(6): 431-35.
10. Jain M, Kapadia R, Jadeja RN, Thounaojam MC, Devkar RV, Mishra SH. Hepatoprotective potential of *Tecomella undulata* stem bark is partially due to the presence of betulinic acid. J Ethnopharmacol, 2012; 143 (1): 194-00.
11. Feng Y, Li M, Liu J, Xu T, Fang R, Chen Q, He G. A novel one-step microbial transformation of betulin to betulinic acid catalysed by *Cunninghamella blakesleeana*. Food Chem, 2013; 136 (1): 73-9.
12. Machado DG, Cunha MP, Neis VB, Balen GO, Colla A, Bettio LEB, Oliveira A, Pazini FL, Dalmarco JB, Simionatto EL, Pizzolatti MG, Rodrigues ALS. Antidepressant-like effects of fractions, essential oil, carnosol and betulinic acid isolated from *Rosmarinus officinalis* L. Food Chem, 2013; 136 (2): 999-05.
13. Zhang M, Zhang Y, Xie J. Simultaneous determination of jujuboside A, B and betulinic acid in Semen ziziphi spinosae by high performance liquid chromatography evaporative light scattering detection. J Pharm Biomed Anal, 2008; 48(5): 1467-70.
14. ICH, Validation of analytical procedures: Text and methodology-ICH harmonized tripartite guideline, 2005.