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Validated HPTLC method for qualitative and quantitative estimation of oleanolic acid in roots of *Cissampelos pareira* Linn. var. *hirsuta*, Menispermaceae

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ABSTRACT

An approach to develop a high performance thin layer chromatography (HPTLC) method in the present study has been made for quantitative estimation of marker compound oleanolic acid in the roots of Cissampelos pareria Linn. var hirsuta (family Menispermaceae). Oleanolic acid was estimated in saponin rich fraction prepared from the powdered roots of C. pareira. Oleanolic acid was separated using silica gel 60 F_{254} HPTLC plates using toluene: ethyl acetate: formic acid (7:3:0.3, %v/v) as mobile phase. The determination was carried out using densitometric absorbance-reflection mode at 530 nm for oleanolic acid in visible mode. The concentration of oleanolic acid in the saponin rich fraction was found to be 0.1316 % mg/mg. The method was validated in terms of linearity, accuracy, precision and specificity according to ICH guideline.

Keywords: *Cissampelos pareira* Linn. var. *hirsuta*, oleanolic acid, HPTLC, validation.

INTRODUCTION

WHO (world health organization) estimates that 80% of the world population currently uses herbal drugs for major healthcare [1]. Medicinal herbs as potential source of therapeutic aids has attained a significant role in health care system all over the world for human beings not only in the diseased condition but also as potential material for maintaining proper health [2]. Quality control of herbal medicines is highly desired in order to make sure the safe use of these medicines and to ensure their authenticity, stability, consistency because of the fact that sufficient bioactive phytochemical content is crucial for therapeutic purpose. The WHO and herbal pharmacopoeia has introduced chromatography for standardization of plant products and is accepted as a strategy for identification and evaluation of the quality of plant medicines [3]. HPTLC provides a simple, fast, efficient and reliable alternative for quantitative determination of natural products. An advantage of HPTLC over other chromatographic technique is several samples can be analyzed simultaneously by eluting with small quantity of mobile phase. This reduces the time and cost of analysis. The present study was undertaken to develop and validate HPTLC method for estimation of bebeerine and oleanolic acid in roots of *Cissampelos pareira* Linn.

Cissampelos pareira Linn. var. *hirsuta* is very variable, lofty, slender, dioecious, perennial climber, commonly distributed throughout tropical and subtropical India, ascending upto an altitude of 2000 m. The roots are known traditionally for treatment of kidney gravels, fever, dysentery, skin eruptions, heart troubles, burning, itching,

vomiting and asthma. Literature survey revealed that the plant contains saponin (oleanolic acid). Oleanolic acid is a triterpenoid compound which exists in natural plants in the form of free acid or aglycone for triterpenoid saponins [4, 5, 6, 7]. Extensive pharmacological studies on the oleanolic acid extracted from plant have proved to have anti-inflammatory, hepatoprotection, antitumor and antihyperlipideamic activity [8]. Hence study was carried out for identification and quantification of oleanolic acid in roots extract using HPTLC. The developed method was validated in terms of system suitability, specificity, linearity, LOD, LOQ according to ICH guidelines.

MATERIALS AND METHODS

Equipment

A CAMAG HPTLC system: CAMAG Linomat V sample applicator fitted with a 100 µl Hamilton syringe, CAMAG- twin trough chamber, CAMAG TLC Scanner 3 with D2 and Hg lamp, Reprostar and winCATS Planar Chromatography Manager and CAMAG integration software and TLC viewing cabinet (all from CAMAG, Muttenz, Switzerland)

Reference compounds and chemicals

Reference standard oleanolic acid (O5504, ≥97% purity) was obtained from Sigma Aldrich Ltd. (Mumbai, India). Toluene, ethyl acetate, formic acid, n-butanol, potassium hydroxide, methanol, anisaldehyde, sulphuric acid (analytical grade solvents) and charcoal were obtained from S.D fine chemicals (Mumbai, India). Precoated silica gel 60 F_{254} HPTLC aluminium plates (10×10 cm, 0.2 mm layer thickness, 5-6 µm particle size; E. Merck, Darmstadt, Germany) were obtained from E. Merck Ltd. (Mumbai, India).

Plant material

Fresh roots were collected from fully grown flowering plants of *Cissampelos pareira* Linn. var *hirsuta* from New Vallabh Vidyanagar, Dist- Anand, Gujarat, India. The plant was identified and authenticated by Senior Scientist, National Research Centre for Medicinal and Aromatic Plants, Boriavi, Anand, Gujarat. The voucher specimen was submitted for future reference (VGP/CP-1/5/ARGH-09). The roots were dried under shade, powdered with the aid of an electrical grinder, stored in an airtight container and used for HPTLC studies.

Preparation of standard oleanolic acid solution:

A stock solution of oleanolic acid was prepared by dissolving 10 mg in methanol (1000 µg/ml) and was further diluted with methanol to give a standard solution of oleanolic acid (100 µg/ml).

Preparation of saponin rich fraction of roots of *C. pareira*

20 gm of powdered roots were extracted with methanol by using Soxhlet's extractor for 6 hours. The methanolic extract obtained was concentrated using rotary vacuum evaporator and refluxed with n- butanol for 2 hours. n-butanol soluble constituents were separated by filtration followed by washing with distilled water, alkali (2% potassium hydroxide) and distilled water again. The n- butanol layer was evaporated and dried. The dried residue was dissolved in water and boiled with charcoal, filtered and filtrate was further dried to give saponin rich fraction. 25 mg of saponin rich fraction was accurately weighed and dissolved in 5 mL of methanol (5000 µg/mL). The solution was filtered through 0.45 µm membrane filter [9, 10].

Chromatographic conditions

The HPTLC analysis was performed using precoated silica gel 60 F_{254} aluminium backed plates in temperature controlled laboratory (22° C and 55% humidity). Linomat V autosampler was used for spotting of standard (oleanolic acid 100 to 500 ng/spot) and sample solution (saponin rich fraction 25µl), operated with settings of a band length 6 mm; distance between bands, 5mm; distance from the plate edge, 10 mm; and distance from the bottom of the plate, 10 mm. The plate was developed to a distance of 80 mm in a CAMAG twin trough chamber presaturated with mobile phase toluene: ethyl acetate: formic acid (7:3:0.3, v/v) for 15 minutes. The developed plate was air dried and scanned for densitometry measurement, spectra recording and data processing. The absorbance/ reflectance measurement mode was used at a scan speed of 20 mm/s. Zones of standard oleanolic acid were scanned from 400 to 600 nm to record their U.V. spectra and for obtaining the wavelength of maximum absorption. Densitogram was recorded at the wavelength of maximum absorption of oleanolic acid followed by recording of U.V. spectra of the respective bands. The plate of oleanolic acid was photographed in the visible mode after derivatising with anialdehyde sulphuric acid reagent using CAMAG Reprostar instrument. The peak areas were recorded for all

concentrations. Calibration curve of standard was plotted as, peak area versus concentration of standard (oleanolic acid, ng/spot) applied in triplicate.

Validation of method

The method was validated according to International Conference on Harmonization guidelines for parameters such as linearity, limit of detection, limit of quantification, precision, accuracy and specificity [11].

Linearity was studied by applying different aliquots of standard stock solution (oleanolic acid 100 to 500 ng/spot). The calibration curve was developed by plotting peak area versus concentrations with the help of the winCATS software. The areas of peaks were treated by least square linear regression analysis.

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the standard deviation (S.D.) of densitometric response and slope of curve (S) using the following equation;

LOD = 3.3 (S.D. / S) …….. (1)

LOQ = 10 (S.D. / S) …….. (2)

The intermediate precision of the method was studied by analyzing aliquots of standard in triplicate at three concentration levels for oleanolic acid on the same day for intraday precision and on different days with freshly prepared samples to determine interday precision. The results were expressed as relative standard deviation (RSD). Repeatability of the HPTLC instrument was assessed by applying the same sample solution 6 times on a plate with the Linomat V using the same syringe and by taking 6 scans of the sample spot for oleanolic acid.

The accuracy of the method was determined from recovery studies at three different levels in triplicate by spiking with various concentrations of standard solution. The recovery of each spiked solution was calculated.

The specificity of the method was ascertained by determining the peak purity of the component by comparing overlain UV spectra of oleanolic acid in the sample extract with the absorption spectra of reference standard at the start, middle and end positions of the bands.

Statistical analysis

Statistical analysis was performed by using Microsoft Excel 2007 which includes computation of linear regression analysis, mean, standard deviation, relative standard deviation.

RESULTS AND DISCUSSION

Different proportions of toluene, ethyl acetate and formic acid was used for separation of different constituents. Well resolved symmetric bands were obtained using precoated HPTLC plates with toluene: ethyl acetate: formic acid (7:3:0.3, v/v) as mobile phase [12, 13]. Oleanolic acid gave purple color with anisaldehyde sulphuric acid reagent in visible mode. Standard oleanolic acid (R_f 0.56) showed single peak in HPTLC chromatogram (Figure 1). Calibration curve was prepared by plotting concentration of standard (oleanolic acid) versus area of the peak (Figure 2).

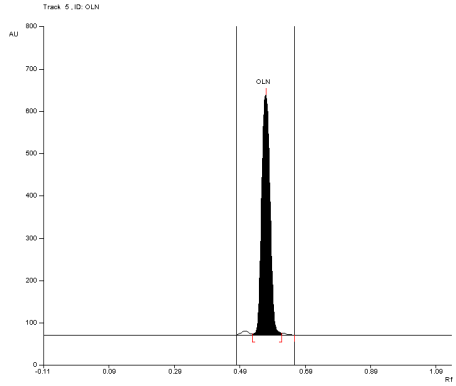

Figure 1: Chromatogram of standard oleanolic acid

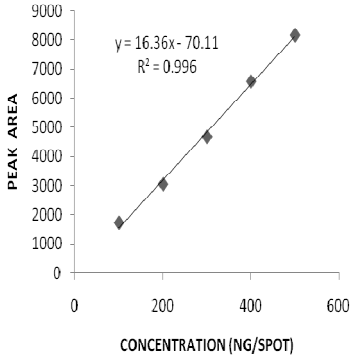

Figure 2: Calibration curve of standard oleanolic acid

Determination of marker in extract

The content of oleanolic acid was calculated by linear regression and mean percentages were calculated from six replicate experiments. Saponin rich fraction was found to contain 0.1316% mg/mg of oleanolic acid. Furthermore, saponin rich fraction showed peak in the chromatogram at same R_f value as standard oleanolic acid (0.56) (Figure 3).

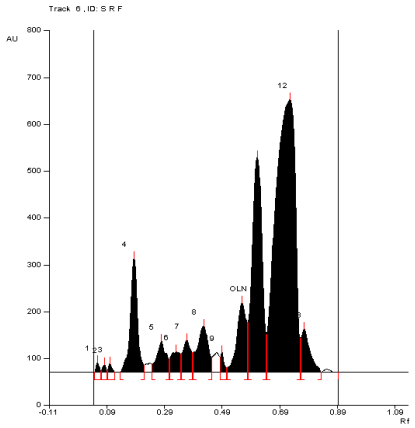

Figure 3: Chromatogram of saponin rich fraction of roots of *C. pareira*

Method validation

The developed HPTLC method for estimation of oleanolic acid had been validated using following parameters according to ICH guideline.

Linearity

Linearity of oleanolic acid was found between the concentration ranges of 100 to 500 ng/spot, with acceptable correlation coefficient 0.996. It exhibits good linearity between concentration and peak area. (Figure 2). Table 1 indicates method validation parameters.

Table 1: Method validation parameters for estimation of oleanolic acid

Parameters	Oleanolic acid
Wavelength, nm	530
Linearity range	100-500 ng/spot
Regression equation	Y=16.24x-52.57
Correlation coefficient	0.996
Limit of detection	4.52 ng/spot
Limit of quantification	13.96 ng/spot
Specificity	Specific
Repeatability of application, % RSD	1.25
Repeatability of measurement, % RSD	1.33

Limit of Detection and Limit of Quantitation

The limit of detection and limit of quantification was found to be 4.52 ng/spot and 13.96 ng/spot, respectively.

Precision

The proposed method was found to be precise as indicated by intermediate precision studies expressed as percent RSD (Relative Standard Deviation) for intra-day and inter-day as shown in Table 2.

Table 2: Intermediate precision studies for oleanolic acid

Marker	Concentration (ng/spot)	Concentration (ng/spot)	Intraday	Concentration (ng/spot)	Interday
		Mean	%RSD	Mean	%RSD
Oleanolic acid	200	202.407	0.644	200.35	0.294
Oleanolic acid	300	300.623	0.629	200.35	0.186
Oleanolic acid	400	402.497	0.659	401.713	0.370

Accuracy

The accuracy of the method was determined from recovery studies. After spiking with varying amount of standard oleanolic acid and analyzing according to the procedure, it afforded recovery of oleanolic acid in the range of 98.71%-100.30%, at three concentration levels. The results are as presented in Table 3.

Table 3: Recovery studies of oleanolic acid in saponin rich fraction

Amount of sample powder (mg)	Amount of oleanolic acid in sample (µg)	Amount of standard oleanolic acid added (µg)	Total amount of oleanolic acid taken (µg)	Total amount of oleanolic acid found (µg)	Percent Recovery (%)
26	34.22	2	36.22	36.11	99.6963
27	35.54	4	39.54	39.03	98.71017
28	36.85	6	42.85	42.98	100.3034

Specificity

The identity of the bands in the sample extracts were confirmed by comparing the R_f and the overlain UV absorption spectra with those of their respective standard. The absorption spectra of standard oleanolic acid (R_f 0.56) and the corresponding spots present in extracts matched exactly, indicating no interference by the other plant constituents (Figure 4).

The purity of the bands due to oleanolic acid in the sample extract was confirmed by overlaying the absorption spectra recorded at start, middle and end position of the band in the sample tracks (Figure 5)

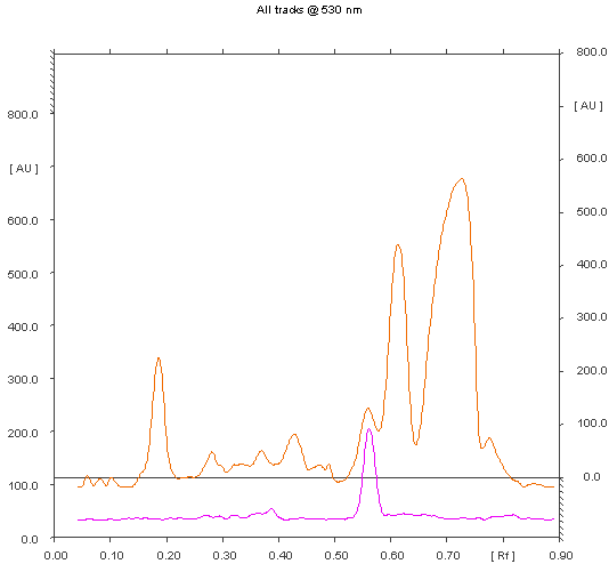

Figure 4: TLC densitograms of saponin rich fraction of roots of *C. pareira* with oleanolic acid standard scanned at 530 nm

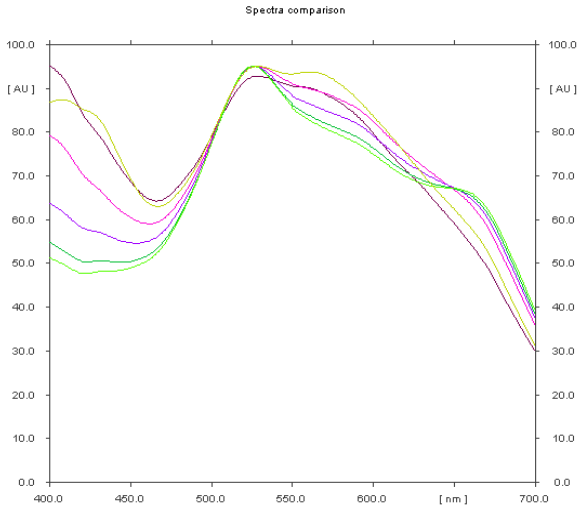

Figure 5: Overlain UV spectra of standard oleanolic acid and saponin rich fraction scanned at 530 nm

CONCLUSION

The proposed HPTLC method was found to be simple, fast, accurate, precise and sensitive for quality control of the raw material. Various other phytoconstituents did not interfere in the estimation of the marker. The method was reliable and suitable since the recovery value of oleanolic acid was found to be 98.71%-100.30%. Since this method resolves and quantifies oleanolic acid effectively, it can be used for standardization of roots of *Cissampelos pareira* Linn.

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