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Research Article

SEPARATION AND DETERMINATION OF TRITERPENE ACIDS BY USING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY FROM STEM BARK OF *MIMUSOPS ELENGI* LINN.

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ABSTRACT

Objectives: To develop and validate high performance thin layer chromatographic (HPTLC) method for the quantitative determination of betulinic acid and ursolic acid present in stem bark of *Mimusops elengi* Linn.

Method: Chromatographic analysis was performed using methanolic extract of dried stem bark powder of *Mimusops elengi* Linn. on silica gel 60 F_{254} TLC plates, using a suitable solvent system. Post derivatization was carried out using anisaldehyde-sulphuric acid reagent. Detection and quantification of betulinic acid and ursolic acid was done by densitometric scanning at λ= 540nm. The developed HPTLC method has been validated using International Conference on Harmonization (ICH) guidelines.

Results: The developed method is rapid, simple and precise and provided good resolution of betulinic acid and ursolic acid from other phyto constituents present in the dried stem bark powder of *Mimusops elengi* Linn.The mean amounts of betulinic acid and ursolic acid from the methanolic extract of dried leaf powder of *Mimusops elengi* Linn. were found to be 4.6409mg/g and 1.3127mg/g respectively.

Conclusion: The developed HPTLC technique is precise, specific and accurate and can be used for the routine quality control analysis and simultaneous quantitative determination of betulinic acid and ursolic acid from the dried powder of stem bark powder of *Mimusops elengi* Linn.

Keywords: *Mimusops elengi* Linn., Betulinic acid, Ursolic acid, Triterpene acids, High performance thin layer chromatography, Sapotaceae.

INTRODUCTION

Mimusops elengi Linn., commonly called as bakul, is a medicinal plant belonging to family Sapotaceae. *Mimusops elengi* Linn. is a small to large evergreen tree which grows upto 15 m in height which has many medicinal properties.

The stem bark of *Mimusops elengi* Linn., is reported to have wound healing activity[1,2], antioxidant activity[3, 4, 5], anti-hyperglycaemic activity[3, 6], anti urolithiatic activity[4] and antibiotic activity[7]. The stem bark is reported to contain many phyto chemicals such as β-amyrin, bassic acid, betulinic acid, lupeol, taraxerone, taraxerol, ursolic acid, α-spinasterol, β-D- glucoside of β-sitosterol, quercitol[8].

Betulinic acid and ursolic acid are triterpene acids. Betulinic acid is reported to slow down the activity of the human immunodeficiency virus (HIV), induce apoptosis and cell death in melanoma cells without impacting healthy tissue [9]. Betulinic acid is useful for its pharmacological properties, especially the treatment of diarrhoea, dysentery and cholera[9]. Ursolic acid has several important biological activities like anti-inflammatory, anti-tumour properties. It also possesses hepatoprotective, anti-ulcer, hypolepidemic and antiatherosleroic activity [10]. It is significant anti tumorigenesis and antioxidant agent [11].

Considering medicinal properties of betulinic acid and ursolic acid, in the present research work, a high performance thin layer chromatographic method was developed for the separation and quantification of betulinic acid and ursolic acid. Some of the high performance thin layer chromatographic methods are reported in literature for quantitation of betulinic acid and ursolic acid.

In literature, a normal phase HPTLC method [12] has been used for the quantification of betulinic acid from rhizome of *Nelumbo nucifera* Gaertn. using mobile phase comprised of chloroform: methanol: formic acid in the volume ratio of 49.0: 1.0 :1.0.

Ursolic acid and oleonic acid were also simultaneously determined from *Salvia officinalis herba*[13]. Pre-chromatographic derivatization was carried out with 1% iodine solution in chloroform. The separation was achieved using petroleum ether: ethyl acetate: acetone in the volume ratio of 8.2: 1.8: 0.1.

Simultaneous quantification of betulinic acid, ursolic acid and oleonic acid from *folium Salviae* (*Salvia officinalis* L.), *folium Plantaginis lanceolatae* (*Plantago lanceolata* L.) and *flos Lamii albi* (*Lamium album* L.) [14] has been reported. The chromatographic separation was achieved with multiple (five step) gradient program of the mobile phase. The ratio of petroleum ether: ethyl acetate was changed after each step.

However, no HPTLC method is reported for the simultaneous quantification of betulinic acid and ursolic acid from the stem bark of *Mimusops elengi* Linn.

Thus, precise and accurate HPTLC method has been developed and validated using International Conference on Harmonization (ICH) guidelines for simultaneous determination and quantification of betulinic acid and ursolic acid from dried stem bark powder of *Mimusops elengi* Linn.

MATERIALS AND METHODS

Experimental Reagents

All solvents, methanol (99.9%), toluene (99.5%), acetone (99.8%), were procured from Qualigens Fine Chemicals (Mumbai, India). TLC plates precoated with silica gel 60F_{254} used were obtained from E. Merck (India).

Reference Standard

The reference standards betulinic acid (purity ≥98.0% HPLC Grade) and ursolic acid (purity ≥90.0% HPLC Grade) were purchased from Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinbeim, Germany).

Plant material

The stem bark of *Mimusops elengi* Linn., was collected from Keshav Srushti, Mumbai, India. Herbarium of the plant was prepared and authenticated from Botanical Survey of India (BSI), Pune, India. A duplicate herbarium was prepared and is preserved in Ramnarain Ruia College.

The stem barks of *Mimusops elengi* Linn., were washed with water to remove soil particles, dried at 45 ± 2° C[16], powdered and then sieved through BSS mesh size no. 85 and stored in an airtight container at room temperature (25 ± 2° C).

Preparation of solutions

Preparation of stock and working standard solutions of betulinic acid

About 10.0 mg of betulinic acid was accurately weighed and transferred to 10.0 mL volumetric flask. 5.0 mL of methanol was added and the contents were sonicated in an ultrasonic bath (Model:TRANS-O-SONIC, Frequency: 50 Hz) for 5 minutes for complete dissolution of betulinic acid . The contents were then diluted up to the mark with methanol to obtain a solution of betulinic acid with concentration of 1000 µg/mL.

2.5 mL of above stock solution of betulinic acid was then transferred to 10.0 mL volumetric flask and the contents of volumetric flask were diluted up to 10.0 mL by methanol to obtain working solution of betulinic acid with concentration of 250.0 µg/ mL.

Preparation of stock and working standard solutions of ursolic acid

About 10.0 mg of ursolic acid was accurately weighed and transferred to 10.0 mL volumetric flask. 5.0 mL of methanol was added and the contents were sonicated for 5 min for complete dissolution of ursolic acid. The contents were then diluted up to the mark with methanol to obtain a stock solution of ursolic acid with concentration of 1000 µg/mL.

1.0 mL of above stock solution of ursolic acid was then transferred to 10.0 mL volumetric flask and the contents of volumetric flask were diluted up to 10.0 mL by methanol to obtain working solution of ursolic acid with concentration of 100.0 µg/ mL.

Preparation of sample solution

About 1.0g of finely powdered stem bark powder of *Mimusops elengi* Linn., was accurately weighed in a 20mL stoppered test tube. 10mL of methanol was added to it and the tube was sonicated in an ultrasonic bath for 15 minutes. The tube was then shaken at 50 rpm, on a test tube rotator overnight at room temperature (25 ± 2ºC). The contents of test tube were filtered through whatmann filter paper no.41. The filtrate was passed through 0.45 µm nylon filters (Millipore) before the analysis.

Preparation of mobile phase

The mobile phase used in the present research work for simultaneous quantification of betulinic acid and ursolic acid from the stem bark powder of *Mimusops elengi* Linn., was prepared by mixing toluene: acetone (9.0:1.0 v/v) in a test tube. It was then sonicated for 5 minutes.

Preparation of derivatizing reagent[16]

Anisaldehyde- sulphuric acid was used as derivatizing reagent. 10 mL of H_2SO_4 was carefully added to ice cooled mixture of 170 mL methanol and 220 mL of acetic acid. To this solution, 1.0 mL of anisaldehyde was added. The solution was stored in refrigerator.

HPTLC conditions

Chromatography was performed on 5.0 cm x 10.0 cm TLC plates which were cut from 20.0 cm x 20.0 cm TLC aluminum plates, precoated with 200 µm layers of silica gel $60F_{254}$ (E. Merck, Mumbai, India). The plates were prewashed with methanol and activated at 105°C-110ºC for 15 minutes before analysis. Standard and sample solutions were applied to the plates as 7mm bands, 6mm apart from each other and 10 mm above from bottom edge of the plate, under a continuous supply of nitrogen by means of a CAMAG (Muttenz, Switzerland) Linomat V Automatic TLC sample applicator fitted with a 100µL syringe (Hamilton, Bonaduz, Switzerland).

Linear ascending development was carried out in a twin-trough glass chamber (Camag, Muttenz, Switzerland) saturated with mobile phase comprising of toluene: acetone (9.0:1.0 v/v). The optimized chamber saturation time for the mobile phase was 20 minutes at room temperature (25±2ºC). The plates were developed to a distance of 90 mm from the bottom edge of the plate. After first development, the plates were dried in air and subjected to redevelopment with the same mobile phase. This process is known as double development of the plate.

Post derivatization of the plate was done by anisaldehyde-sulphuric acid reagent. The plate was dried, and then heated to 110º C for 2-3 min. The densitometric scanning was then performed at λ=540 nm in reflectance/absorbance mode using CAMAG TLC scanner III with Win CATS software version 1.4.2.

Method validation

Linearity

Preparation of calibration curve of betulinic acid

The linear working range of betulinic acid was obtained by applying 8µL, 12µL, 16µL, 20µL and 24µL from standard solution of betulinic acid with concentration of 250.00µg/mL, as bands on same TLC plate to obtain concentrations in the range of 2.0 µg /band to6.0 µg/band.

Preparation of calibration curve of ursolic acid

Linear working range for ursolic acid was obtained by applying 6µL, 10µL, 14µL, 18µL and 22µL from standard solution ursolic acid with concentration of 100.00µg/mL, of on same TLC plate to obtain concentrations in the range of 0.80µg/band to 2.40µg/band.

The calibration curves of both betulinic acid and ursolic acid were obtained by plotting graphs of mean peak areas vs. corresponding concentrations. The results listed in Table 1.0. For both the standards, within the concentration range indicated, there was a good correlation between mean peak area and concentration of standards.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) were determined at signal to noise ratios of 3:1 and 10:1, respectively. The LOD and LOQ values obtained for both the components are listed in Table 1.

System suitability

System suitability was carried out to verify that resolution and reproducibility of the system were acceptable for the analysis. System suitability test was carried out by applying standard solutions of betulinic acid and ursolic acid concentrations of 5.0µg /band and 1.2µg /band respectively, on same TLC plates in six replicates under specified chromatographic conditions. The chromatograms for both standards were recorded. The values of percent relative standard deviations of peak area and retention factors of standards were taken as an indicator of system suitability and are less than 2, indicating that the method is suitable for analysis.

Precision

The method was validated in terms of repeatability and intermediate precision.

The repeatability was evaluated by triplicate analysis of three sample solutions i.e. 10 µL of methanolic extract of the stem bark powder of *Mimusops elengi* Linn., was applied on the TLC plate in triplicates on the same day in the same laboratory under the specified chromatographic conditions. The peak areas of betulinic acid and ursolic acid were recorded.

The intermediate precision of the method was evaluated by analyzing the sample solution in triplicate on three different days, under the specified chromatographic conditions. The peak areas of betulinic acid and ursolic acid were recorded.

The precision results were expressed as percentage relative standard deviations of peak areas of betulinic acid and ursolic acid and are listed in Table 1. The results indicate that the proposed method is precise and reproducible.

Stability of the standard betulinic acid and ursolic acid solution

The stabilities of standard betulinic acid and ursolic acid solutions were determined by comparing the peak areas of standard solution of betulinic acid and ursolic acid at different time intervals, for a period of minimum 48 hrs. Standard solution of betulinic acid with concentration of 5.0µg/band and ursolic acid with concentration of 1.20µg/band, were applied as bands on the same TLC plate and analyzed under the optimized chromatographic conditions. The results showed that the peak areas of betulinic acid and ursolic acid almost remained unchanged (values of percent relative standard deviation were less than 2) over a period of 48 hrs, and no significant degradation was observed within the given period, indicating the stability of standard solutions of betulinic acid and ursolic acid for minimum 48 hrs.

Robustness

Robustness tests examine the effect of the operational parameters on the analysis results. By introducing small changes in the mobile phase composition, the effects on the results were examined. The mobile phase in the developed method was toluene: acetone in the volume ratio of 9.0: 1.0. The composition of the mobile phase was altered to toluene: acetone (9.1: 0.9) and toluene: acetone (8.9: 1.1).

The amounts of betulinic acid and ursolic acid from dried stem bark powder of *Mimusops elengi* Linn. obtained by altered method to that obtained by normal method was found to be similar. The modifications did not affect the system suitability criteria. From the observations, it was concluded that the method is robust as the above mentioned deliberate changes made the method did not affect the results.

Assay procedure

The developed and validated HPTLC method was used for quantification of betulinic acid and ursolic acid from the methanolic extract of dried stem bark powder of *Mimusops elengi* Linn. 10µL of methanolic extract of the dried stem bark powder of *Mimusops elengi* Linn., was applied as a band on the same TLC plate (n=7). The plate was developed and scanned under the optimized chromatographic conditions. The chromatograms were recorded. Amounts of betulinic acid and ursolic acid present in the sample solution were determined from the calibration curve, by using the peak area of betulinic acid and ursolic acid in the sample. The amounts of betulinic acid and ursolic acid were found to be 4.6409mg/g and 1.3127mg/g respectively.

Accuracy

The accuracy of the method was established by performing recovery experiment by using standard addition method at three different levels. To accurately weighed about1.0g of dried stem bark powder of. *Mimusops elengi* Linn., known amounts of standard betulinic acid and ursolic acid i.e. 0.1mg, 0.2mg, 0.3mg were added, and extracted using methanol. Betulinic acid and ursolic acid contents were quantified by the proposed method and the percentage recovery was calculated. The values of percent recoveries obtained were 97.58 and 99.54 for betulinic acid and ursolic acid respectively. The results of accuracy are listed in Table 2.

RESULTS

Different mobile phases were tried for simultaneous HPTLC separation of betulinic acid and ursolic acid from other components of the dried stem bark powder of *Mimusops elengi* Linn. and good separation was achieved by using toluene : acetone (9.0:1.0v/v) as mobile phase with double development of the plate. Detection was carried out densitometrically using a CAMAG TLC Scanner at λ = 540 nm as both betulinic acid and ursolic acid showed maximum response at this wavelength. The identity of the bands of betulinic acid and ursolic acid in the sample solutions was confirmed by comparing their Rf values in sample with that of reference standards. Figure 1 represents a typical TLC plate showing separation of betulinic acid and ursolic acid. The Rf values for betulinic acid and ursolic acid were 0.60 and 0.48 respectively. Figure 2 shows typical HPTLC chromatograms of standard betulinic acid, standard ursolic acid and methanolic extract of dried stem bark powder of *Mimusops elengi* Linn.

A good linear relationship was observed for betulinic acid and ursolic acid in the concentration in the range of 2.0µg/band to 6.0µg/band and 0.8µg/band to 2.4µg/band respectively with correlation coefficient of 0.999 for both the components (Table 1). When the method was validated for repeatability and intermediate precision, the values of percentage relative standard deviations were less than 2, indicating the proposed method is precise and repeatable (Table 1). The mean amounts of betulinic acid and ursolic acid from the methanolic extract of dried leaf powder of *Mimusops elengi* Linn. were found to be 4.6409mg/g and 1.3127mg/g respectively. The values of percent recoveries of betulinic acid and ursolic acid at three levels were 97.58 and 99.54 respectively indicating accuracy of the method (Table 2).

Table 1: Method validation data for simultaneous quantification of betulinic acid and ursolic acid

Parameters	Results	
	Betulinic acid	Ursolic acid
Linear range (n=3) (µg/band)	2.0-6.0	0.8-2.4
Correlation coefficient (r)	0.999	0.999
LOD (µg/ band)	0.50	0.20
LOQ (µg/ band)	2.0	0.80
Repeatability (% R.S.D.) (n=3) (on the same day)	1.20	1.08
Intermediate precision (% R.S.D.) (n=9) (For three successive days)	1.28	1.21

Table 2: Results of recovery study for simultaneous HPTLC quantification of betulinic acid and ursolic acid from methanolic extract of stem bark powder of. *Mimusops elengi* Linn.

Level	Amount of sample (g)	Amount of standard added to sample (mg)	Mean amount of standard found (mg) ±S.D*	Percent recovery
Betulinic acid				
0	1.003	0.0000	4.6545± 0.0441	97.58
1	1.002	0.100	4.7323± 0.0295	
2	1.001	0.200	4.8536± 0.0338	
3	1.003	0.300	4.9460± 0.0304	
Ursolic acid				
0	1.003	0.0000	1.3069± 0.0116	99.54
1	1.002	0.1000	1.4075± 0.0089	
2	1.001	0.2000	1.5062± 0.0120	
3	1.003	0.3000	1.6058± 0.0097	

*Mean ±S.D. (n=7)

Fig. 1: HPTLC plate showing separation of methanolic extract of dried stem bark powder of *Mimusops elengi* Linn. (1), standard ursolic acid (2) and standard betulinic acid (3)

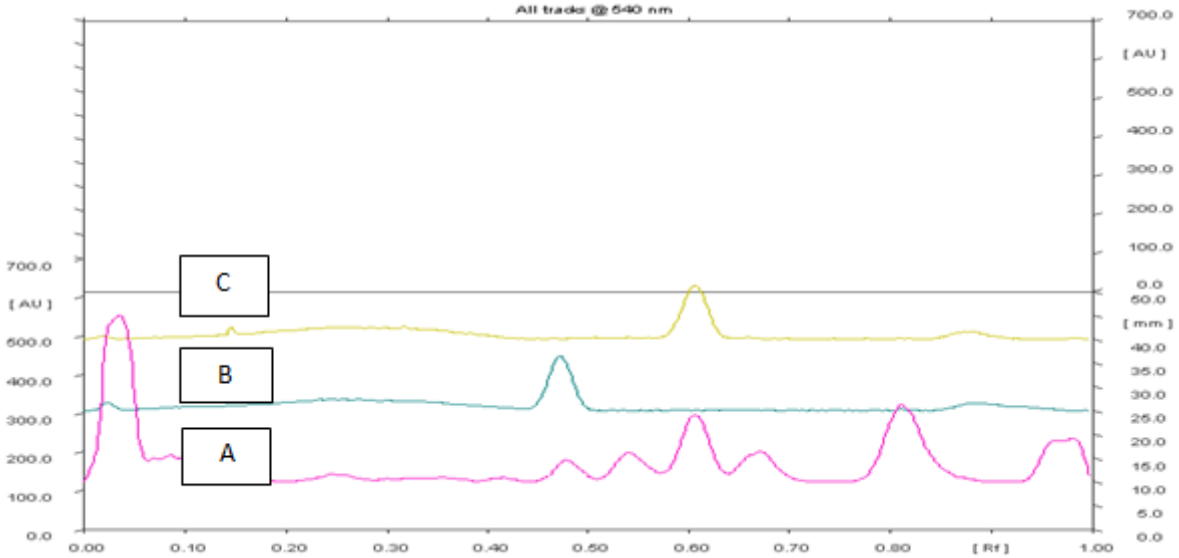

Fig. 2: HPTLC chromatograms obtained for methanolic extract of dried stem bark powder of *Mimusops elengi* Linn. (A), standard ursolic acid (B) and standard betulinic acid (C)

DISCUSSION

In the literature survey, a normal phase HPTLC method has been used for quantifications of betulinic acid from rhizome of *Nelumbo nucifera* Gaertn. [12] using mobile phase comprised of chloroform: methanol: formic acid in the volume ratio of 49.0: 1.0: 1.0. Post chromatographic derivatization was carried out using anisaldehyde-sulphuric acid reagent and detection was done at λ=420nm.

Ursolic acid and oleonic acid were also simultaneously quantitated from *Salvia officinalis herba*. [13] Pre-chromatographic derivatization was carried out with 1% iodine solution in chloroform on a distance of 1.2 cm, and the next start zone was covered with a glass strip and the plates were placed in dark for 10 minutes. After the completion of reaction the plate was dried in a steam of warm air to remove excess of iodine. The separation was achieved using petroleum ether: ethyl acetate: acetone in the volume ratio of 8.2:1.8:0.1. The post chromatographic derivatization was carried out by spraying the air plate with 10% ethanolic H_2SO_4. The plate was dried for 10 min and then heated to 120 ºC for 3 minutes. The derivatized compounds was detected at λ=530nm.

Simultaneous determination of betulinic acid, ursolic acid and oleonic acid from *folium Salviae* (*Salvia officinalis* L.), *folium Plantaginis lanceolatae* (*Plantago lanceolata* L.) and *flos Lamii albi* (*Lamium album* L.) The chromatographic separation was achieved with multiple (5 step) gradient program of the mobile phase. The ratio of petroleum ether: ethyl acetate was changed after each step. The mixture of cyclohexane: ethyl acetate: formic acid in the volume ratio of 7.5:2.5:0.05 was used in the last (fifth) step to improve the shape of the chromatographic bands. Post chromatographic derivatization was carried out by using 10% ethanolic H_2SO_4. The detection was carried at λ = 515 nm [14]. The present developed method is isocratic which requires less and minimum amount of solvents compared to gradient method reported above.

The mobile phase selected for the present research work is toluene: acetone (9.0:1.0 v/v). The mobile phase helped to separate betulinic acid and ursolic acid with a very little resolution, so the double development of the plate was performed to improve the resolution between them and chromatographic peak shape. The double development of the plate also helped for the separation of betulinic acid and ursolic acid from the matrix of stem bark of *Mimusops*

elengi Linn. The developed method for the separation of betulinic acid and ursolic acid in the present research work is advantageous as compared to the reported methods as mobile phases is very simple, with less solvents used and the technique of double development is less time consuming.

CONCLUSION

The developed HPTLC technique is precise, specific and accurate and can be used for the routine quality control analysis and simultaneous quantitative determination of betulinic acid and ursolic acid from the dried powder of stem bark powder of *Mimusops elengi* Linn.

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