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Validated HPTLC Method for Quantification of Variability in Content of Oleanolic Acid in Different Variety of *Lantana camara*.

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ABSTRACT

Oleanolic acid belongs to pentacyclic triterpenes and are common constituents of many medicinal herbs and plants including *Lantana camara*. They have many important pharmacological effects i.e., anti-inflammatory, hepatoprotective, anti-tumour, anti-HIV, anti-microbial, antifungal, anti-ulcer, gastroprotective, hypoglycemic and antihyperlipidemic properties. The literature reveals there is no high-performance thin-layer chromatographic method for determination of oleanolic acid in different varieties of *Lantana camara*. Thus a simple, sensitive, precise and accurate stability-indicating high performance thin-layer chromatographic method for analysis of pentacyclic triterpenoid "Oleanolic acid" (the main active constituent of *Lantana camara*) was done. The different parts aerial shoots and root parts of *Lantana camara* were well shade dried and powdered. The powdered material was extracted by using extraction method developed by Banik and Pandey (2009). The separation was achieved on TLC aluminum plates precoated with silica gel $60F_{254}$ using chloroform-methanol (95:5, v/v/) as a mobile phase. Densitometric analysis was performed at 530 nm. This system was found to have compact spot of oleanolic acid at R_F value of (0.5). For the proposed procedure, linearity show in the range 200-1000 ng ($r^2 = 0.99$), limit of detection (50 ng/spot), limit of quantification (170 ng/spot), recovery (100.43%) and precision (2.25%) were found to be satisfactory. Statistical analysis reveals that the content of oleanolic acid in different variety of *Lanana camara* varied significantly. Oleanolic acid was quantified in different parts of the *Lantana camara* plants varieties. It was found that oleanolic acid content in the roots was higher than the aerial shoots. The highest and lowest concentration of oleanolic acid in *Lantana camara* was found to be present in the wild variety i.e., yellow color flower variety followed by pink color flower variety and was present lowest in the cultivated variety yellow color flower variety followed by white color flower variety of *Lantana camara*. Thus, we can conclude that the wild yellow variety was the elite variety among the *Lantana camara*.

Key words: *Lantana camara*, HPTLC, oleanolic acid, chromatography

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INTRODUCTION

Lantana camara Linn. (Fam. Verbinaceae) is a hairy shrub native of tropical America. It is cultivated as an ornamental or hedge plant. Different parts of the plant are used in folklore remedies and traditional systems of medicine for the treatment of various human ailments. Three varities of *Lantana camara* viz. var aculeate, var mista, var nivea have been reported from India. Out of which *Lantana camara Var aculeate* found abundantly. Morphologically, it can be identified with flowers small, usually orange sometimes varying from white to red. In traditional medicine, it was reported to use against bilious fever, catarrhal affections, eczema eruptions, rheumetism, malaria and tetanus. The major secondary metabolites of this plant were reported mostly with Oleanone type pentacyclic triterpene skeleton with' oleanolic acid' as the major constituent[1]. Oleanolic acid is the predominant compound found in the stem and root parts of *Lantana camara*[2]. In the literature there are numerous data on their anti-inflammatory, hepatoprotective, anti-tumour, anti-HIV, anti-microbial, antifungal, anti-ulcer, gastroprotective, hypoglycemic and antihyperlipidemic properties[3,4,5,6]. They are relatively non-toxic and have been used in cosmetics and health products, e.g., oleanolic acid is marketed in China as an oral drug for human liver disorders[7]. The most explored method in the determination of these compounds is HPLC[8,9] but TLC is still an important tool of phytochemical investigations[10]. Its advantages are the low amount of organic solvent used in the separation process and possibility of application of samples without any pretreatment. The literature reveals there is no high-performance thin-layer chromatographic method for

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determination of oleanolic acid in *Lantana camara* plant powder. A sensitive, simple, rapid, economical, precise and accurate HPTLC method has therefore been established. The method can be used for phytochemical profiling of the plant and screening of the elite *Lantana camara* variety for the micropropagation technique, thus aiding identification of the plant material and quantification of oleanolic acid.

MATERIAL AND METHODS

Plant and its cultivation: The *Lantana camara* plant as shown in Fig. 1, available locally in Varanasi (25'18"N, 83'03"E) was collected and used for this study. Its identity was confirmed by taxonomists and voucher specimen (No. 010105) was kept stored in the School of Biochemical engineering, Institute of Technology, Banaras Hindu University, Varanasi, India. The plants were grown in the field. The experimental location experiences semi-arid tropical climate. The soil of experimental field was sandy loam texture; organic electrical conductivity 0.42 dS m^{-1}, available carbon 0.38 %, available nitrogen 180 kg ha^{-1}, available phoshorus 21 kg ha^{-1}, pH 7.3]. Plants used in the study were propagated by stem cuttings from a yellow variety (wild) *Lantana camara* , yellow variety (cultivated), Pink variety (wild) and white (cultivated) respectively. Stem cuttings were initially grown in medium size (20×20×20 cm) earthern pots filled with the field soil, kept under partial shade and regularly watered. One cutting was planted in each pot for rooting in the first week of August 2008. Healthy, profusely rooted, 30 days old cuttings were transplanted in the field in defined row spacing of 30 cm between plants. After one year plants were harvested from the field. The aerial and root parts of the plants were separated and the roots were washed with tap water, shade dried and kept in cellulose bags for the analysis.

Fig. 1: *Lantana camara* (wild Yellow) plant in natural condition

Extraction of oleanolic acid in different varieties of *Lantana camara*: The different parts aerial shoot and root parts of *Lantana camara* were well shade dried and powdered in a polarizer. The powdered material was extracted by using solid-solvent extraction method for oleanolic acid developed by Banik and Pandey[11](2009). The most suitable condition for extraction of oleanolic acid was found to be a single step extraction at extraction temperature 35°C, extraction time 55 min, solvent-solid ratio 55:1, mean particle size 0.5 mm and solvent composition 52.5% methanol in a methanol-ethyl acetate mixture. At these optimum extraction parameters, the maximum yield of oleanolic acid obtained.

A Preliminary TLC patterns of the different parts of extract was performed for the identification of oleaniolic acid by using aluminium backed plates precoated with 0.2 mm layers of silica gel 60 F_{254} and solvent system chloroform and methanol (95:5).

HPTLC condition: Chromatography was performed on aluminum HPTLC plates coated with silica gel 60 F_{254}. Before use, plates were pre-washed with methanol and dried in an oven at 105°C for 2 h. Standard (5 µL) and Samples (4 µL) were spotted as 6 mm bands, starting 15 mm from the edge of the plates, by means of a Camag Linomat IV sample applicator and the plates were developed to a distance of 90 mm above the position of sample application in a Camag twin-trough chamber previously equilibrated with mobile phase for 20 min. The mobile phase was Chloroform-methanol, 9.5+0.5 (v/v). After development, the plates were dried under a current of air at room temperature, derivatized with freshly prepared Anisaldehyde-sulphuric acid reagent in a derivatization chamber for 1.0 min and dried at room temperature. After drying, plates were heated in oven at 105°C for 10 min before densitometric scanning. Densitometric evaluation of the plates was performed at λ = 530 nm using a Camag Scanner III with tungsten lamp in conjunction with CATS III software for quantification. The wavelength used for densitometry was selected after acquiring the in-situ spectra of the oleanolic acid.

Test sample solutions: All solvents and reagents were pro analysis grade from HPLC grade Reagents. Oleanolic aid standards were purchased from Sigma-Aldrich chemicals.

To quantification 1.00 g of different parts of dry *Lantana camara* was extracted by the method developed by

Banik and Pandey[11]. The obtained extract was evaporated to dryness and the residue was dissolved in methanol to a fixed volume (10 mL).

Standard solutions: Stock solutions of oleanolic acid were prepared by dissolving 2.0 mg of oleanolic acid compound in 10 mL of methanol (final concentration: 200 μg mL^{-1}).

Sample application: Different concentrations of standard and test samples were applied in different tracks as bands by Linomat IV applicator in different studies. Solutions of oleanolic acid of seven different concentrations 1, 2, 3, 4 and 5 μL (0.2, 0.4, 0.6, 0.8 and 1.0 μg $spot^{-1}$) were prepared in methanol by serial dilution of the stock solution of the drug. Each of these solutions (10 μL) was applied to a plate, the plate was developed, the spots were derivatized and the detector response for the different concentrations was measured. A graph was plotted of drug peak area against amount of oleanolic acid and the plot was found to be linear over the range 0.2 to 1.0 μg. The experiment was performed three times. The data were analyzed by linear regression least-squares fitting.

Scanning: The plate was kept in the above mentioned solvent system and allowed to run up to a distance of 8 cm. After drying , it was scanned densitometrically at 530 nm.

Method validation: The method was validated by determining linearity, peak purity, limit of detection, repeatibility (Table 1), percentage recovery (Table 2), intra-day (Table 3) and intermediate precision (Table 4) of oleanolic acid from shoot and root samples. Each of the standard solutions of oleanolic acid (0.2, 0.4, 0.6, 0.8 and 1.0 μg per band) was applied in triplicate. The calibration plot was prepared by plotting peak area against the amount of oleanolic acid and linearity range was determined. Instrument precision was checked by scanning the same oleanolic acid band (1 μg) six times. The mean, standard deviation and coefficient of variation [%] were calculated for peak area and R_F. The results obtained from study of spotting repeatability/instrument precision are listed in Table 1. Repeatability was tested by analyzing the oleanolic acid band after application of standard solution to the plate (n=3) and calculating %CV. The accuracy of the method was tested by determination of recovery at three levels, after addition of 50, 100 and 150% oleanolic acid to the sample. Known amounts of standard oleanolic acid was added to 100 mg powdered plant material (containing approximately 1.67 mg oleanolic acid), the sample was extracted and the amounts of oleanolic acid were determined as described above. Recovery was calculated for each of the three levels Table 2. Precision was studied by analyzing three bands of sample solution per plate on three plates (intra-day precision) and by analyzing three bands of sample solution per plate on second day (intermediate precision) and calculating % CV (Table 3, 4). The specificity of the method was determined by absorbance spectrum of oleanolic acid standard and the corresponding peak in the test samples in the range 200-800 nm. Different dilutions of the standard solutions were applied with methanol as blank and the Limits of Detection (LOD) and Quantification (LOQ) were determined.

Determination of oleanolic acid in samples: Sample extracts and five levels of standard solution (0.2, 0.4, 0.6, 0.8, 1.0 μL) were applied to 10×10 cm silica gel $_{254}$ TLC plates and analyzed as described above. Peak areas were recorded and a calibration plot was obtained by plotting

Table 1: Method validation parameters for estimation of oleanolic acid by HPTLC

Parameters	Value
Instrument precision [%CV, n = 10]	2.25
Repeatabilty [%CV, n = 10]	0.58
Retention factor (R_f)	0.5
Linear regression equation	Y = 374.5X+35.4
Limit of detection (ng/band)	50
Limit of quatification (ng/band)	170
Linearity range (ng)	200-1000
Correlation coefficient	0.998

Table 2: Results from study of the recovery of oleanolic acid by HPTLC

	Level			
Parameters	0	1	2	3
Amount of oleanolic acid in 100 mg plant material [mg] (A)	1.67	1.65	1.653	1.659
Amount of oleanolic acid added [mg](B)	0.00	0.80	1.600	2.400
Amount of oleanolic acid found in the mixture [mg](C)	1.67	2.46	3.250	4.068
Recovery [%]* (D)	100.00	101.25	99.800	100.400

•D: [(C-A)/B] ×100, Average recovery: 100.43%

Table 3: Results from determination of intra-assay/within day precision

Observation	Amount of drug (μg)			Samples prepared on the diffrent day					
No. for day 1	1	2	3	1	2	3	Mean	SD	CV (%)
1	0.4	0.4	0.4	2113	2116	2111	2114.3	2.52	0.119
2	0.6	0.6	0.6	3564	3566	3568	3566	2.00	0.056
3	0.8	0.8	0.8	4768	4770	4772	4769.3	2.00	0.042

Table 4: Results from determination of intermediate precision

Observation	Amount of drug (μg)			Samples prepared on the diffrent day					
No. for day 2	1	2	3	1	2	3	Mean	SD	CV (%)
1	0.4	0.4	0.4	2113	2117	2115	2115.00	2.00	0.094
2	0.6	0.6	0.6	3564	3567	3569	3566.67	2.52	0.070
3	0.8	0.8	0.8	4768	4773	4775	4772.00	3.61	0.075

peak area against the amount of standard oleanolic acid applied. The amounts of oleanolic acid present in the samples were calculated using the calibration plot.

RESULTS AND DISCUSSION

Validation: The HPTLC method was validated for precision, accuracy and repeatability (Table 2). The method is specific for oleanolic acid because it resolved the compound (R_f = 0.5) well in the presence of other components of *Lantana camara* (Fig. 2, 3). A linear relationship was obtained between response (peak area) and amount of oleanolic acid in the range 0.2-1.0 µg per band and the correlation coefficient was 0.99 (Fig. 4) (Table 2). Instrument precision was studied by scanning the same spot of oleanolic acid ten times (%CV = 2.25). The repeatability of the method was tested by analyzing eleven applications of the same standard solution (%CV = 0.58). The accuracy of the method was determined at three levels (50, 100 and 150%) by adding known amounts of oleanolic acid to root extract. Recovery at the three levels was found to be 101.0, 99.8 and 100.5%, respectively (Table 3, 4). Repeatability and precision were assessed by measurement of intra-day and inter-day variation. Little intra-day and inter-day variation was observed. The %CV were 0.18 and 0.07%, respectively (Table 3, 4). The specificity of the method was determined by acquiring the spectrum of oleanolic acid standard and the corresponding peak in the test samples in the range 200-800 nm. The spectra (Fig. 5) obtained from the pure marker and the marker present in root powder matched exactly, indicating no interference from other plant constituents. The Limits of Detection (LOD) and Quatification (LOQ) were calculated by use of equations LOD = 3×N/B and LOQ= 10×N/B, were N is the standard deviation of the peak area of the

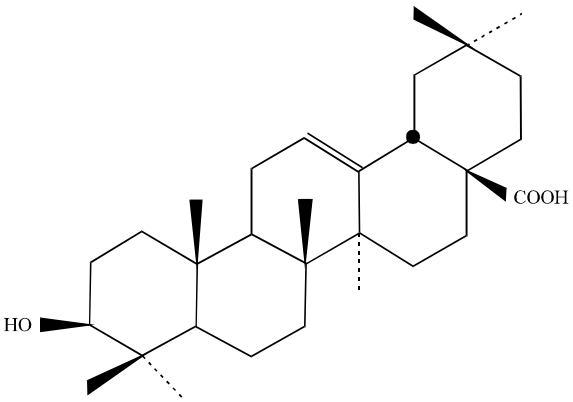

Fig. 2: Chemical structure of Oleanolic acid

Fig. 3: TLC of standard Oleanolic acid (OA), root sample and stem sample (Solvent system-Chloroform: methanol-95:5)

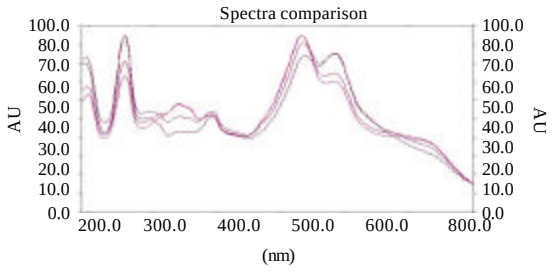

Fig. 4: Overlay spectra of oleanolic acid at 530 nm

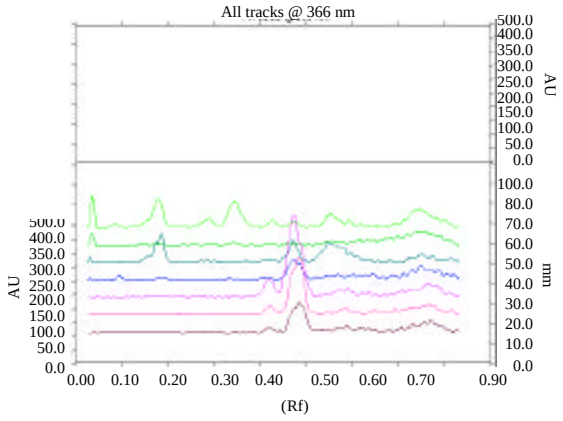

Fig. 5: HPTLC of standard oleanolic acid (1,2,3), root samples of pink (wild),yellow(wild), white (cultivated) and yellow (cultivated) varities of *Lantana camara*

Table 5: Levels (%) of oleanolic acid of dry weight of *Lantana camara*

Lantana camara varieties	Oleanolic acid (% w/w dry weight)	
	Root	Aerial shoot
Wild yellow flower	1.67	1.21
Wild pink flower	1.45	1.11
Cultivated yellow flower	1.36	0.89
Cultivated white flower	1.14	0.58

satandard (n = 3), taken as a measure of the noise and B is the slope of the corresponding calibration plot. The LOD and LOQ were 50 ng per band and 170 ng per band, respectively.

Determination of oleanolic acid in samples: Oleanolic acid (Fig. 2) in the different parts of *Lantana camara* was quantified by HPTLC analysis and high Oleanolic acid yielding part were identified. Its identity was confirmed by comparing its R_f value by reference standard oleanolic acid. Preliminary TLC studies revealed well resolved spots with R_f 0.50 for the marker and well resolved spots for test samples (Fig. 3). The initial HPTLC finger printing studies were done on the bioactive marker (Oleanolic acid standard) and parameters were optimized. The finger printing patterns of the standard oleanolic acid and high oleanolic acid containing root samples was given in Fig 5 and b. Under identical parameters finger printing patterns of the test samples (different parts of plant material) were recorded. The three dimensional patterns of the standards and test samples reveal the presence of super imposable peaks (R_f 0.50 oleanolic acid). Further, spectrum studies reveal that the peaks corresponding to R_F 0.50 of both standard oleanolic acid and test samples are identical as they showed similar patterns with λ_{max} at 530 nm (Fig. 4).The analytes in solution and during analysis are highly stable as evident from their absorption spectra which are consistent throughout the analysis.

The peak purity test was done by comparing the spectra of the standards and its corresponding peaks in test samples. The correlation coefficients were found to be 0.9990 for oleanolic acid, which indicates its purity. The percentage of the bio-active marker was determined by calculation mode using peak area parameter and the data is presented in Table 5.

The result shows that oleanolic acid accumulates highest in root part followed by stem part while oleanolic acid is found to be absent in leaves and fruit part. In case of oleanolic acid, the Limit of Detection (LOD) and Limit of Quantification (LOQ) were found to be 50 and 170 ng, respectively and show linearity in the range 200-1000 ng. Further the recovery values for oleanolic acid were found to be 97-98%, which shows the reliability and suitability of the method. The present HPTLC method is rapid, simple and accurate for quantitative monitoring of *Lantana camara* plant with respect to oleanolic acid.

CONCLUSION

The present investigation describes a simple, cost-effective and easily adaptable HPTLC method for simultaneous screening and quantitative determination of oleanolic acid from different varieties of *Lantana camara*. The method has been validated and found to be selective, linear, repeatable and accurate within established ranges. The method can be utilized effectively to explore the qualitative components of large number of root samples and also to screen and identify elite varieties whose propagation (conventional commercial propagation as well as *in vitro* multiplication) may play a significant role in the improvement of commercial trade across the globe.

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