



Received on 31 August 2021; received in revised form, 31 October 2021; accepted, 17 November 2021; published 01 June 2022

## IDENTIFICATION AND QUANTIZATION OF OLEANOLIC ACID IN STEM AND ROOTS OF *ACHYRANTHES ASPERA* USING VALIDATED HPTLC METHOD

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### Keywords:

In-situ gel, Acyclovir, Anti-viral, HPMC E50 LV, Pluronic F-127 HPTLC, *Achyranthes aspera*, Petroleum ether extract, Oleanolic acid

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**ABSTRACT: Objective:** Active chemical constituent in plants exhibits pharmacological action. Hence this research work aimed to identify and quantitate oleanolic acid. **Methods:** A simple, high-performance thin-layer chromatography (HPTLC) is developed and validated to identify and quantify oleanolic acid. The extracts were spotted as bands on aluminum TLC plates which were pre-coated with silica gel. Different compositions of mobile phase tried for high resolution and reproducible results. Mobile phase composition toluene: methanol: ethyl acetate (8.5:1:0.5 V/V) utilized. Plates were derivatized with 10% sulphuric acid and scanned. Oleanolic acid was detected quantitatively at 529nm. **Result:** In the selected mobile phase, R<sub>f</sub> value for oleanolic acid was found to be 0.35, which indicated a good separation. The Chromatography method was validated with respect to Linearity range (ng), Intraday and Interday precision, Instrumental Precision, Quantitation Limit *i.e.*, LOQ (ng), Detection Limit, *i.e.*, LOD (ng), Repeatability for oleanolic acid as per ICH guidelines. Oleanolic acid calibration graph in 200-1200 ng range per band was found linear with correlation coefficient value 0.998. All the parameters obeyed acceptable criteria as per the ICH guidelines. In the extract, the oleanolic acid was found 0.29% w/w. Hence the recently developed HPTLC technique was found prompt, cost-effective, precise, simple, accurate, reproducible, and rapid for analysis of quantity and quality of oleanolic acid in stem and roots petroleum ether extract of *Achyranthes aspera*. For other chromatographic techniques, this validated method of HPTLC may be useful as an economical alternative.

**INTRODUCTION:** *Achyranthes aspera* (Aghada) family *Amaranthaceae*, is also called as Apamarga in Sanskrit, is a tiny herb found throughout India, and it possesses many medicinal properties and is effective in bronchitis, rheumatism, and cough, malarial fever, hypertension, diabetes, asthma, and dysentery <sup>1-3</sup>.

The plant is a useful medicinal herb and is found as a weed in all regions of India. Around 80% of World's population relies on traditional herbs for initial medical care. Now a day's peoples prefer medicines obtained from herbs to avoid the side effects of allopathic medicines <sup>4</sup>.

In traditional Chinese medicine, plant extract prepared by hot water was used for spermicidal action; seed oil also has antimicrobial activity <sup>5</sup>. *Achyranthes aspera* leaf extract has cytotoxic and thrombolytic properties <sup>6</sup>. *Achyranthes aspera* aqueous extract possesses antioxidant, anti-inflammatory, and antibacterial activity <sup>7</sup>. Infusion of roots is effective as mild astringent.

<p><b>QUICK RESPONSE CODE</b></p> 	<p><b>DOI:</b> 10.13040/IJPSR.0975-8232.13(6).2399-04</p>
<p>This article can be accessed online on <a href="http://www.ijpsr.com">www.ijpsr.com</a></p>	
<p>DOI link: <a href="http://dx.doi.org/10.13040/IJPSR.0975-8232.13(6).2399-04">http://dx.doi.org/10.13040/IJPSR.0975-8232.13(6).2399-04</a></p>	

The seeds and leaves of plant *Achyranthes aspera* is emetic and are helpful to treat hydrophobia. Chewing and rubbing of spikes of the herb are good for the health of teeth and gums<sup>8</sup>. Herb *Achyranthes aspera* also shows anti-inflammatory and anti-arthritic and hypoglycemic Activity<sup>9, 10</sup>. Various extracts obtained from *Achyranthes aspera* roots show spermicidal activity in rats and human sperms<sup>11</sup>. Extract of the herb also shows wound healing and immunomodulatory activities<sup>12, 13</sup>. *Achyranthes aspera* leaves extract antiperoxidative and prothyroic activity<sup>14</sup>. High-Performance Thin Layer Chromatography is an automated and sophisticated form of Thin Layer Chromatography. It is characterized by better separation efficiency, accurate sample application, standardized chromatogram development, and software-controlled evaluation<sup>15</sup>. HPTLC is a versatile, economic and flexible process. Drug extracts could be spotted directly on HPTLC plates<sup>16</sup>. HPTLC can accomplish the assay of various components in the multi-component formulation. Identification of various species of plants, evaluation of stability and consistency of their preparations is possible by collaboration with chromatography<sup>17</sup>. Oleanolic acid is the chief active constituent present in *Achyranthes aspera* and is isolated from different plant parts. It is a pentacyclic triterpenoid abundantly present in plants of the Oleaceae family, such as olive plant<sup>18</sup>. Hence, this work aimed to detect the concentration of oleanolic acid in stems and roots extract of *Achyranthes aspera* using HPTLC.

#### EXPERIMENTAL WORK:

**Plant Material:** *Achyranthes aspera* roots and stem was collected in November month from Sinnar and Nashik Road region of Nashik, Dist: Nashik, Maharashtra, India.

The collected *Achyranthes aspera* plant was taxonomically identified by the Head of Department Botany, MVP Samaj's K.T.H.M. College, Nashik, with specimen No. 13-14/01. Roots and stems were separated, washed and dried in the shade and powdered. Dried powder of the roots and stems of *Achyranthes aspera* was extracted by sequential Soxhlet method using petroleum ether as solvent. The extract was collected and preserved in a tightly closed container.

**Reagents and Chemicals:** Oleanolic acid the main active constituent was purchased from Sigma-Aldrich Pvt. Ltd., Mumbai, India. Petroleum ether, toluene, methanol, and ethyl acetate are HPLC grade while sulphuric acid, ethanol (90%), and all other chemicals are analytical grade.

**Oleanolic Acid Stock Solution Preparation in Petroleum Ether:** For preparing a stock solution, 1 mg oleanolic acid was dissolved in petroleum ether. This mixture is sonicated until oleanolic acid gets dissolved. In 10ml of the volumetric flask by adding petroleum ether, the final volume was adjusted up to 10ml to get a concentration 100µg/ml.

**Sample Solution Preparation:** The successive Soxhlet method extracted 80 gm of stem and root powder using petroleum ether as solvent. The extract was evaporated on a water bath. Weighed 10mg of solid residue, transferred it into the 10 ml volumetric flask. Added 4-5 ml of petroleum ether in a flask and sonicated the flask. The final volume was adjusted up to a total 10 ml by adding petroleum ether. Using Whatman filter paper, the prepared solution was filtered to separate the undissolved particles.

**Chromatographic Conditions:** Chromatography was performed on HPTLC silica gel 60 F<sub>254</sub> aluminium backed plates, pre-washed using methanol, and dried for about 20 min at 105°C in oven. Samples (10µl) were spotted as 6 mm bands, starting from 45 mm from the edge of the plates, using a Camag Linomat V sample applicator. The plates developed in the twin trough chamber of Camag, which was previously equilibrated with mobile phase toluene: methanol: ethyl acetate (8.5:1:0.5V/V). Sample spots were given to a distance of about 80 mm above a position with the help of Camag Linomat V Applicator. Chromatographic plates after development were dried at normal temperature. Before densitometry scanning, plates were heated in the oven for 5 minutes at 105°C. Plates were subjected for densitometry evaluation at 529 nm with Camag Scanner III and tungsten lamp and CATS software for quantification. Slit dimension was 5.00X0.30 mm. Plates scanned in absorbance mode.

**Quantification of Oleanolic Acid in Roots and Stem Extract:** 10 µl roots and stem extract and

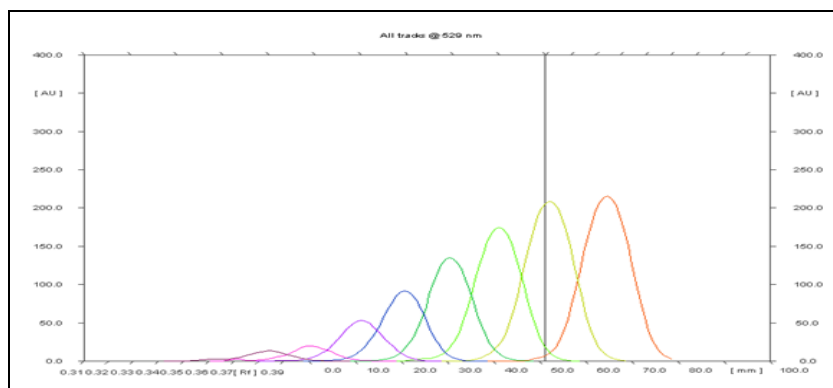
oleanolic acid solution was applied in linear range on plate and developed according to the method described in the manuscript. Using line equation oleanolic acid concentration in stem and roots extract was calculated.

**Linearity:** On the plate, oleanolic acid solutions having different concentrations (200-1200ng ml<sup>-1</sup>) were applied, and the plate was developed, spots were derivatized and detector responses obtained for different concentrations were measured at 529 nm. The results were obtained in triplicates. A graph of oleanolic acid concentration versus peak

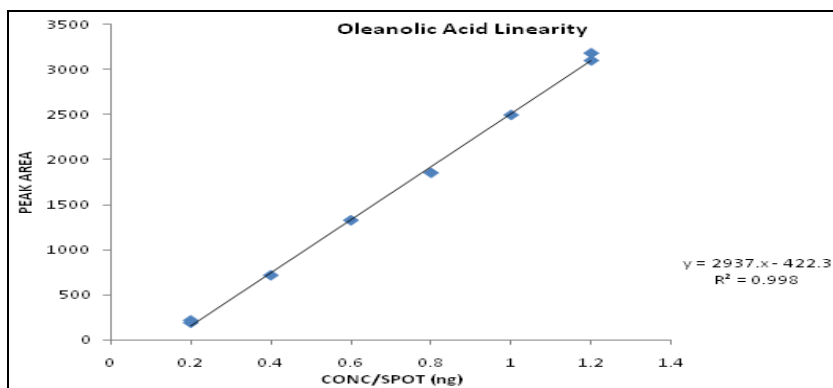
area was plotted. Data were analyzed using linear regression least-squares fitting as indicated in **Table 1** and **Fig. 1**.

**TABLE 1: LINEARITY OF OLEANOLIC ACID**

S. no.	Conc/ spot (ng)	Peak area
1	200	187.7
2	200	215
3	400	715.4
4	600	1325.8
5	800	1952.4
6	1000	2494.8
7	1200	3098.9
8	1200	3180.5



**FIG. 1: 3-D VIEW OF PEAKS SHOWING LINEARITY**



**FIG. 2: OLEANOLIC ACID CALIBRATION CURVE**

**Detection of Oleanolic Acid Content in *Achyranthes aspera* Extract:** Oleanolic acid content in extract was detected by spotting extract

along with a standard oleanolic acid solution on HPTLC plate. The recorded peaks are as provided in **Table 2**.

**TABLE 2: DETECTION OF OLEANOLIC ACID CONTENT IN *ACHYRANTHES ASPERA* EXTRACT**

S. no.	Conc/ spot (ng)	Peak area
1	200	215
2	400	715.4
3	600	1325.8
4	800	1952.4
5	1000	2494.8
6	1200	3098.9
7	Sample	205.4
8	Sample	320.1

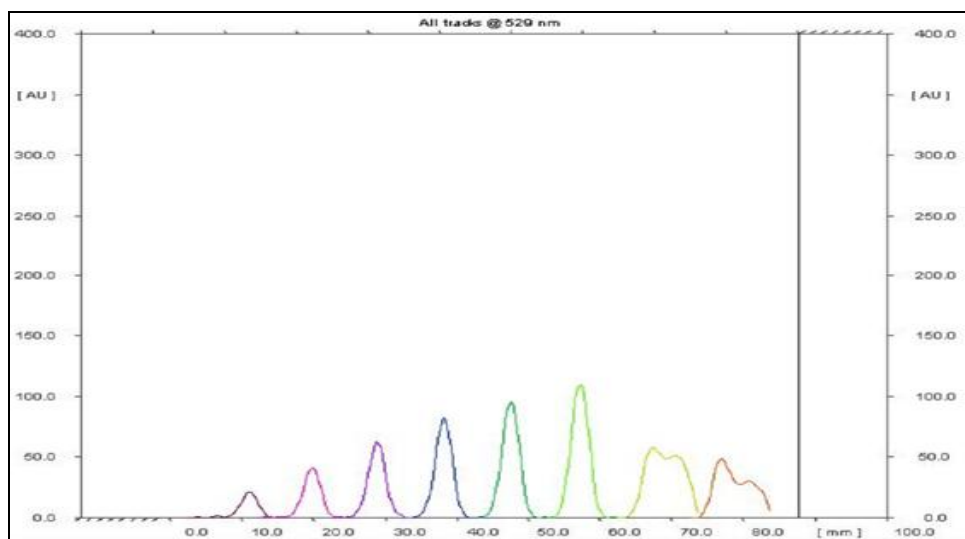


FIG. 3: 3-D VIEW OF PEAKS SHOWING OLEANOLIC ACID QUANTIFICATION IN ROOTS AND STEM EXTRACT

**Precision:** 10 µl Oleanolic acid solutions in six bands were applied on the plate. After the development of chromatographic plates, spots detection was performed at 529nm. Mean standard deviation and % RSD were calculated. To study

interday and intraday precision, oleanolic acid 200, 400, 600 ng/spot aliquots were used. The experiment was repeated and performed thrice. Obtained results are mentioned in **Table 3** and **Table 4**.

TABLE 3: INTRADAY PRECISION

S. no.	Concentration (ng)	Set 1	Set 2	Set3	Mean	SD	% RSD
1	200	215	217	213	215	1.24	0.57
2	400	715	716	715.4	715.2	0.78	0.11
3	600	1324.1	1325.2	1325	1325.1	0.68	0.051

TABLE 4: INTERDAY PRECISION

S. no.	Concentration (ng)	Set 1	Set 2	Set3	Mean	SD	% RSD
1	200	213	214.8	214.6	214.1	1.24	0.57
2	400	714.2	716	715.4	715	0.78	0.12
3	600	1323.5	1327.2	1324	1325.3	0.68	0.053

**LOD:** Detection Limit is the lowest concentration present in sample that gives a measurable response. Detection Limit can be calculated by formula:

$$\text{Quantization Limit} = 10\sigma/S$$

Where  $\sigma$  = standard deviation of response  
 S = slope of calibration curve

**LOQ:** Quantitation Limit is the lowest concentration of an oleanolic acid, which gives response that can be quantified.

$$\text{Quantization Limit} = 10\sigma/S$$

Where  $\sigma$  = standard deviation of response.

S = slope of the calibration curve.

From the calibration curve of the oleanolic acid slope, S can be calculated.

**RESULTS AND DISCUSSION:** Various compositions of mobile phases like toluene: methanol: chloroform (8:1:1 V/V), methanol: chloroform: ethyl acetate (7:2:1V/V), Toluene: acetic acid: chloroform (9:0.5:0.5V/V), Toluene: chloroform (9:1 V/V), Toluene: methanol: ethyl acetate (8.5:1:0.5 V/V) were used and tested for oleanolic acid HPTLC detection in stem and roots petroleum ether extract of plant *Achyranthes aspera*. Toluene: methanol: ethyl acetate (8.5:1:0.5 V/V) mobile phase fulfilled desired objective by giving a peak at  $R_f$  0.35 as depicted in **Fig. 4** for oleanolic acid.

As depicted in **Fig. 1**, oleanolic acid was observed linear 200-1200 ng range per band. The correlation coefficient was found 0.998. ( $Y=2937X-422.3$ ). Oleanolic acid concentration in extract found was 0.29% w/w. The chromatography method was

validated by determining linearity, inter-day and intra-day precision, detection limit, repeatability, quantitation limit for the oleanolic acid.

Limit of Detection and Quantitation Limit was found at 6.93ng and 21.3ng, respectively.

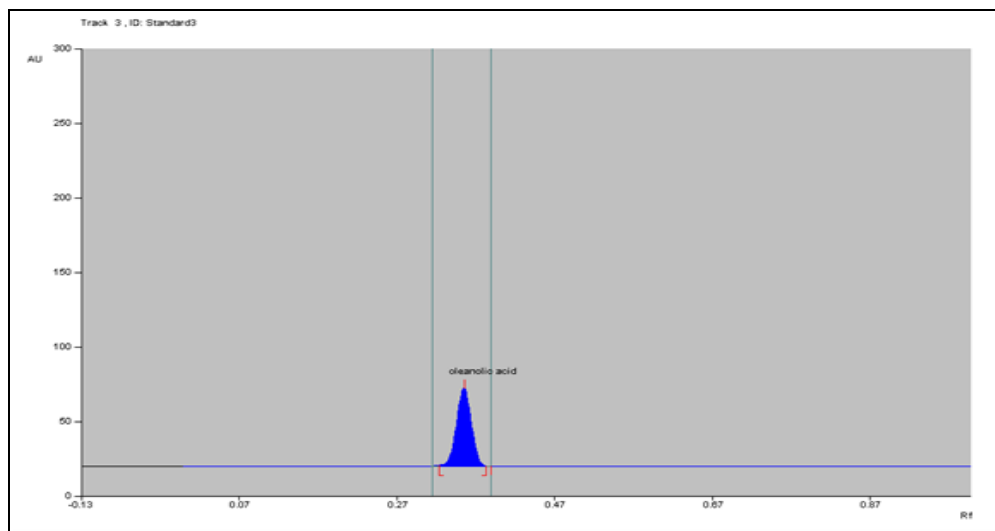


FIG. 4: R<sub>t</sub> VALUE FOR OLEANOLIC ACID

**Repeatability:** Estimating the repeatability analysis for oleanolic acid application showed % RSD for peak area, which was found 0.55 as indicated in Table 5 and Fig. 5. This method is possibly used to determine the oleanolic acid concentration present in extract and formulations.

TABLE 5: REPEATABILITY STUDIES

S. no.	Conc/spot (ng)	Peak area
1	200	217
2	200	215
3	200	216.6
4	200	218.2
5	200	223
6	200	220
	Mean	215.2
	SD	1.243
	% RSD	0.557

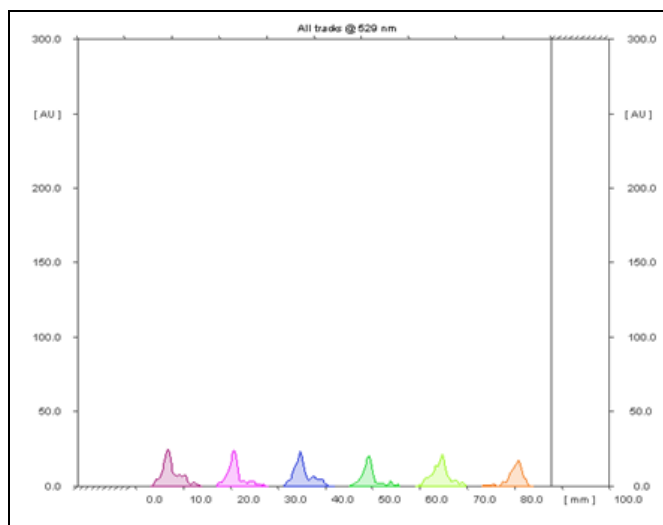


FIG. 5: 3-D VIEW OF PEAKS SHOWING REPEATABILITY

**Summary of Validation Parameters:**

S. no.	Parameter	Oleanolic acid	ICH guideline limits <sup>19,20</sup>
1	Instrumental precision (% CV, n=7)		RSD<2%
2	Repeatability (% CV, n=5)	0.557% RSD	RSD≤2%
3	Coefficient of determination (r <sup>2</sup> )	0.998	Correlation coefficient – NLT 0.999
4	Linearity range (ng)	200-1200	r <sup>2</sup> ≥0.99, similar response ratios
5	LOD (ng)	6.93	>2 times base line or S/N > 2 or 3
6	LOQ (ng)	21.3	Signal-to-Noise=10:1 or S/N > 10
7	Intra-day precision (% CV, n=9)	0.051 to 0.57%RSD	RSD<2%
8	Inter-day precision (% CV, n=9)	0.053 to 0.57%RSD	RSD<2%
9	Specificity	0.557% RSD	No interference

**CONCLUSION:** An original HPTLC method became advanced for identification and for determination of the quantity of oleanolic acid. The

main characteristics of this method found are satisfactory accuracy, precision, faster speed, and economical. The developed method was

successfully validated as stated by ICH guidelines. The oleanolic acid concentration in plants and herbal formulations can be possibly detected using this method.

**ACKNOWLEDGEMENT:** We are thankful to Dr. R. S. Bhambar, Principal, M.G.V's Pharmacy College, Panchavati, Nashik, for providing us facilities for this work and research.

**CONFLICTS OF INTEREST:** The authors have no conflicts of interest to declare.

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#### How to cite this article:

Jitendra YN, Varsha CF and Minal RN: Identification and quantization of oleanolic acid in stem and roots of *Achyranthes aspera* using validated HPTLC method. Int J Pharm Sci & Res 2022; 13(6): 2399-04. doi: 10.13040/IJPSR.0975-8232.13(6).2399-04.

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