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DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR IDENTIFICATION AND QUANTIFICATION OF URSOLIC ACID IN THE LEAVES OF *ALSTONIA SCHOLARIS*

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ABSTRACT: Herbs have been used since antiquity for treatment and management of various disease conditions. Their ability to cure or prevent diseases is due to storehouses of various phytoconstituents like alkaloids, flavonoids, glycosides, terpenoids, steroids, tannins, phenols, *etc*. present in them. Pentacyclic triterpenoids have gained greater attention of researchers due to its wide array of pharmacological actions. Thus, a simple and precise HPTLC method has been developed for identification and quantification of one such ubiquitous pentacyclic triterpenoid- ursolic acid in the leaves of *Alstonia scholaris*. The optimized mobile phase Toluene: Ethylacetate (7:3v/v) gave a better resolution of bands of ursolic acid at 540 nm after derivatizing plate with ASR. The R_f value for ursolic acid was found to be 0.46. The amount of ursolic acid quantified in ethanol, and chloroform extracts of leaves of *Alstonia scholaris* were 2.34 and 2.38 %w/w, respectively. The developed method was validated in terms of linearity, range, specificity, precision, accuracy, LOD, and LOQ following ICH guidelines. The calibration curve of ursolic acid was linear between 500-900 ng/band with a correlation coefficient (r^2) 0.9990. Inter-day and intra-day precision were assessed in terms of %RSD, which was less than 2% in both the cases, thus indicating good precision of the method. LOD and LOQ were 56.79 and 172.10 ng/band, respectively. Thus, the developed method was found to be quick, simple, specific, precise, and accurate for identification and quantification of ursolic acid in the leaves of *Alstonia scholaris* and can be used for routine quality control of the plant.

INTRODUCTION: In recent times, there have been increased waves of interest in investigation of natural products as a source of potential drug substance. This resurgence of interest can be attributed to un-met therapeutic needs and loads of side effects from chemically synthesized products.

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At the same time, findings of the presence of rich amount of pharmacologically active phyto-constituents in natural products has led to development of novel techniques to detect, quantify, isolate, purify and characterize these potential phytoconstituents [1, 2].

Amongst various phytoconstituents of plant origin, Pentacyclic triterpenoids have received much attention during the last few decades, and several of its derivatives are being marketed as therapeutic agents or as dietary supplements across the globe [3]. They form the common and natural constituent of the human diet since they are found in many

vegetable oils, fruits, and cereals [4]. They exert array of pharmacological actions like anti-inflammatory [5], anti-oxidant [6], anti-viral [7], anti-diabetic [8], anti-tumor [9], hepatoprotective [10], cardio-protective [11,] and anti-urolithiasis [12] activities. They have the potential to restore vascular disorders associated with hypertension, obesity, diabetes, and atherosclerosis [13]. Due to their wide therapeutic applications, they have attracted the attention of medical professionals, marketers, and researchers, leading to their testing in clinical trials [14, 15, 16, 17].

Pentacyclic triterpenoids are commonly divided into 3 subgroups oleanane, ursane, and lupane. Ursolic acid (also known as urson, prunol, micromerol and malol) is one such pentacylic triterpenoid acid that is derived from ursane subgroup. It is a secondary plant metabolite, usually present in stem, bark, leaves, and fruit peels. It was considered to be pharmacologically inactive for a long time and was used as an emulsifying agent in cosmetics. However, on examination, this ubiquitous triterpenoid acid was found to be pharmacologically active internally as well as topically [18].

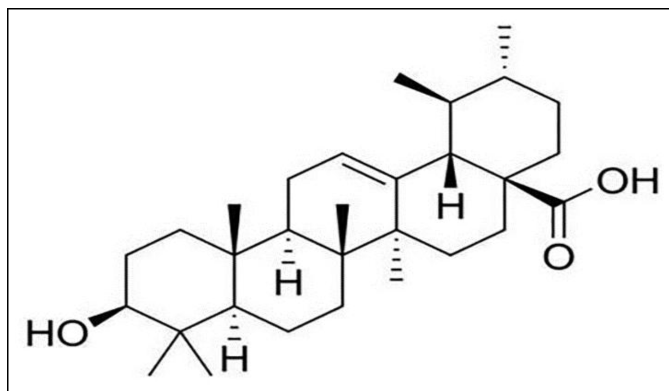

FIG. 1: CHEMICAL STRUCTURE OF URSOLIC ACID

Alstonia scholaris Linn. R. Br. (Apocynaceae) popularly known as "Saptaparni" is an evergreen tropical tree widely distributed in India. Upon investigation leaves of *A. scholaris* have shown presence of pentacyclic triterpenoids in good amount [19]. Thus, efforts have been made in this study to develop HPTLC (High-Performance Thin Layer Chromatography) method so as to identify and quantify ursolic acid (UA) in ethanolic and chloroform extracts of leaves of *A. scholaris* and to validate the method following ICH guidelines with extract having a higher quantified amount of UA in it.

MATERIALS AND METHODS:

Plant Material: The fresh leaves of *A. scholaris* were procured from Joginder Nursery, Delhi. Leaves were shade dried, crushed, and ground to obtain a coarse powder. This powder was subjected to Soxhlet extraction using ethanol and chloroform as solvents to get ethanolic (EtAS) and chloroform (ChAS) extracts of leaves of *A. scholaris*. Extraction was continued till the siphon tube had colorless solvent, after which extracts obtained were collected. The excess of solvent from each extract was evaporated on an electronic water bath to obtain semi-solid mass. All extracts were stored in an airtight container in the refrigerator at 2-8 °C.

Fresh leaves of *A. scholaris* with flowers were authenticated at St. Xavier's Blatter Herbarium (Fort, Mumbai, India) under specimen number NI-1417 of N. A. Irani.

Site and Year of Experimentation: The stated research study was performed in Prin. K.M. Kundnani College of Pharmacy and Anchrom Laboratories, Mumbai, India; in the year 2018.

Chemicals and Reagents: Standard ursolic acid (purity >95%) was procured from Yucca enterprises (Mumbai, India).

Methanol, toluene, and ethyl acetate were of analytical grade and purchased from S.D. Fine Chem Ltd (Mumbai, India). Anisaldehyde sulfuric acid reagent (ASR) was freshly prepared and used.

All the other chemicals used were of analytical grade and procured from authorized vendors.

Instruments: Linomat V sample applicator, Twin trough developing chamber (20 × 10 × 4cm), Chromatogram immersion device III, TLC plate heater, TLC plate scanner all from Camag (Muttenz, Switzerland) and 100 µL Hamilton syringe (Sigma-Aldrich, United States).

Standard Stock Solution Preparation: 10 mg of standard UA was accurately weighed and transferred to a 10 ml volumetric flask. This UA was initially dissolved in 5 ml of methanol, sonicated at 2500-3000 rpm for 30 min, and then diluted up to the mark with methanol, which gave the stock solution of 1 mg/ml.

Sample Solution Preparation: 100mg of EtAS and ChAS was accurately weighed and transferred to a 10 ml volumetric flask each. The extracts were initially dissolved in 5ml of methanol, sonicated at 2500-3000 rpm for 30 min, filtered through Whatman filter paper no. 41 and diluted up to the mark with methanol, which gave the stock solution of 10mg/ml.

Method Development:

Identification: The presence of UA in the leaves extract of *A. scholaris* was confirmed by loading plate with sample solutions EtAS and ChAS (5 and 10µl; 10mg/ml) and standard solution of UA (5 and 10µl; 1mg/ml). Chromatographic conditions like mobile phase composition, plate loading volume, chamber saturation time were optimized to obtain better resolution and separation of a band of UA from other phytoconstituents of the extract.

TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS FOR IDENTIFICATION AND QUANTIFICATION OF UA IN EtAS AND ChAS

Parameters	Description
Stationary Phase	Aluminum silica gel 60 F_{254} TLC plates from Merck
TLC plate size	20 × 10cm
Mobile Phase	Toluene: Ethylacetate (7:3 v/v)
Standard concentration	1mg/ml (Identification); 0.1-0.45mg/ml (Quantification)
Sample concentration	10mg/ml
Sample Applicator	Camag Linomat 5
Syringe	Hamilton syringe (100µl)
Band length	8mm
Development distance	80mm
Developing Chamber	Camag Twin Trough Chamber
Chamber saturation time	20 min
Visualizer	Camag TLC Visualizer.
Derivatizing Reagent	Anisaldehyde Sulphuric acid Reagent (ASR)
Immersion Device	Camag Chromatogram Immersion Device III
Plate heater	Camag TLC Plate Heater
Plate heating	At 110°C for 3-5 min
Scanner	Camag TLC Scanner
Mode	Absorbance
Lamp	Tungsten
Scanning wavelength	540nm
Software	Vision CATS; version 2.4.17207.2

Preparation of Calibration Curve for Quantification: Quantification study was carried out by external standard method by applying different concentrations of standard UA (2µl; 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4 and 0.45 mg/ml) so as to form a calibration curve and same concentration of sample EtAS (3µl; 10mg/ml) and ChAS (3µl; 10mg/ml) in triplicate. Plates were developed and scanned according to the optimized chromatographic conditions, as mentioned below in **Table 1**. Calibration curve of UA was obtained by plotting peak areas ($\times 10^6$) *vs.* concentration of UA. Amount of UA present in each extract was calculated from the calibration curve.

Method Validation: The developed HPTLC method was validated for identification and quantification of UA in ChAS as per the guidelines laid down by International Conference on Harmonisation (ICH) with respect to linearity and range, specificity, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ) [20].

Linearity and Range: The stock standard solution (1mg/ml) was diluted with methanol to obtain series of concentrations such as 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45mg/ml, application volume was 2µl. Concentrations applied on plate were 200, 300, 400, 500, 600, 700 800, 900 ng/band. Regression equation, correlation coefficient (r^2), coefficient of variation (% CV) or relative standard deviation (% RSD) of the calibration curve were estimated to determine method linearity. Range of the analytical procedure was determined from the concentrations of calibration curve which fall under linearity.

Specificity: Specificity of the method was verified by applying bands of standard UA, ChAS, diluent (Methanol), and mobile phase (Toluene: Ethylacetate 7:3 v/v) and was developed and scanned according to chromatographic conditions mentioned in **Table 1**.

Precision: Precision was determined in terms of intra-day precision (Repeatability) and inter-day precision (Intermediate precision). Intra-day precision was determined in triplicate with the same method on same day by applying 8 bands each of 3µl at 100% concentration of standard UA (1mg/ml). Inter-day precision was performed on 3 consecutive days with the same method as described for intra-day precision.

Accuracy: Accuracy of the method was determined by spiking triplicate bands of sample with 80%, 100% and 120% of standard UA. The percent recoveries and average percent recovery was calculated.

Limit of Detection (LOD): LOD or the lowest level of analyte that can be detected in the sample, but not necessarily quantified under the stated experimental condition was determined on the basis of signal to noise ratio by using following formula

$$LOD = 3.3(\delta/S)$$

δ is standard deviation of the response and S is slope of calibration curve.

Limit of Quantitation (LOQ): LOQ or the lowest amount of analyte that can be detected and quantified with acceptable accuracy, precision and variability was determined on the basis of signal to noise ratio by using following formula.

$$LOQ = 10\ (\delta/S)$$

δ is standard deviation of the response and S is slope of calibration curve.

RESULTS AND DISCUSSION:

Method Development:

Identification: Optimized mobile phase Toluene: Ethyl acetate (7:3 v/v) gave good resolution of bands with sharp and symmetrical peaks. The RF value of standard UA was found to be 0.461. Extracts EtAS and ChAS also displayed peak at RF 0.463 and 0.466 respectively, thus indicating presence of UA in both the extracts. Densitogram of standard UA and UA in EtAS and ChAS at 540 nm is shown in **Fig. 2**.

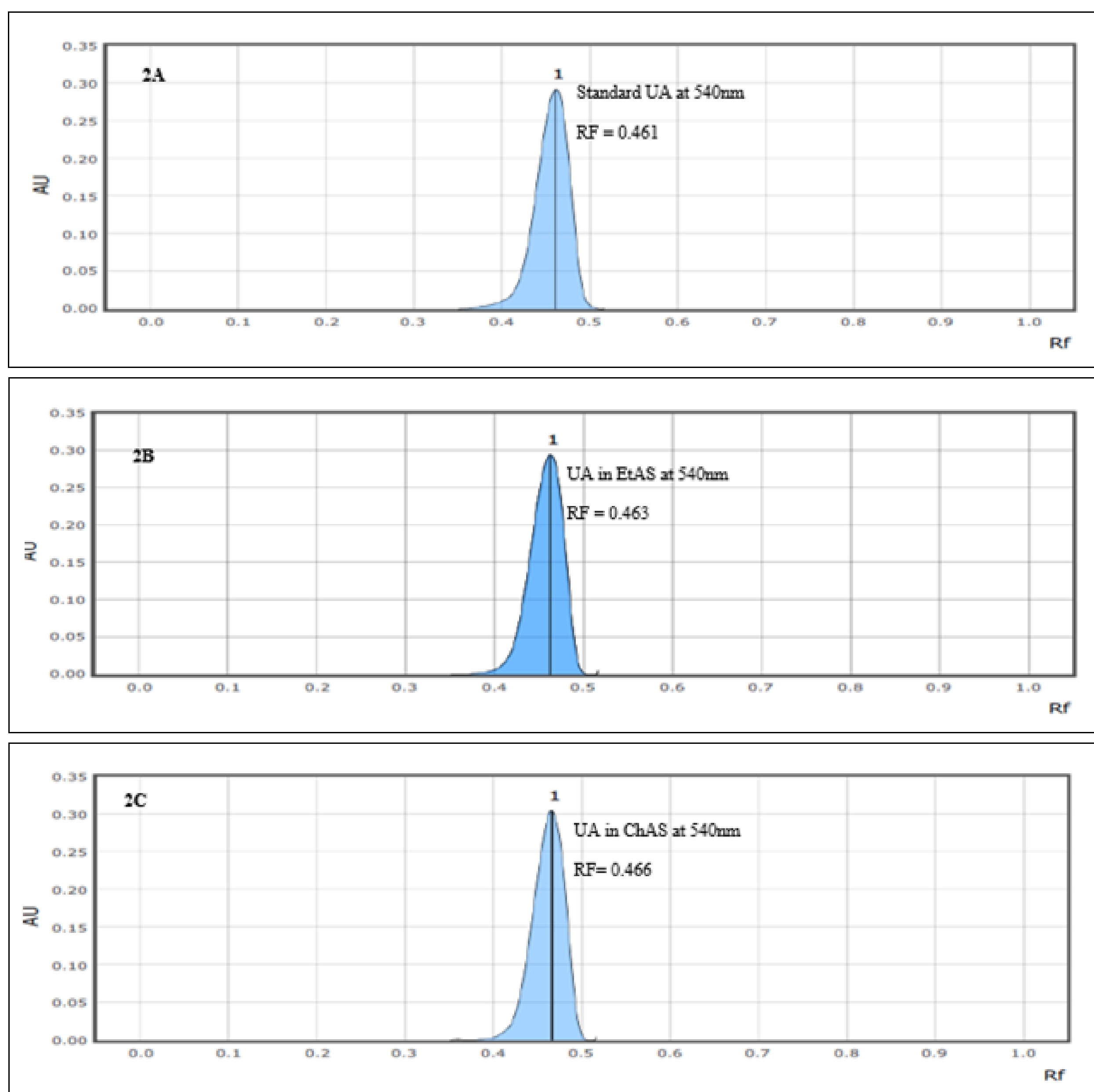

FIG. 2: DENSITOGRAM OF STANDARD UA (2A), UA IN EtAS (2B) AND ChAS (2C)

Quantification: The developed HPTLC method precisely quantified the amount of UA in EtAS and ChAS. The quantified amount of UA in EtAS and ChAS was found to be 2.34% w/w and 2.38% w/w, respectively. [Refer **Table 2**] Other components in the extract did not interfere with the analysis **Fig. 3.**

TABLE 2: QUANTIFIED AMOUNT OF UA IN 10mg OF EtAS AND ChAS

Track no.	Sample	Volume applied (µl)	Amount of sample (µg /band)	Rf	Quantified amount of UA (ng/band)	Concentration of UA (µg /10mg of extract)	Mean concentration (µg /10mg of extract)
1	EtAS	3	30	0.46	672.9	224.3	
2	EtAS	3	30	0.46	711.8	237.3	234.7
3	EtAS	3	30	0.46	727.2	242.4	
4	ChAS	3	30	0.46	712.7	237.6	
5	ChAS	3	30	0.46	719.5	239.8	238.5
6	ChAS	3	30	0.46	714.2	238.1	

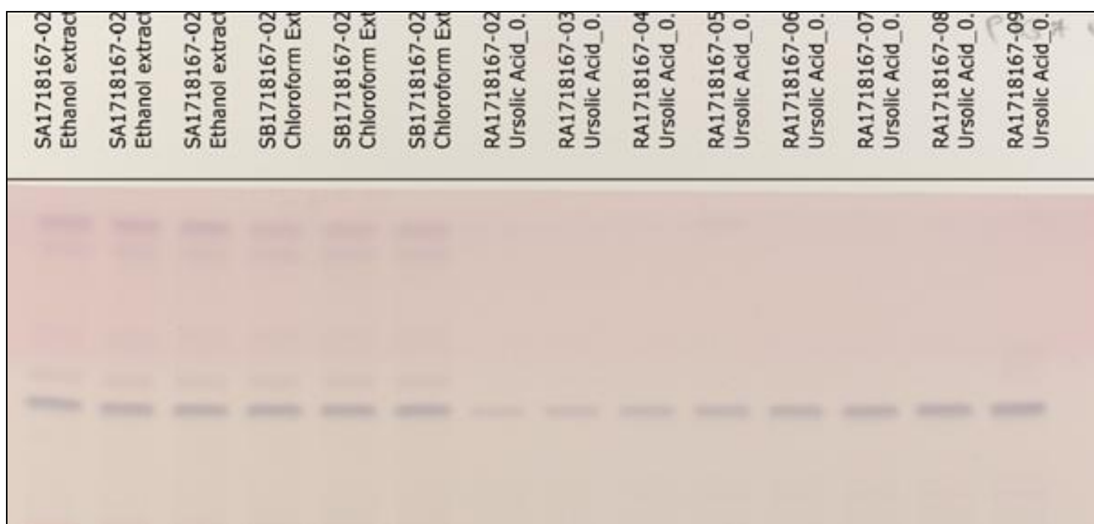

FIG. 3: HPTLC PLATE USED FOR QUANTIFICATION OF UA IN EtAS AND ChAS

Method Validation: Validation of the above-stated method was carried out by using ChAS extract as it had a greater amount of UA in it.

Linearity and Range: Linearity of UA was validated by the linear regression equation and correlation coefficient. The calibration curve was found to be linear over the concentration range of 500-900ng/band, and the correlation coefficient (r^2) was found to be 0.99908 **Fig. 4, Table 3**.

Specificity: Peak purity was assessed by comparing peak apex, peak start, and peak end of an extract with that of standard. The R_f of extract and standard UA was found to be 0.46. There was no other interfering peak of other phytoconstituents of extract around the retention time of UA. Also, the mobile phase and diluent did not show any interference. Thus the method was found to be quite specific for the determination of UA in ChAS **Fig. 5**.

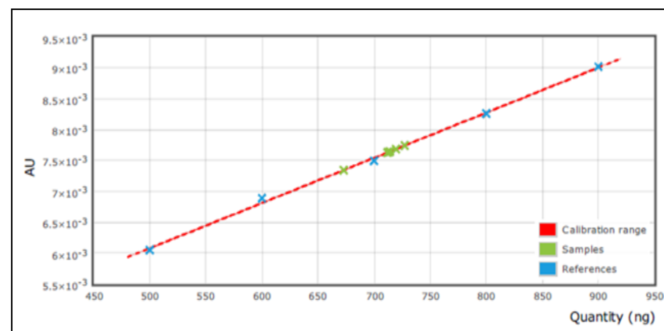

FIG. 4: CALIBRATION CURVE OF STANDARD UA FOR QUANTIFICATION OF UA IN EtAS AND ChAS

TABLE 3: LINEARITY AND RANGE OF CALIBRATION CURVE

Parameters	Result
Range of linearity	500-900 ng/band
Regression equation	$Y = 7.312 \times 10^{-9}x + 2.426 \times 10^{-3}$
Correlation coefficient (r^2)	0.99908607
Coefficient of variation (%CV) or Relative Standard Deviation (% RSD)	0.586%

FIG. 5: HPTLC PLATE FOR ASSESSING SPECIFICITY OF THE PROPOSED METHOD

Precision: Intra-day and inter-day precision were assessed in terms of %RSD. 3 µl of standard UA was loaded on the plate. % RSD of intra-day and inter-day precision where n=8 was found to be 1.24%, 1.28%, 1.41% and 1.24%, 1.33% and 1.54% respectively. %RSD values of intra-day and inter-day precision were less than 2% in all cases, which dictates good precision of the proposed method, **Table 4**.

Accuracy: Average percent recovery was found to be 85.56% when the sample was spiked with 80%, 100%, and 120% of standard UA **Table 5**.

TABLE 4: PRECISION OF UA IN TERMS OF %RSD

Track no.	Quantity of UA (µg/band)	Peak area ($\times10^6$) for Intra-day precision			Peak area ($\times10^6$) for Inter-day precision		
		1	2	3	Day 1	Day 2	Day 3
1	3	0.01645	0.01561	0.01815	0.01645	0.01690	0.01895
2	3	0.01649	0.01549	0.01843	0.01649	0.01721	0.01939
3	3	0.01615	0.01552	0.01866	0.01615	0.01712	0.01900
4	3	0.01589	0.01540	0.01895	0.01589	0.01684	0.01882
5	3	0.01606	0.01530	0.01894	0.01606	0.01729	0.01954
6	3	0.01611	0.01494	0.01892	0.01611	0.01750	0.01965
7	3	0.01599	0.01553	0.01870	0.01599	0.01699	0.01888
8	3	0.01602	0.01550	0.01853	0.01602	0.01745	0.01917
Mean		0.01614	0.01541	0.01866	0.01614	0.01716	0.01917
S.D.		0.000201	0.000198	0.000264	0.000201	0.000229	0.000295
% R.S.D.		1.249%	1.285%	1.419%	1.249%	1.334%	1.541%

TABLE 5: % RECOVERY OF UA IN ChAS WHEN SPIKED WITH 80%, 100% AND 120% OF STANDARD UA

Sample volume (µl)	% UA spiked	UA spiked (µl)	Peak area ($\times10^6$)	% Recovery	Average % recovery
3	80	1.6	0.010314	81.42	
3	100	2.0	0.010864	76.18	85.56
3	120	2.4	0.011555	99.08	

LOD and LOQ: The LOD and LOQ were found to be 56.79 ng/band and 172.10 ng/band, respectively.

CONCLUSION: A new HPTLC method was developed for the identification and quantification of UA in the leaves extract of *A. scholaris*. The reliability of the method was confirmed by assessing validation parameters as per ICH guidelines. The proposed HPTLC method was found to be quick, simple, specific, sensitive, precise, and accurate for identification and quantification of UA in the leaves extract of *A. scholaris*. Thus, the developed and validated method can be used for standardization, quality control analysis, and quantification of UA in the leaves of *A. scholaris.* Also, as UA has shown its therapeutic potential in treatment and management of various medical conditions and the leaves of *A. scholaris* contain a good amount of this triterpenoid acid; thus, leaves of *A. scholaris* can be used as a potential source for isolation of this therapeutically active pentacyclic triterpenoid acid which can be in turn quantified and characterized with the help of developed HPTLC method.

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CONFLICTS OF INTEREST: Authors of this article declare no potential conflict of interest.

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