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

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Keywords:

Alstonia scholaris, Pentacyclic triterpenoid, Lupeol, HPTLC, Quantification, Validation

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ABSTRACT: In ancient literature plant *Alstonia scholaris* (Apocynaceae) has been considered medicinally important in the treatment of various ailments. Extracts of this plant have shown wide spectra of pharmacological activities like anti-cancer, hepatoprotective, anti-inflammatory, anti-diabetic, *etc*. These activities can be attributed to the depositories of various phytoconstituents like alkaloids, flavonoids, phenols, triterpenoids, saponins, *etc*. present in it. Thus, a simple and precise HPTLC method has been developed for identification and quantification of a pentacyclic triterpenoid- lupeol in the leaves of *Alstonia scholaris*. The developed method was validated following ICH guidelines concerning linearity, range, specificity, precision, accuracy, LOD, and LOQ. After derivatization, the optimized mobile phase Toluene: Ethyl acetate: Formic acid (7:2:1 v/v/v) exhibited good resolution of bands at 580 nm. The R_f value of lupeol was found to be 0.63 and amount quantified in ethanol and chloroform extracts of leaves was 0.31 and 0.35% w/w respectively. The calibration curve of lupeol was linear between 200-1000 ng/band with correlation coefficient 0.9990. LOD and LOQ were 55 and 166.69 ng/band respectively. Recovery was 91.88%. Thus the developed method was found to be specific, precise, and accurate for identification and quantification of lupeol in the leaves of *Alstonia scholaris* and can be used for routine analysis of the plant.

INTRODUCTION: Since antiquity, natural products have played a crucial role in the treatment and prevention of various health conditions of mankind across the globe. In recent years, there has been a witness of the increased resurgence of interest in natural products for treatment and management of diseases, which is evident from the greater inclination of developed countries towards the use of herbal drugs and remedies [1]. About 50% of the approved drugs during the last few years are either directly or indirectly derived from natural products [2].

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The global market of products derived from plants is estimated to be 83 billion dollars and continues to grow [3]. According to reports of WHO, between 65-80%populations of developing countries use the medicinal plant as remedies [4]. One such medicinal plant, *Alstonia scholaris* Linn. R. Br. (Apocynaceae) popularly known as "Saptaparni" has been used since long back for treatment of many disease conditions. It is known for its pharmacological activities like anti-oxidant [5], anti-bacterial [6], Broncho-vasodilatory [7], hepatoprotective [8], anti-cancer [9], anti-diabetic [10], anti-stress [11], anti-urolithiasis [12], analgesic and anti-inflammatory [13] activities. These activities can be attributed to the rich amount of various secondary metabolites like alkaloids, glycosides, flavonoids, triterpenoids, steroids, saponins, and phenols present in them [12].

Amongst various phytoconstituents, pentacyclic triterpenoids have received greater attention of

researchers during the last few decades due to its wide spectrum of pharmacological activities. Many of these triterpenoids and their derivatives are being marketed as therapeutic agents or as a dietary supplement around the world [14]. Lupeol (also known as fagarasterol) is one such active pentacyclic triterpenoid and phytosterol derived from lupane subgroup. It is reported to be non-toxic in animals up to 2000 mg/kg [15] and well known for its beneficial activity against inflammation, cancer, arthritis, diabetes, cardiovascular diseases, renal disorder and hepatic toxicity [16].

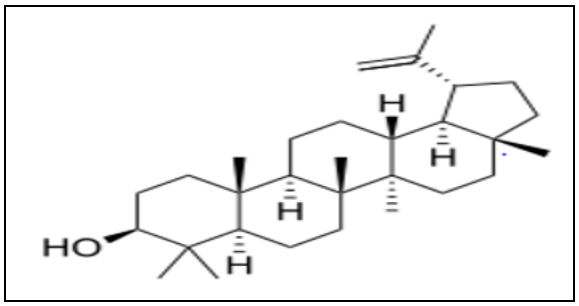

FIG. 1: STRUCTURE OF LUPEOL

Upon investigation, a good amount of active pentacyclic triterpenoids has been reported to be present in leaves of *A. scholaris* [17]. Thus, attempts have been made in the present study to develop quick and simple HPTLC method to identify and quantify lupeol in the ethanol (EtAS), and chloroform (ChAS) extracts of leaves of *A. scholaris*. Also, the developed method was validated following ICH guidelines for the extract possessing greater amount of lupeol.

MATERIAL 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 EtAS and ChAS extracts of leaves of *A. scholaris*. Extraction was continued till the siphon tube showed colorless solvent, after which extracts obtained were collected. Excess of solvent from each extract was evaporated on an electronic water bath to obtain semi-solid mass. All extracts were stored in air tight container in the refrigerator at 2-8 °C.

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

Chemicals and Reagents: Standard Lupeol (purity ≥94%) was procured from Sigma Aldrich (USA). Methanol, toluene, ethyl acetate, and formic acid were of analytical grade and purchased from S.D. Fine Chem Ltd., (Mumbai, India). Anisaldehyde sulphuric acid (ASR) reagent 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 × 4 cm), Chromatogram immersion device III, TLC plate heater, TLC plate scanner used were from Camag (Muttenz, Switzerland) and 100 µL Hamilton syringe (Sigma-Aldrich, United States).

Standard Stock Solution Preparation: 10 mg of standard lupeol was accurately weighed and transferred to the 10 ml volumetric flask. This 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 a stock solution of 1 mg/ml.

Sample Solution Preparation: 100 mg of EtAS and ChAS were accurately weighed and transferred to 10 ml volumetric flask each. The extracts were initially dissolved in 5 ml 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 a stock solution of 10 mg/ml.

Year of Experimentation and Site: Experiment was performed in the year 2018 at Prin. K. M. Kundnani College of Pharmacy and Anchrom Laboratory, Mumbai, India.

Method:

Identification of Lupeol: Extracts EtAS and ChAS (2 and 5µl each; 10 mg/ml), standard lupeol (2 and 5µl; 1 mg/ml) were loaded on Aluminium silica gel 60 F_{254} TLC plate. Also, extracts (5 µl each; 10 mg/ml) were spiked with standard (5 µl; 1 mg/ml) on same TLC plate.

Chromatographic conditions like mobile phase composition, plate loading volume, chamber saturation time were optimized to obtain better resolution and separation of a band of lupeol from other phytoconstituents present in extracts.

Preparation of Calibration Curve for Quantification: Amount of lupeol present in EtAS and ChAS was determined by constructing a calibration curve of standard lupeol. Different concentrations of standard lupeol 0.1, 0.2, 0.3, 0.4, 0.5 mg/ml (2 µl each) and extracts EtAS and ChAS (10 µl each; 10 mg/ml) were loaded on TLC plate in triplicate. The plate was developed and scanned according to the chromatographic conditions mentioned in **Table 1**. Calibration curve of lupeol was obtained by plotting peak areas ($\times 10^6$) *vs.* concentration of lupeol applied. Amount of lupeol in each extract was calculated from this calibration curve.

TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS FOR IDENTIFICATION AND QUANTIFICATION OF LUPEOL IN EXTRACTS 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: Formic acid (7:2:1v/v/v)
Standard concentration	1mg/ml (Identification); 0.1-0.5mg/ml (Quantification)
Sample concentration	10 mg/ml
Sample Applicator	Camag Linomat 5
Syringe	Hamilton syringe (100µl)
Band length	8 mm
Development distance	80 mm
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	580nm
Software	Vision CATS; version 2.4.17207.2

Validation: The developed HPTLC method was validated for quantification of lupeol in extract ChAS as per the guidelines laid down by International Conference on Harmonization (ICH) concerning linearity and range, specificity, precision, accuracy, the limit of detection (LOD) and limit of quantitation (LOQ) [18].

Linearity and Range: The stock standard solution (1mg/ml) was diluted with methanol to obtain 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml concentration solutions, which gave 200, 400, 600, 800, 1000 ng/band on plate with 2µl of application volume. 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 the calibration curve.

Specificity: Specificity of the method was verified by applying bands of standard lupeol, ChAS, diluent (Methanol), and a mobile phase (Toluene: Ethyl acetate: Formic acid 7:2:1 v/v) which was developed and scanned according to optimized 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 by applying 6 bands each of 3 µl at 100% concentration of standard lupeol (1mg/ml) with the same method on the same day but with different time intervals. 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 the sample with 80%, 100% and 120% of standard lupeol. The percent recoveries and average percent recovery were calculated.

Limit of Detection (LOD): LOD or the lowest amount of analyte that can be detected in the sample, but not necessarily quantified under the

stated experimental conditions was determined based on signal to noise ratio by using the following formula

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

δ is sthe tandard deviation of the response and S is sthe lope of cthe alibration 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 based on signal to noise ratio by using the following formula

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

δ is sthe tandard deviation of the response and S is sthe lope of cthe alibration curve.

RESULTS AND DISCUSSION:

Identification of Lupeol by Developed Method: HPTLC method for detection and quantification of lupeol from extracts EtAS and ChAS was standardized for composition of mobile phase, mobile phase saturation time, and plate loading volume. Optimized mobile phase Toluene: Ethyl acetate: Formic acid (7:2:1 v/v/v) exhibited good resolution of bands as indicated by sharp and symmetrical peaks.

The R_f value of standard lupeol was found to be 0.631. Extracts EtAS and ChAS also displayed a peak at R_f 0.632 and 0.629 respectively, thus denoting the presence of lupeol. Densitogram of standard lupeol and lupeol in extracts EtAS and ChAS at 580 nm is shown in **Fig. 2**.

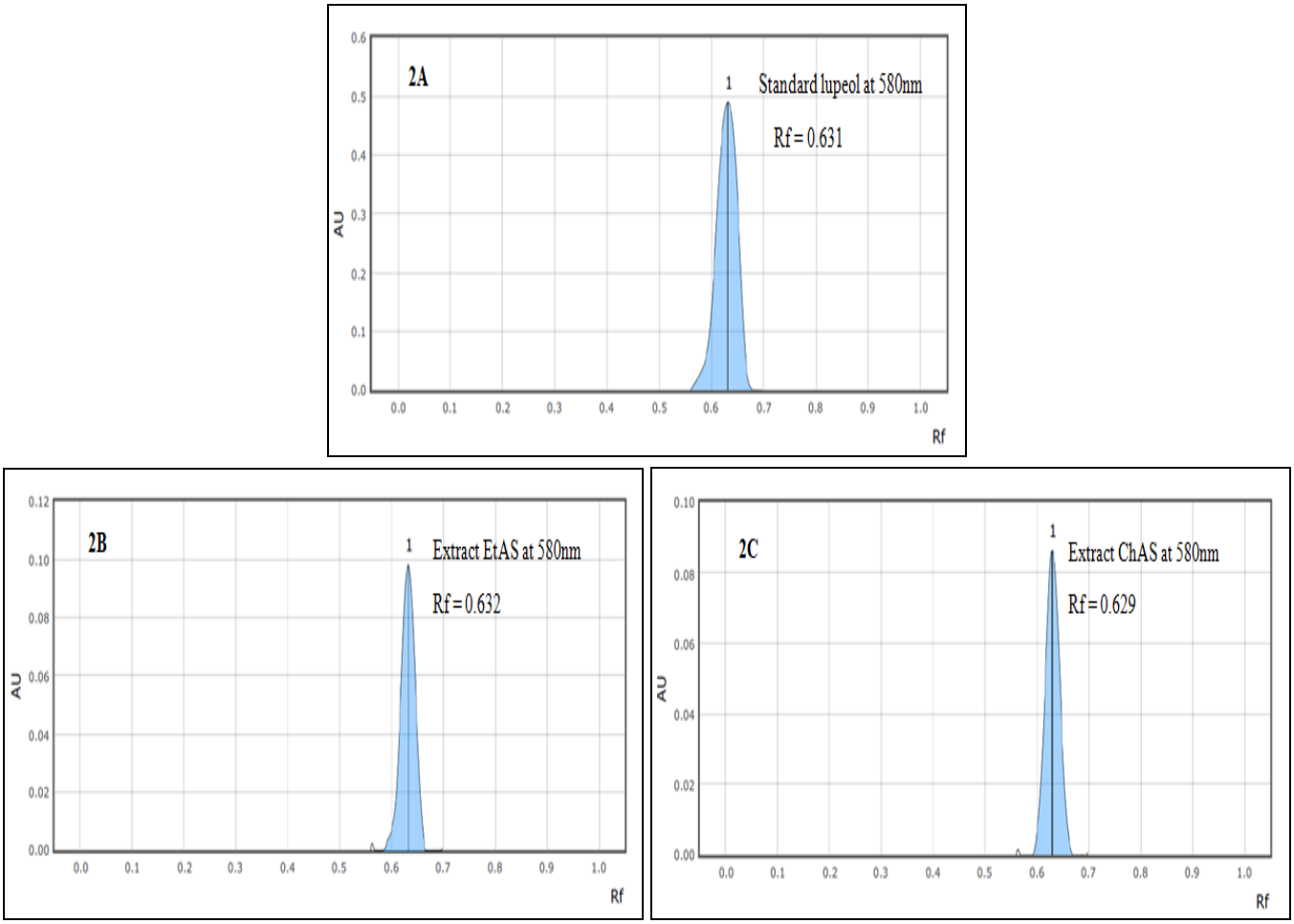

FIG. 2: DENSITOGRAM OF STANDARD LUPEOL, EXTRACT EtAS AND ChAS. Fig. 2A, 2B and 2C represent chromatogram or densitogram of standard lupeol, extract EtAS and ChAS respectively at 580nm. The Rf values were found to be 0.631, 0.632, and 0.629 for standard lupeol, extract EtAS and ChAS, respectively.

Quantification: Amount of lupeol present in extracts EtAS and ChAS was quantified successfully with the developed HPTLC method. The amount of lupeol quantified in extracts EtAS and ChAS was found to be 0.31% and 0.35% w/w respectively. [Refer to **Table 2**] Other components in the extract did not interfere with the analysis. [Refer to **Fig. 3**].

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

Track no.	Sample	Volume applied (µl)	Amount of sample (µg/band)	R_f	Amount of lupeol (ng/band)	Concentration of lupeol (µg/10mg of extract)	Mean concentration/ 10mg of extract
1	EtAS	10	100	0.63	317.1	31.71	
2	EtAS	10	100	0.63	318.2	31.82	31.64µg
3	EtAS	10	100	0.63	314.0	31.4	
9	ChAS	10	100	0.63	352.6	35.22	
10	ChAS	10	100	0.63	349.4	34.94	35.26µg
11	ChAS	10	100	0.63	356.3	35.63	

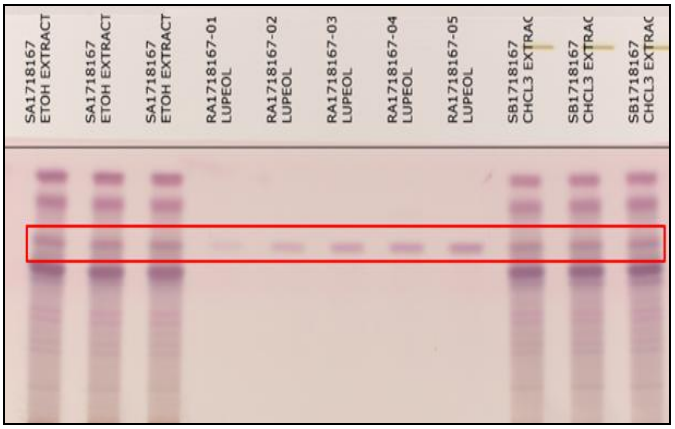

FIG. 3: QUANTIFICATION PLATE. It represents HPTLC fingerprint profile used for quantification of lupeol in extracts EtAS and CHAS concerning standard lupeol used in the concentration range of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml.

Method Validation: The proposed method was validated using extract ChAS, which exhibited significant high content of lupeol in it.

Linearity and Range: Linearity of lupeol was validated by the linear regression equation and correlation coefficient. The calibration curve was found to be linear over the concentration range of 200-1000 ng/band, and correlation coefficient (r^2) was found to be 0.999018 [Refer **Fig. 4**, **Table 3**].

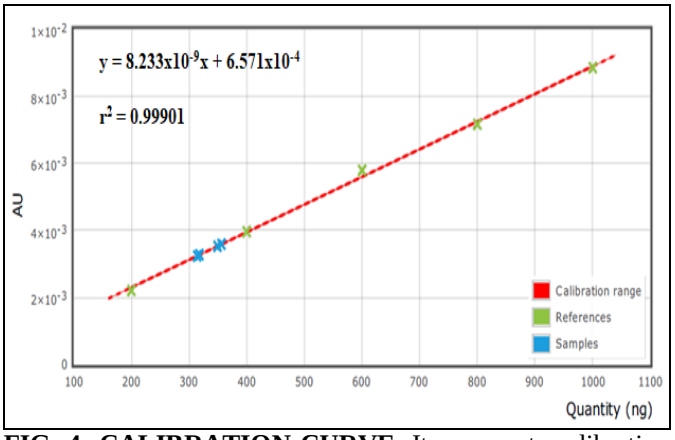

FIG. 4: CALIBRATION CURVE. It represents calibration curve for quantification of lupeol in extracts EtAS and ChAS where the x-axis represents the quantity of lupeol in ng and y-axis represents area under the curve

TABLE 3: LINEARITY AND RANGE OF CALIBRATION CURVE

Parameters	Result
Range of linearity	200-1000 ng/band
Regression equation	$y = 8.233\times10^{-9}x + 6.571\times10^{-4}$
Correlation coefficient (r^2)	0.99901812
Coefficient of variation (%CV/% RSD)	1.85%

Specificity: Peak purity was assessed by comparing peak start, peak apex, and peak end of an extract with that of standard. The R_f of extract and standard lupeol was found to be 0.63. There was no other interfering peak by other phytoconstituents of extract around the retention time of lupeol. Also, mobile phase and diluent did not show any interference. Thus, the method was found to be quite specific for the determination of lupeol in ChAS [Refer **Fig. 5**].

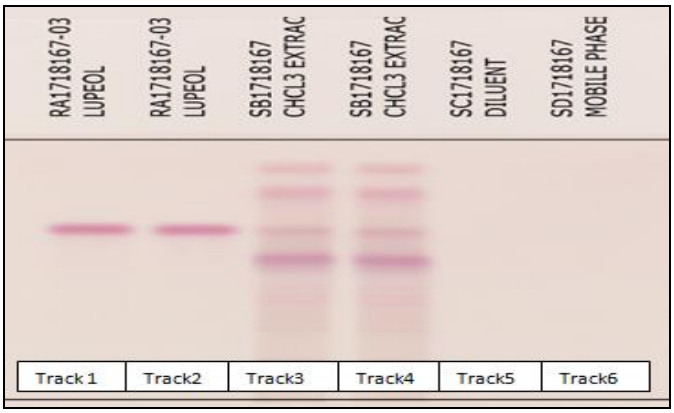

FIG. 5: SPECIFICITY PLATE. It represents a TLC plate used for assessment of specificity of the proposed method. Track 1 and 2 represents standard lupeol, 3 and 4 represents extract ChAS and 5, and 6 represents diluent (methanol) and mobile phase Toluene: Ethyl acetate: Formic acid (7:2:1 v/v/v).

Precision: Intra-day and inter-day precision were assessed in terms of %RSD. 3µl of standard lupeol was loaded on the plate. Intra-day and inter-day precision where n=6 was found to be 1.10%, 1.23%, 0.82% and 1.10%, 1.21% and 1.07% respectively. The values were less than 2% in all cases, which indicates good precision of the proposed method [Refer to **Table 4**].

TABLE 4: INTRA-DAY AND INTER-DAY PRECISION OF LUPEOL

Track no.	Quantity of lupeol (µg/band)	Peak area (x10^6) for Intra-day precision			Peak area (x10^6) for Inter-day precision		
		1	2	3	Day 1	Day 2	Day 3
1	3	0.01434	0.01559	0.01475	0.01434	0.01603	0.01459
2	3	0.01445	0.01581	0.01491	0.01445	0.01624	0.01435
3	3	0.01437	0.01535	0.01512	0.01437	0.01654	0.01450
4	3	0.01469	0.01550	0.01463	0.01469	0.01592	0.01463
5	3	0.01428	0.01572	0.01489	0.01428	0.01634	0.01437
6	3	0.01471	0.01593	0.01506	0.01471	0.01627	0.01470
	Mean	0.01447	0.01565	0.01489	0.01447	0.01622	0.01452
	S.D.	0.00016	0.00019	0.00016	0.00016	0.00020	0.00012
	% R.S.D.	1.105%	1.214%	1.074%	1.105%	1.233%	0.826%

Accuracy: Average percent recovery was found to be 91.88% when extract ChAS was spiked with 80%, 100% and 120% of standard lupeol [Refer **Table 5**, **Fig. 6**].

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

Sample volume (µl)	% lupeol spiked	Lupeol spiked in µl	Peak area (×10^6)	% Recovery	Average recovery
3	80	1.6	0.010592	96.15	
3	100	2.0	0.010985	85.53	91.88%
3	120	2.4	0.012794	93.97	

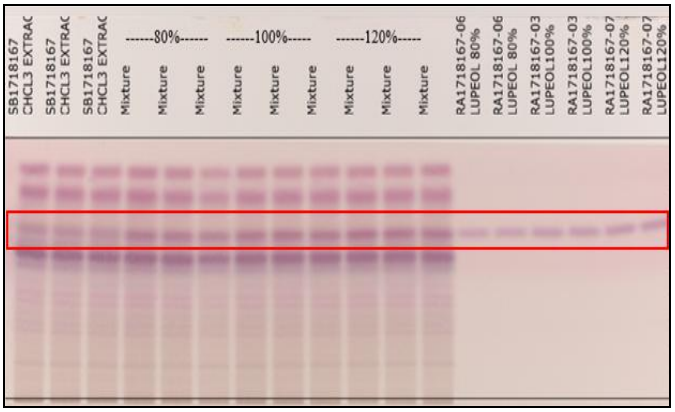

FIG. 6: RECOVERY/ACCURACY PLATE. It represents a TLC plate used for determination of % recovery when extract ChAS was spiked with 80%, 100% and 120% of standard lupeol.

LOD and LOQ: The LOD and LOQ for extract ChAS were found to be 55.0 ng/band and 166.69 ng/band respectively.

CONCLUSION: A new HPTLC method was developed for the identification and quantification of lupeol in the leaves 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 simple, specific, sensitive, precise and accurate for identification and quantification of lupeol in the leaves of *A. scholaris*. Thus, the developed and validated method can be used for standardization, quality control analysis and quantification of lupeol in leaves of *A. scholaris.*

Also, as leaves of *A. scholaris* possess therapeutic potential in treatment and management of various medical conditions and they possess good content of triterpenoid, thus can be used as a potential source for isolation of one of the pharmacologically important pentacyclic triterpenoid-lupeol.

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CONFLICT OF INTEREST: The authors of this research article declare no potential conflict of interest.

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