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Development and Validation of HPTLC Method for Quantitative Estimation of Oleanolic acid as Marker in Total Methanolic extract of Fruits of *Randia dumetorum* Lamk.

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ABSTRACT

The objective of the present investigation was to develop a validated HPTLC method for the determination of oleanolic acid as marker in the Methanolic extract of fruits of *Randia dumetorum* Lamk. Analysis of oleanolic acid was performed on TLC aluminium plates pre-coated with silica gel 60F-254 as the stationary phase. The mobile phase consists of Toluene: Ethyl acetate: Glacial acetic acid (7:3:0.1 v/v/v). Linear ascending development was carried out in twin trough glass chamber. The plate was sprayed with 10% sulphuric acid, heated at 110°C and immediately scanned at 540nm using Camag TLC scanner III. The system was found to give compact spots for oleanolic acid (R_f value of 0.58 ± 0.01). The linear regression analysis data for the calibration plots showed good linear relationship with r^2 = 0.9922 ± 0.0002 in the concentration range 50-500ng per spot. The mean value (± S.D) of slope and intercept were 5.989 ± 0.0491 and 211.547 ± 4.5092 respectively. According to ICH guidelines the method was validated for precision, recovery, robustness and ruggedness. The limits of detection and quantification were 10 ng/spot and 30 ng/spot respectively. The oleanolic acid content of methanolic extracts was 3.45%. Recovery values from 99.38 – 100.79 % showed excellent reliability and reproducibility of the method. Statistical analysis of the data showed that the method is reproducible and selective. Since the proposed mobile phase effectively resolves oleanolic acid, the developed HPTLC method can be applied for identification and quantification of oleanolic acid in herbal extracts and formulations.

KEYWORDS: HPTLC, Oleanolic acid, *Randia dumetorum*

INTRODUCTION

The art of herbal medicine is extremely ancient, probably predates modern Homo sapiens. Traditional medicine is an evolutionary process as communities and individuals continue to discover new techniques to heal the aliment that can renovate into medical practices (1). The WHO estimates that about three-quarters of the world's population currently use herbs and other forms of traditional medicines to treat their diseases.

Most of the herbal extracts are made from crude herbs; they can vary in percentage of active constituents, which further influences the therapeutic activity of herbs. A chemical marker is a particular phytochemical, invariably presents in the plant or plant part and is the main source of determining its identity as well as its efficacy. Standardization for the level of active components or key biological markers, is the only real means to assure an appropriate delivery of an effective dosage for the user. Correct identification and quality assurance of the starting material is therefore, an essential prerequisite to ensure reproducible quality of herbal medicine, which contributes to its safety and efficacy (2). The utilization of modern scientific methods in exploring possibilities of obtaining better medicaments from the traditional system has therefore become an important task in herbal medical

research (3). In the past few years, HPTLC is emerging as a powerful tool for qualitative and quantitative analysis. HPTLC provides a chromatographic fingerprint. It is therefore suitable for monitoring the identity and purity of drugs and for detecting adulteration and substitutions.

In Indian system of medicine *Randia dumetorum* Lamk. (Rubiaceae) is an important medicinal plant and popularly known as emetic nut. It is found in waste places & jungles all over India, extending northwest to the Bias river & ascending to outer Himalaya to 4000 ft. Ceylon, Java & South China. Also found in Gonda, Garhwal & Baraitch division of Oudit forest (4–5). Literature survey reveals that the fruit is emetic, purgative, carminative, alexiteric, anthelmintic, abortifacient, antipyretic; cures abscess, ulcers, inflammations, wounds, tumors, skin diseases, piles and have antibacterial activity (6). It contains triterpenoidal saponins (2–3%) in fresh & 10% in dried fruit. They are mostly concentrated in pulp. A mixture of two saponin Randia or neutral saponin (mp-289-90°C) & Randia acid (mp-260°C) which occurs at all stage of ripening. These all saponins yield oleanolic acid as sapogenin on hydrolysis (7). In later investigation urosaponin was also isolated (8). It also contains essential oil, veleric acid, tannins and resin. There are no chromatographic methods available for quantitative estimation of oleanolic acid in extract of *R. dumetorum* and different marketed formulation. Today HPTLC is rapidly becoming a routine analytical technique due to its advantages of low operating costs, high sample throughput and need for minimum sample preparation. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase-unlike HPLC thus reducing the analysis time and cost per analysis (9). Therefore the extract was subjected to HPTLC analysis by developing a method for quantification of oleanolic acid. The proposed method was validated according to the ICH guidelines (10–11).

MATERIAL AND METHODS

Equipment:

A Camag HPTLC system equipped with a sample applicator Linomat V, Twin trough plate development chamber, Camag TLC Scanner III and Wincats an integration software 4.02 (Switzerland).

Materials:

Standard oleanolic acid was a gift sample from PERD Research Centre, Ahmadabad, India. All the chemicals used in the experiments are of analytical grade (Merck, India). TLC aluminum plates pre-coated with silica gel 60 F 254 (10× 10 cm, 0.2 mm thick) used were obtained from E. Merck KGaA Ltd (Mumbai, India).

Plant material:

Fruits of *Randia dumetorum* were collected during November from Botanical garden of M.S.U. Baroda and were identified by Head of Botany department, M. S. University, Baroda. A voucher specimen has been deposited in the museum of department of Pharmacognosy, M.S.U., Baroda. Voucher specimen (PH-805) was deposited in the herbarium of Pharmacy Department of M.S.U., Baroda.

Plant extract:

The fruits were dried in sunlight and reduced to a coarse powder. The powdered materials were subjected to qualitative tests for the identification of various phytoconstituents like alkaloids, glycosides, steroids, terpenoids and flavanoids. Then the powder was subjected to soxhlet extraction with methanol for 72 hours at a temperature of 50–60°C. The extract was concentrated and the solvent was completely removed. They were freeze dried and stored in the vacuum dessicator until further use.

Preparation of Standard solution:

Standard stock solution of oleanolic acid was prepared by dissolving 5 mg of standard oleanolic acid in 10 ml of Chloroform: Methanol (1:1). From this, working standard stock solution of concentration 50 ng/µl was prepared and 1, 2, 4, 6, 8, and 10 µl of this solution were applied using LINOMAT 5 applicator with the band width of 8 mm., which gave different concentration 50, 100, 200, 300, 400 and 500 ng/spot respectively on HPTLC plate (10× 10 cm, 0.2 mm thick silica gel 60 F 254 Merk KGaA by CAMAG).

Preparation of Sample solution:

10 mg of dried methanol extract was dissolved in 10 ml of mixture of equal volume of Methanol and Chloroform (5:5) (1000 µg/ml). From this 5 and 10 µl of the solution was applied to the plate with the band width of 8 mm.

Development:

The plate was developed in a twin tough chamber with the mobile system as Toluene: Ethyl acetate: Glacial acetic acid. (7: 3: 0.1). The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2°C) at relative humidity of 60% ± 5%. Ascending mode was used for development of thin layer chromatography.

Detection and Quantification:

Following the development the TLC plates were dried in a current of air with the help of an air dryer in a wooden chamber with adequate ventilation. The plate was sprayed with 10% sulphuric acid reagent, heated at 110°C for 10 minutes and immediately scanned and quantified at 540 nm using CAMAG TLC SCANNER -3. Data of peak area of each band were recorded. Calibration curve was obtained by plotting peak area Vs concentration and peak height Vs concentration of oleanolic acid and checked for reproducibility, linearity and validating the proposed method. The correlation coefficient, coefficient of variance and the linearity of results were calculated. Spectra of samples and standard oleanolic acid were matched.

RESULTS AND DISCUSSION

Both standard and sample were tried in different solvent system. The mobile phase consisting of Toluene: Ethyl acetate: Glacial acetic acid. (7: 3: 0.1) gave better resolution. To improve the resolution of the spots, the plate was run in the same mobile phase twice.

Standard oleanolic acid showed single peak in HPTLC chromatogram (Fig. 1). The system was found to give compact spots for oleanolic acid (R_f value of 0.58 ± 0.01). After development the plate was scanned at 540 nm. The calibration curve was linear and prepared by plotting the different concentration of oleanolic acid (50ng – 500ng/spot) versus average area of the peak (Fig. 2). Peak area and concentration were subjected to linear regression analysis to calculate calibration equation and correlation coefficients. Methanolic extract of fruits was analyzed by the proposed method (Fig. 3). To identify the oleanolic band in the sample solution was confirmed by overlaying the UV absorption spectrum of the sample with that from the reference standard oleanolic acid, using Camag TLC Scanner. The amount of oleanolic acid was computed from calibration curve. The oleanolic acid content of Methanolic extracts was 3.45% (Table 1).

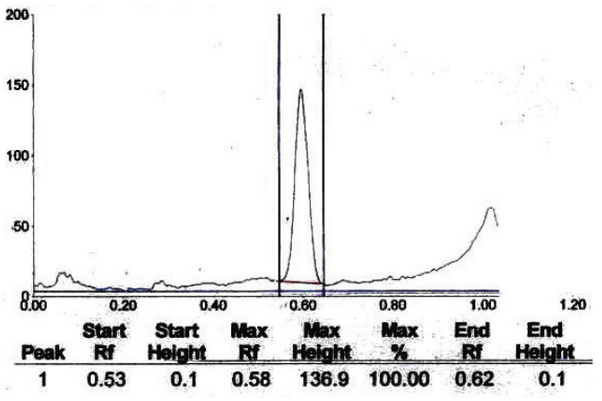

Figure 1: HPTLC chromatogram of Oleanolic acid (Standard)

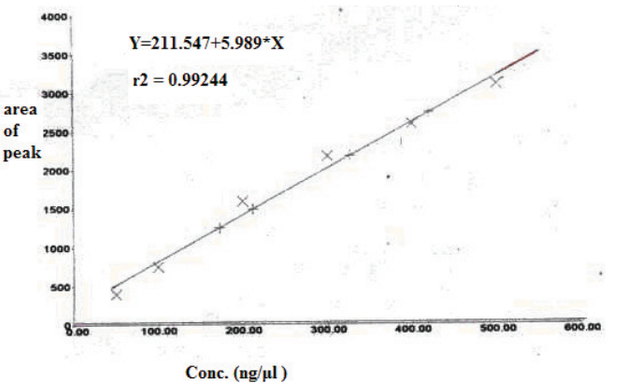

Figure 2: Calibration curve of Oleanolic acid.

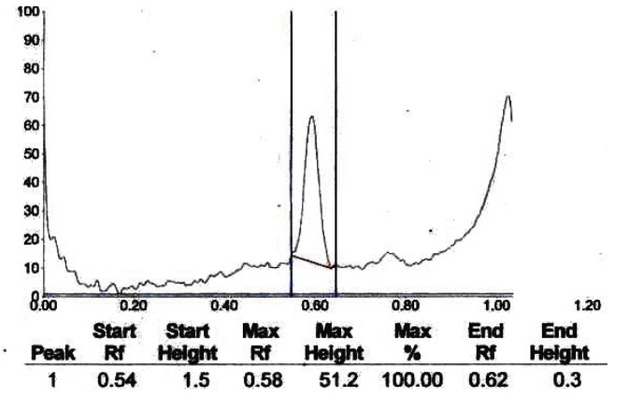

Figure 3: HPTLC chromatogram of *R. dumetorum* (sample)

Table 1: Average percentage content of oleanolic acid in Methanolic extract of dry fruit powder of *Randia dumetorum,* by the proposed HPTLC method

Sample	Weight of sample (ng)	Average* content of oleanolic acid found in sample	% RSD	Average percentage content of oleanolic acid
Dried methanolic extract of fruits of *R. dumetorum*	5000	172.55±0.27	0.16	3.45

*Average ± S. D. (n=6)

Validation of HPTLC method

1) Linearity

A calibration curve of standard oleanolic acid was obtained by plotting peak area of oleanolic acid against the different concentration. Stock solutions of oleanolic

Table 2: Regression analysis for calibration plots. (n=3)

Parameters	Values
Linearity Range	50ng/µl -500ng/µl
Coefficient of Determination (Height)	0.98995
Coefficient of Determination (Area)	0.99224
Regression equation (Height)	Y=12.113+0.262*X
Regression equation (Area)	Y=211.547+5.989*X
Slope (Height)	12.113
Slope (Area)	211.547
Intercept (Height)	0.262
Intercept (Area)	5.989

acid was prepared in equal volume of chloroform and methanol and different amounts 50, 100, 200, 300, 400 and 500 ng/spot of these were loaded onto a TLC plate, using ATS4 for preparing six points calibration curves. According to the area of peak, the regression equation and correlation coefficient were from calibration curves, Y = 211.547 + 5.989*X and r^2 = 0.99224. There was a good linear relationship between peak area and concentration in the range 50–500 ng per zone (Table 2). There was no significant difference between the slopes of standard plots (ANOVA, P > 0.05).

2) Accuracy

To the pre-analyzed sample, 100 and 300 ng of oleanolic acid was added and the mixture was analyzed by the proposed method. The experiment was conducted in triplicate to check recovery and accuracy of the system. The results are summarized in Table 3, showing the accuracy (expressed in recovery) of the method as the mean values of oleanolic acid. The % recovery of oleanolic acid was found to be 99.38 – 100.79, which are highly satisfactory.

3) LOD and LOQ

The LOD was found to be 10 ng/spot with signal/noise ratio of 3:1 while the LOQ was seen to be 30 ng/spot with signal/noise ratio of 7:1.

4) Precision

The repeatability of sample application and measurement of peak area have been expressed in terms of RSD%.

Table 4: Intra- and inter-day precision of HPTLC method. (n=6)

Amount of oleanolic acid (ng/spot)	Mean area of peak	S.D.	% RSD
Intra-day precision			
100	742.445	3.86	0.52
200	1580.435	5.23	0.33
Inter-day precision			
100	743.945	5.46	0.73
200	1579.718	7.98	0.51

Table 5: Robustness testing

Parameters	SD of peak areas	% RSD
Mobile phase composition	1.32	0.58
Time from spotting to chromatography	0.76	0.42
Time from chromatography to scanning	0.95	0.72

The results are depicted in Table 4 shows that there was no significant intra- and inter-day variation has been observed in the analysis of oleanolic acid at two different concentrations level 100 and 300 ng/spot.

5) Specificity

It was observed that other constituents present in the extract did not interfere either with the peak of oleanolic acid. Therefore the method was specific. The spectrum of standard oleanolic acid spot and spot present in the samples was found to be similar or overlap. Good correlation was (r^2=0.9922) also obtained between standard and overlay spectra of oleanolic acid.

6) Robustness of the method

The low values of % RSD obtained after introducing small changes in mobile phase composition indicated the robustness of the method (Table 5).

CONCLUSION

The developed HPTLC method was precise, specific, accurate and robust for determination of oleanolic acid in the extracts of fruits of *R. dumetorum*. The activity of plant extract is always influenced by the quantity of active principle present in the extract. Since fruits of *R.*

Table 3: Recovery studies

Amount of oleanolic acid present in Methanolic extract (ng)	Amount of oleanolic acid added (ng)	Average* amount of oleanolic acid recovered	Recovery (%)	% RSD
172.55	100	270.86±1.97	99.38	0.73
326.65	200	525.86±3.01	99.85	0.57
498.840	300	805.15±3.64	100.79	0.45

*Average ± S. D. (n=6)

dumetorum is used in various diseases, it is very essential to develop a standardization method from which one can optimize its quantity in extracts as well as its formulations. HPTLC densitometry is a rapid, reproducible, accurate, and selective alternative to HPLC for the separation of oleanolic acid in fruits of *R. dumetorum*. The recovery values of oleanolic acid were found to be about 99.38 – 100.79 %, which shows the reliability and suitability of the method. Running time and cost per analysis are relatively low in comparison with other methods.

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