CHAPTER - 7

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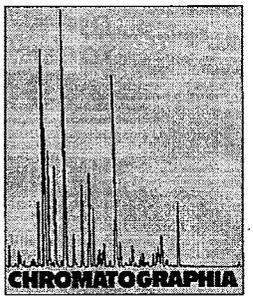
TLC Determination of Betulinic Acid from *Nymphodies macrospermum*: A New Botanical Source for Tagara



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Abstract

The accepted botanical source of Tagara, an ayurvedic drug is *Valeriana jatamansi* Jones. In South India, a drug by the name of Granthika Tagara is used as Tagara in several therapeutic preparations. Currently, no analytical procedures appear to be available for quality control purposes. A sensitive, selective and precise thin-layer chromatographic method has been developed and validated for the analysis of betulinic acid in *Nymphoides macrospermum*. Separation and quantification was achieved by TLC using ternary mobile phase of hexane:ethyl acetate:acetic acid (7:3:0.03, *v/v*) (R_F 0.60) on precoated silica gel $60F_{254}$ aluminium plates and densitometric determination was carried out after derivatization with anisaldehyde-sulphuric acid reagent in the reflection/absorption mode at 540 nm. The calibration curve was linear in the concentration range of 100–600 ng spot^{-1}. The method was validated for precision, repeatability and accuracy. The proposed method was found to be simple, precise, specific, sensitive and accurate for the quantification of betulinic acid. This is the first TLC report for the identification and quantification of betulinic acid in *N. macrospermum* and may be useful for the routine quality control of Granthika Tagara.

Keywords

Thin layer chromatography
Betulinic acid
Nymphoides macrospermum

Introduction

Tagara mentioned in the ayurvedic classics forms an important ingredient of several preparations used in the treatment of diseases, such as anaemia, jaundice, bleeding, haemorrhoids, tuberculosis, mental disorders, epilepsy, fever, cough, asthma and as a brain tonic. The accepted botanical source of Tagara, is *Valeriana jatamansi*, belonging to the Valerianaceae family and infrequently from *V. hardwickii*. A literature search revealed that in Karnataka and other centres in South India a drug under the name of Granthika Tagara (Kannada) is available in the market in place of the ayurvedic drug Tagara which is been identified as *Nymphoides macrospermum* [1].

Tagara is fast diminishing due to indiscriminate exploitation and the literature survey on *N. macrospermum* revealed no information on its phytochemical aspects hence it was thought worth if this drug is investigated for its phytochemical aspects. Preliminary phytochemical screening, thin-layer chromatographic (TLC) fingerprinting and co-TLC studies with betulinic acid and other available markers in our laboratory revealed the presence of a fairly high content of terpenoids and an identical spot as that of standard betulinic acid was observed; further it was confirmed by R_F comparison, multi wavelength scanning, and spectral overlay.

Betulinic Acid

3β-Hydroxy-lup-20(29)-en-28-oic acid, a naturally occurring triterpene was originally extracted from the bark of an African tree, *Ziziphus mauritiana* [2], later

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0009-5893/08/11

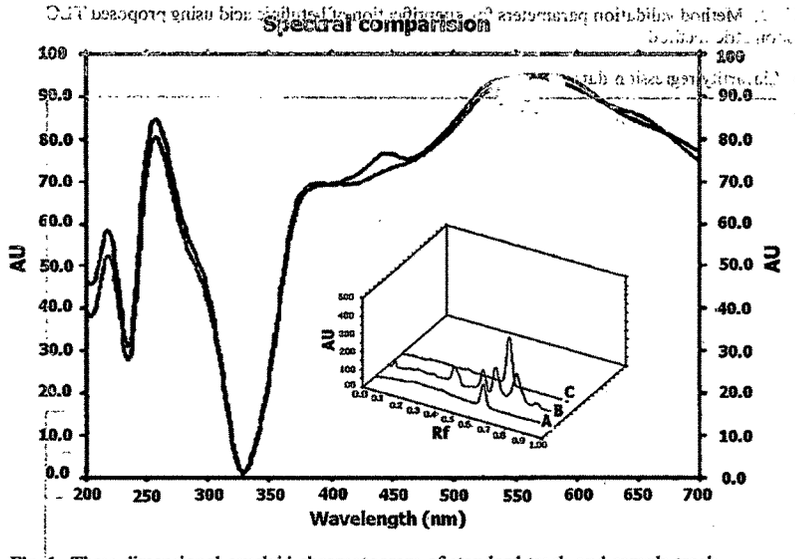

Fig. 1. Three-dimensional overlaid chromatogram of standard track and sample track

from other plants like *Trifillum peltatum*, *Ancistocladuis heyeneaus*, *Diospyros leucomelas*, *Tetracera boliviana*, *Sizigium formosanum*, [3] *Chaenomeles sinensis* [4]. Betulinic acid and its derivatives have been discovered as a new class of compounds as potential anti-cancer and anti-HIV agents [5, 6]. Previously, betulinic acid has been determined quantitatively in white birch bark by LC [7] while there is another report of a TLC method for its estimation in an ayurvedic preparation using chloroform:methanol:formic acid (98:2:2) solvent system [8].

Considering the wide therapeutic applications of betulinic acid and also as one of the marker constituent to ensure identity and quality of this plant a simple, sensitive, specific and reproducible TLC method was developed for the quantification of betulinic acid in *N. macrospermum* roots.

Experimental

Reagents and Chemicals

Pure Betulinic acid was obtained from Sigma-Aldrich Chemicals, (Steinheim, Germany) other solvents and chemicals were of analytical grade and TLC plates, silica gel $60F_{254}$ (20 cm × 20 cm), were purchased from E Merck (Darmstadt, Germany).

Plant Material

Roots of *N. macrospermum* were procured from the local market of Udupi, Karnataka, India, and authenticated in the Botany Department of The M. S. University of Baroda, Vadodara, India. A voucher specimen (No.Pharmacy/NM/05-06/04/KM) has been deposited in the Pharmacy Department of The M. S. University of Baroda, Vadodara, India.

Preparation of Crude Extract

Accurately weighed 2.5 g of the coarse powder of *N. macrospermum* roots were extracted separately with methanol (4 × 25 mL) under reflux (30 min each time) on a water bath. The combined extracts were filtered and concentrated, and transferred to a 25 mL volumetric flask and the volume was made up with methanol.

Preparation of Standard Solution

A stock solution of betulinic acid (100 μg mL^{-1}) was prepared by dissolving 1 mg of accurately weighed betulinic acid in methanol and making up the volume of the solution to 10 mL with methanol.

Chromatography

A Camag TLC system equipped with Camag Linomat V an automatic TLC sample spotter, Camag glass twin trough chamber (20 × 10 cm), Camag scanner 3 and integrated winCATS 4 software was used for the analysis. Chromatography was performed on a pre-coated TLC plate, silica gel $60F_{254}$ (20 cm × 20 cm). Samples and standards were applied on the 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 presaturated with 20 mL mobile phase hexane:ethyl acetate:acetic acid (7:3:0.03 *v/v*) for 30 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 in current air with the help of a hair dryer. The post chromatographic derivatization was carried out in anisaldehyde and sulphuric acid followed by heating at 110 °C for 3 min [9]. Quantitative evaluation of the plate was performed in absorption–reflection mode at 540 nm, using a slit width of 6 × 0.45 mm and data resolution 100 μm $step^{-1}$ and scanning speed 20 mm s^{-1} with a computerized Camag TLC scanner-3 integrated with winCATS 4 software. Quantification of betulinic acid in the extract of *Nymphoides* roots was performed by external standard method, using pure betulinic acid as standard.

Calibration Curve for Betulinic Acid

Stock solution of betulinic acid (100 μg mL^{-1}) was prepared in methanol and different amounts (100–600 ng $spot^{-1}$) were applied on a TLC plate, using Linomat V for preparing six point calibration graphs of peak area versus concentration. The regression equation for betulinic acid was $39.366 + 12.165x$ and co-relation coefficient (r) was 0.998.

Quantification of Betulinic Acid in Test Sample

Ten microlitres of sample solution were applied in triplicate on a TLC plate and developed, scanned as above. Peak areas were recorded and the amount of betulinic acid was calculated using the calibration plot.

Specificity

Specificity of the method was determined by analysing a sample of the standard betulinic acid and the unknown sample. The spot for betulinic acid in the sample was confirmed by comparing the R_F and spectra of the spot with that of the standard. The peak purity of betulinic acid was assessed by comparing the spectra at three different levels, i.e. peak start, peak apex and peak end positions of the spot.

Method Validation

The method was validated for precision, accuracy [10] and repeatability. Instrumental precision was checked by repeated scanning of the same spot 100 and 600 ng five times and was expressed as coefficient of variance (% RSD). Method precision was studied by analysing the standards 100 and 600 ng per spot under the same analytical procedure and lab conditions on the same day and on different days (inter-day precision) and the results were expressed as % RSD. Accuracy of the method was tested by performing the recovery studies of the pre-analysed sample with standard at three levels (24.4, 30.6 and 36.7 μg mL^{-1}), % recovery and average % recovery were calculated.

Results and Discussion

TLC Separation Optimization

Different compositions of the mobile phase were tested and the desired resolution of betulinic acid with symmetrical and reproducible peaks was achieved by using a mobile phase of hexane:ethyl acetate:acetic acid (7:3:0.03 *v/v*) with 30 min of chamber saturation with the mobile phase and 18 min of development. A peak corresponding to betulinic acid was seen at R_F 0.60. The methanolic extract of the roots of *N. macrospermum* when subjected to TLC as per the methodology described above, showed the presence of betulinic acid peaks. A comparison of the spectral characteristics of the peaks for standard betulinic acid and that of the sample revealed the identity of betulinic acid present in the sample. It can be seen from Fig. 1 that good separation can be achieved by the conditions described above. Peak purity test of betulinic acid was done by comparing its UV–visible spectra in standard and sample track.

Table 1. Method validation parameters for quantification of betulinic acid using proposed TLC densitometric method

(a) Lineartity regression data

Sl no.	Parameter	Results
1	R_F	0.60
2	Dynamic range (ng $spot^{-1}$)	100–600
3	Equation	$Y = 39.366 + 12.165x$
4	Slope	12.165
5	Intercept	39.366
6	Limit of detection	15.24 ng
7	Limit of quantification	50.82 ng
8	Linearity (correlation coefficient)	0.998
9	Specificity	Specific

(b) Precision studies data

Concentration (ng $spot^{-1}$)	Instrumental precision (% RSD)	Method precision (% RSD)	
		Intra-day	Inter-day
100	1.89	1.59	2.0
600	1.38	1.32	0.86

(c) Recovery studies of betulinic acid

Sl no.	Amount of betulinic acid present in the sample (μg)	Amount of betulinic acid added (μg)	Amount of betulinic acid found (μg)	Recovery (%)
1	30.6	24.4	57.9	98.99–105
2	30.6	30.6	60.9	
3	30.6	36.7	66.6	

System Suitability Test

Linearity and Detection Limit

Linearity was checked by applying standard solutions of betulinic acid at six different concentration levels. The calibration curve was drawn in the concentration range of 100–600 ng $spot^{-1}$. The equation for the calibration curve of betulinic acid is $Y = 39.366 + 12.165x$ and the correlation coefficient of the calibration plot was 0.998 indicating good linearity.

Results of regression analysis on the calibration curve and detection limits are presented in Table 1a.

Precision Studies

Instrumental precision was checked by repeated scanning of the same spots (100 and 600 ng $spot^{-1}$) of standard betulinic acid five times and the RSD values were 1.89 and 1.38 for 100 and 600 ng $spot^{-1}$, respectively. To determine the precision of the developed assay method 100 and 600 ng $spot^{-1}$ of the betulinic acid standard was analysed five times within the same day to determine the intra-day variability. The RSD values were 1.59 and 1.32 for 100 and 600 ng $spot^{-1}$, respectively. Similarly the inter-day precision

was tested on the same concentration [illegible] levels on 2 days and the RSD values were 2.0 and 0.86, respectively (Table 1b).

Sample Analysis and Recovery Studies

This developed TLC method was subsequently applied for the analysis of betulinic acid in methanolic extract of *N. macrospermum* and the free betulinic acid content of the roots by this proposed method was found to be 0.030%.

For the examination of recovery rates, 80, 100 and 120% of pure betulinic acid were added in pre-analysed samples and quantitative analysis was performed. The recoveries were between 98.99 and 105% (Table 1c).

Conclusion

The TLC method developed here for the quantification of betulinic acid in [illegible] simple, rapid, cost-effective and easily adaptable for the screening and quantitative determination than any other analytical technique.

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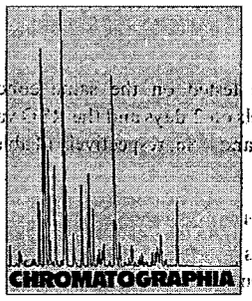
Quantification of β-Sitosterol in *Mucuna pruriens* by TLC



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Abstract

Mucuna pruriens Linn. one of the popular and important medicinal plants of India is a constituent of more than 200 indigenous drug formulations. β-Sitosterol is one of the most prevalent phytosterols which is ubiquitous throughout the plant kingdom. A sensitive, selective and precise thin-layer chromatographic method has been developed and validated for the analysis of β-sitosterol in *Mucuna pruriens* roots. Separation and quantification was achieved by TLC using ternary mobile phase of toluene: chloroform; methanol (4:4:1 *v/v*) (R_F 0.55) on precoated silica gel $60F_{254}$ aluminium plates and densitometric determination was carried out after derivatization with anisaldehyde-sulphuric acid reagent in reflection/absorption mode at 527 nm. The calibration curve was linear in the concentration range of 100–600 ng spot^{-1}. The method was validated for precision, repeatability and accuracy. The proposed method was found to be simple, precise, specific, sensitive and accurate for the quantification of β-sitosterol.

Keywords

Thin layer chromatography
β-Sitosterol quantification
Mucuna pruriens

Introduction

Mucuna pruriens one of the popular and important medicinal plants indigenous to tropical countries like India is a constituent of more than 200 indigenous drug formulations. It is commonly known as common Cowitch, Velvet bean and Cowhage and belongs to family Leguminoseae [1].

Mucuna finds traditional use in a number of diseases. Roots are used in the treatment of nephropathy, strangury, dysmenorrhoea, amenorrhoea, elephantiasis, dropsy, neuropathy, ulcers, and fever and as febrifuge and tonic. Leaves are aphrodisiac, tonic, and are useful in ulcers, inflammation, helminthiasis, cephalalgia and general debility. Seeds are used in snakebite, sexual debility, cough, tuberculosis, impotence, rheumatic disorders, muscular pain, gonorrhea, sterility, gout, delirium, dysmenorrhea, diabetes, and cancer [2, 3].

Preliminary phytochemical screening, TLC fingerprinting and co-TLC studies (with β-sitosterol) of *Mucuna* roots revealed the presence of a fairly high content of phytosterols and an identical spot as that of standard β-sitosterol was observed. Further it was confirmed by RF comparison, multi wavelength scanning and spectral overlay.

β-Sitosterol is one of the most prevalent vegetable-derived phytosterols, which is found in numerous plants including rice, wheat, corn, nut, peanut, etc. It is structurally related to cholesterol [4]. β-Sitosterol has an amazing array of scientifically acknowledged benefits for key areas of health in immune dysfunctions, inflammatory disorders and rheumatoid arthritis [5], hypercholesterolemia [6], breast cancer [7], colon cancer [8] and benign prostatic hypertrophy [9, 10]. Previously, β-sitosterol has been quantified by liquid chromatography and tandem mass

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spectrometry using atmospheric pressure photoionization (APPI–LC–MS–MS) [11] while there are other reports using liquid chromatography (LC) with evaporative light scattering detection (ELSD) [12] online liquid chromatography–gas chromatography (LC–GC) [13] and gas chromatography [14].

Hence considering its wide therapeutic applications, alternative quantification techniques and, as one of the marker constituent to ensure identity and quality of this plant a simple, sensitive, specific and reproducible TLC method was developed for the quantification of β-sitosterol in *Mucuna pruriens* roots.

Experimental

Reagents and Chemicals

Pure β-sitosterol was obtained from Acros Organics (NJ, USA), other solvents and chemicals were of analytical grade and TLC plates silica gel $60F_{254}$ (20 cm × 20 cm) were purchased from E. Merck (Darmstadt, Germany).

Plant Material

Roots of *Mucuna pruriens* were collected from the outfield of Vadodara city, Gujarat, India and were authenticated from Botanical Survey of India, Southern Circle, Coimbatore. A voucher specimen (No.Pharmacy/MP/05-06/05/KM) has been deposited in the Pharmacy Department of The M. S. University of Baroda, Vadodara, India.

Preparation of Crude Extract

Accurately weighed 5 g of the coarse powder of *Mucuna pruriens* roots were extracted separately with methanol (4 × 50 mL) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated, and transferred to a 25-mL volumetric flask and the volume was made up with methanol.

Solutions

A stock solution of β-sitosterol (100 μg mL^{-1}) was prepared by dissolving 1 mg of accurately weighed sitosterol in methanol and making up the volume of the solution to 10 mL with methanol.

Chromatography

A Camag TLC system equipped with Camag Linomat V an automatic TLC sample spotter, Camag glass twin trough chamber (20 × 10 cm), Camag scanner 3 and integrated winCATS 4 Software were used for the analysis. TLC was performed on a pre-coated TLC plate silica gel $60F_{254}$ (20 cm × 20 cm). Samples and standards were applied on the 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 were 15 mm of the plate. The linear ascending development was carried out in a Camag twin trough chamber (20 cm × 10 cm) which was presaturated with 20 mL mobile phase toluene: chloroform: methanol (4:4:1 *v*/*v*) 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 in current air with the help of an hair dryer. The post chromatographic derivatization was carried out in anisaldehyde–sulphuric acid followed by heating at 110 °C for 3 min [15]. Quantitative evaluation of the plate was performed in absorption-reflection mode at 527 nm, using a slit width of 6 × 0.45 mm and data resolution 100 μm step and scanning speed 20 mm s with a computerized Camag TLC scanner-3 integrated with winCATS 4 software. Quantification of β-sitosterol in the extract of *Mucuna* root was performed by external standard method, using pure β-sitosterol as standard.

Calibration Curve for β-Sitosterol

Stock solution of β-sitosterol (100 μg mL^{-1}) was prepared in methanol and different amounts (100–600 ng $spot^{-1}$) were applied on a TLC plate, using Linomat V for preparing six point calibration graph of peak area versus concentration.

Quantification of β-Sitosterol in Test Sample

Five microliters of sample solution was applied in triplicate on a TLC plate, developed and scanned as above. Peak areas were recorded and the amount of β-sitosterol was calculated using the calibration plot.

Specificity

Specificity of the method was determined by analyzing sample of standard β-sitosterol and the unknown sample. The spot for β-sitosterol in the sample was confirmed by comparing the R_F and spectra of the spot with that of the standard. The peak purity of β-sitosterol was assessed by comparing the spectra at three different levels, i.e., peak start, peak apex and peak end positions of the spot.

Method Validation

The method was validated for precision, accuracy and repeatability [16]. Instrumental precision was checked by repeated scanning of the same spot 200 and 600 ng three times and was expressed as coefficient of variance (%RSD). Method precision was studied by analyzing the standards 200 and 600 ng $spot^{-1}$ under the same analytical procedure and lab conditions on the same day and on different days (inter-day precision) and the results were expressed as % RSD. Accuracy of the method was tested by performing the recovery studies of preanalyzed sample with standard at three

Table 1. Method validation parameters for quantification of β-sitosterol by densitometric method

Linearity regression data		
Sl No.	Parameter	Results
1	R_F	0.55
2	Dynamic range (ng spot^{-1})	100–600
3	Equation	$Y = 59.708 + 9.816x$
4	Slope	9.816
5	Intercept	59.708
6	Limit of detection	5.55 ng
7	Limit of quantification	18.5 ng
8	Linearity (correlation coefficient)	0.9998
9	Specificity	Specific

Precision studies data			
Concentration (ng spot^{-1})	Instrumental precision (% RSD)	Method precision (% RSD)	
		Intra-day	Inter-day
200	0.46	0.78	0.69
600	0.56	0.55	0.60

Recovery studies of β-sitosterol				
Sl No.	Amount of β-sitosterol in the sample (μg)	Amount of β-sitosterol added (μg)	Amount of β-sitosterol found (μg)	Recovery (%)
1	76	60.8	140.4	102.63
2	76	76.0	150.6	99.07
3	76	91.2	168.0	100.47

levels (136.8, 152 and 167.2 μg mL^{-1}), and % recovery was calculated.

Results and Discussion

TLC Separation Optimization

Different compositions of the mobile phase were tested and the desired resolution of β-sitosterol with symmetrical and reproducible peaks was achieved by using mobile phase of toluene: chloroform: methanol (4:4:1 *v*/*v*) with 20 min of chamber saturation with the mobile phase and 10 min of development. A peak corresponding to β-sitosterol was seen at R_F 0.55. The methanolic extract of the roots of *Mucuna*, when subjected to TLC as per the methodology described above, showed the presence of β-sitosterol peaks. A comparison of the spectral characteristics of the peaks for standard β-sitosterol and that of the sample revealed the identity of β-sitosterol present in the sample and a good separation can be achieved by the conditions described above. Peak purity test of β-sitosterol was done by comparing its UV-visible spectra in standard and sample track.

System Suitability Test

Linearity and Detection Limit

Linearity was checked by applying standard solutions of β-sitosterol at six different concentration levels. The calibration curve was drawn in the concentration range of 100–600 ng spot^{-1}. The equation for the calibration curve of β-sitosterol is $Y = 59.708 + 9.816x$ and the correlation coefficient of the calibration plot was 0.9998 indicating good linearity.

Results of regression analysis on calibration curve and detection limits are presented in Table 1.

Precision Studies

Instrumental precision was checked by repeated scanning of the same spots (200 and 600 ng spot^{-1}) of standard β-sitosterol three times and the RSD values were 0.46 and 0.56 for 200 and 600 ng spot^{-1}, respectively. To determine the precision of the developed assay method 200 and 600 ng spot^{-1} of β-sitosterol standard was analyzed three times within the same day to determine the intra-day variability. The RSD values were 0.78 and 0.55 for 200 and 600 ng spot^{-1}, respectively. Similarly, the inter-day precision was tested on the same concentration levels on two days and the RSD values were 0.69 and 0.60, respectively (Table 1).

Sample Analysis and Recovery Studies

This developed TLC method was subsequently applied for the analysis of β-sitosterol in the methanolic extract of *Mucuna pruriens* rooots. The β-sitosterol content of the roots by this proposed method was found to be 0.076%.

For the examination of recovery rates, 80, 100 and 120% of pure β-sitosterol were added to preanalyzed sample and quantitative analysis was performed. The recoveries were between 99.07 and 102.63% (Table 1).

Conclusion

The TLC method developed here for the quantification of β-sitosterol in *Mucuna pruriens* roots is simple, rapid, cost-effective and easily adaptable for

screening and quantitative determination than any other analytical technique.

Acknowledgments

One of the authors, Mr. Krishna Murthy, would like to thank the University Grants Commission, New Delhi, for providing the financial assistance to carry out this work.

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