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HP-TLC DENSITOMETRIC ANALYSIS OF LUPEOL FROM *ELEPHANTOPUS SCABER* [LINN.]

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ABSTRACT

A high performance thin layer chromatography (HP-TLC) method was developed for identification, quantification and validation of lupeol from methanolic and hydro-methanolic extract of *Elephantopus scaber* L. TLC Si gel 60 F_{254} precoated plate was used as a stationary phase. Toluene: Ethyl acetate: Formic acid (12:7:1 v/v/v) was used as a mobile phase for better separation and resolution. Quantification was performed by densitometric scanning at 540 nm after derivatizating with anisaldehyde sulphuric acid reagent (ASR). Compact spot at R_f 0.67 was obtained corresponding to standard lupeol in the plant sample. According to the ICH guidelines, this method was validated in terms of specificity, precision and accuracy assay. Linearity range for lupeol was 100 – 800 ng/band with correlation of coefficient (r^2) = 0.99319. The LOD and LOQ were found to be 9.34 and 28.31 ng/band, respectively. The lupeol content in methanol and hydro-methanol extract were found to be 386.0 and 301.3 µg/mg, respectively. The HP-TLC method was found to be rapid, simple, specific, precise, accurate and convenient method for screening of active principles present in the plant extracts. This method can also be used for routine analysis of quality control of herbal formulations containing *E. scaber*.

KEYWORDS: *Elephantopus scaber* L., HP-TLC, Lupeol, Method development, Quantification, Validation.

INTRODUCTION

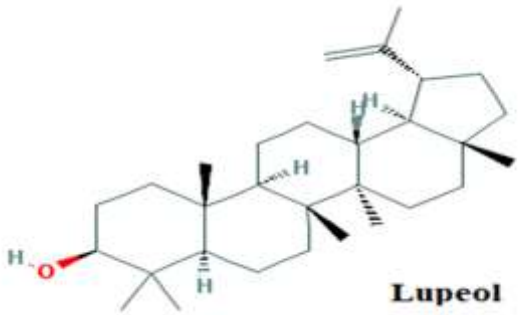
Elephantopus scaber L., family Asteraceae, is a small herb found in Neotropics, Europe, Asia, Africa and Australia. *E. scaber* L. has been matter of research interest for many workers as it has been reported to have anticancer characteristics (Huang et al., 2010).

Phytoconstituents such as Deoxyelephantopin (sesquiterpene lactones), Lupeol (triterpenoids), Stigmasterol (sterols), Quercetin (flavonoids), Stearic acid (fatty acid) and Vanillic acid (phenolic acids) are some of the constituents reported in various parts of the plant (Ahmad et al., 2009; Chang et al., 2012). Many other biological activities such as anti-microbial, anti- viral, anti-oxidant, anti-cancer, anti-diabetic and hepatoprotective have been reported in various research papers (Farha et al., 2014).

The advancement in analysis and quality control of the herbal formulations has progressed to an integrative and comprehensive direction, in order to learn their complex nature. Thus, chromatographic techniques and spectral fingerprints play an important role in such analysis (Gong et al., 2005). High performance thin layer chromatography (HP-TLC) is one of the sophisticated analytical technique due to its advantages of dependability in quantification of biomolecules. HP-TLC has major advantage in reduction of analysis time. HP-TLC has capability of detecting more compounds with samples having high matrix interference. Compounds like sugars, amino acids which do not have UV absorption, are easily detected detected with the help of derivatizing reagents. The instrumental TLC chromatogram patterns are used for fingerprinting of the active compounds in plants. These active compounds can also be extracted using preparative HP-TLC method. Furthermore, HP-TLC provides a large feedback at UV (254 nm), visible light and fluorescence (366 nm) in the plant extracts, that are obtained due to different biomolecules reacting differently to derivatizing reagents. Simultaneous analysis of multiple samples in a short duration can be carried out.

Phytochemical lupeol is an active triterpenoid. It has potential medicinal properties such as anti- coagulant activity (Sankaranarayanan et al., 2010), anti-snake venom activity (Mors et al., 2000), anti- mutagenic activity (Ragasa et al., 1995) and anti-cancer activity (Pitchai et al., 2014).

Literature survey has revealed that no proper method has been reported for quantification and validation of lupeol in *E. scaber*. Hence, in this present work a densitometric HP-TLC method has been developed and validated for standard lupeol in methanol and hydro-methanol extract of *E. scaber*. The following method has been validated as per ICH guidelines (ICH, 1994, 2002, 2005).



MATERIALS AND METHODS

Plant Material

The leaves of *Elephantopus scaber* (healthy and disease free plant sample) was collected from Matheran, Raigad district, MS, India in the month of August 2017. The plant was taxonomically authenticated from Department of Botany, Blatter Herbarium, St. Xavier's College, Mumbai (Voucher specimen no. 2943 of H. Santapau). The plant material was washed, cleaned, dried under shade and finely powdered for further use.

Equipments and Chemicals

CAMAG make HPTLC instrument includes – Linomat 5, Twin Trough Chamber, Derivatizer, TLC Plate Heater III, Visualizer, TLC Scanner-4 along with VisionCats – 2.4 version software was used for analysis. All chemicals like Toluene, Ethyl acetate, Formic acid, Water and Methanol were HPLC grade and Merck make with 99.8% purity. Standard lupeol was procured from Sigma-aldrich. Standard volumetric flask, pipettes and beakers of borosil make were used for analysis.

Sample Preparation

As per the USP 203 chapter, sample extraction procedure was followed. Leaf extract was prepared using leaf powder in methanolic and hydro-methanolic (60% methanol) solvents with concentration of 100 mg/ml.

Standard Solution of Lupeol

Stock solution of lupeol was prepared with 1 mg/ml (1000 ppm) concentration in methanol. Working solution of lupeol (0.1 mg/ml; 100 ppm) was prepared with 1ml of stock solution diluted upto to 10 ml with methanol. This working solution was used further for HP-TLC densitometric analysis.

HPTLC instrumentation and conditions

A high-performance thin layer chromatography (HPTLC) was used to standardize the methanol and hydro-methanol extract of *E. scaber*. The chromatography was performed on 20×10 precoated silica gel 60 F_{254} TLC plates, using lupeol as a marker compound. To get good separation and resolution of various compounds present in *E. scaber*, different combinations of mobile phases was tried. Based on our observations, Toluene: Ethyl acetate: Formic acid (12:7:1 v/v/v) were selected as mobile phase to accomplish the standardization. With help of CAMAG automatic TLC sampler-4, samples along with the marker compound of different concentration was applied on the TLC plate. A constant application rate of 150 nL/s was employed with band width of 8 mm. CAMAG twin trough developing chamber was used to develop TLC plate with pre-saturation of 20 min at temperature (21^0C) and relative humidity of 40% RH. The developed plate was dried and derivatized by spraying anisaldehyde sulphuric acid reagent (ASR) with post-heating at 110^0C for 3 min and scanned with CAMAG scanner-4 (λ = 540 nm). The scanning speed was employed at 20 mm/s and the slit dimension was kept at 6.0×0.45 mm, micro (Table 1).

Table 1: HPTLC - chromatographic conditions.

Parameters	Description
Stationary phase	20×10 TLC Silica gel 60 F_{254}
Mobile phase	Toluene: Ethyl acetate: Formic acid (12:7:1 v/v/v)
Temperature and humidity	21^0C, 40%
Band length	8 mm
Application position (X,Y)	20 mm, 8 mm
Dosage speed	150 nL/s
Chamber saturation	20 min
Solvent front	70 mm
Derivatizing reagent	Anisaldehyde Sulphuric acid Reagent (ASR)
Plate heating (after derivatization)	3 min at 110^0C
Scanning	540 nm (Tungsten lamp)
Slit	6.0×0.45 mm, micro

Estimation of lupeol in herbal extract

Calibration curve plot was used to determine the content of lupeol in the plant sample with standard concentration ranged from 100-1000 ng/spot. Lupeol was applied on the TLC plate of concentration 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ng/spot. Along with the standard, 5 µl of methanol and hydro-methanol extract were applied in triplicates. Each concentration peak area was plotted against the concentration of lupeol spotted.

Validation of HP-TLC method

According to the ICH guidelines, the developed method was validated for hydro-methanolic extract (ICH, 2005). Limit of detection (LOD) and limit of quantification (LOQ) was determined for standard lupeol. The specificity of the method was ascertained by comparing R_f value after derivatization and visible light spectra (peak area) of lupeol in sample, standard, mobile phase, methanol and hydro-methanol chromatogram. For the precision studies, intra-reproducibility and inter-reproducibility were determined. Accuracy was carried out by recovery studies after spiking known concentration of standard to pre-analyzed samples.

RESULTS AND DISCUSSION

Optimization of solvent system

Different trials were made for the development of the mobile phase using many solvents in different proportions. When the mobile phase consisting petroleum ether: ethyl acetate: formic acid (10:8:2 v/v/v) was used, spot was observed at R_f value 0.73 for lupeol. But it was found that the compactness and sharpness of peak was poor. Thus, in order to improve the resolution of the peak, a new mobile phase with composition of toluene: ethyl acetate: formic acid (12:7:1 v/v/v) was used. This achieved a very compact spot at R_f value 0.67 for lupeol in standard and as well as in samples with better resolution (Fig. 1 and 2A, B, C).

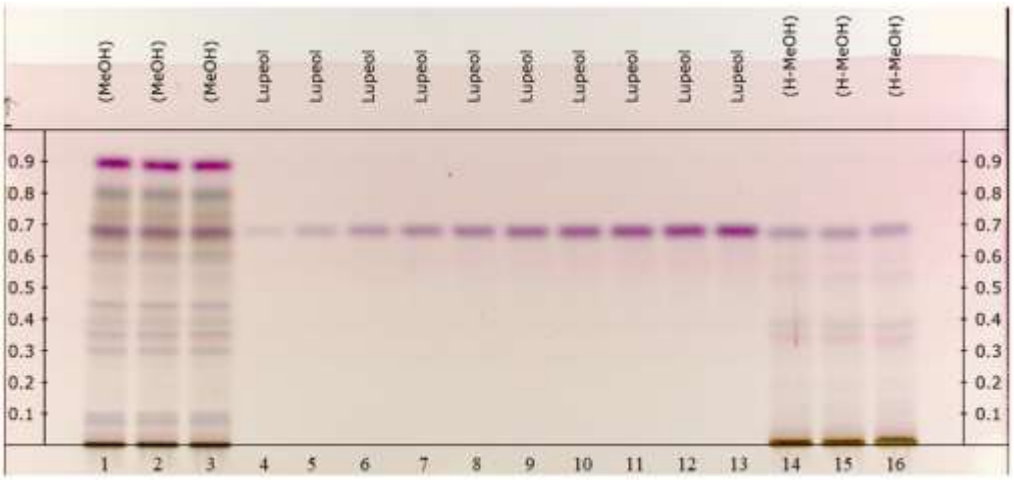


Fig. 1: HP-TLC chromatoplate of lupeol, methanol and hydro-methanol extract of *E. scaber* after derivatization (540 nm).

Track 1-3 is methanol extract 5 µl/spot; track 4-13 is standard lupeol of concentration 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 ng/spot and track 14-16 is hydro-methanol extract 5 µl/spot.

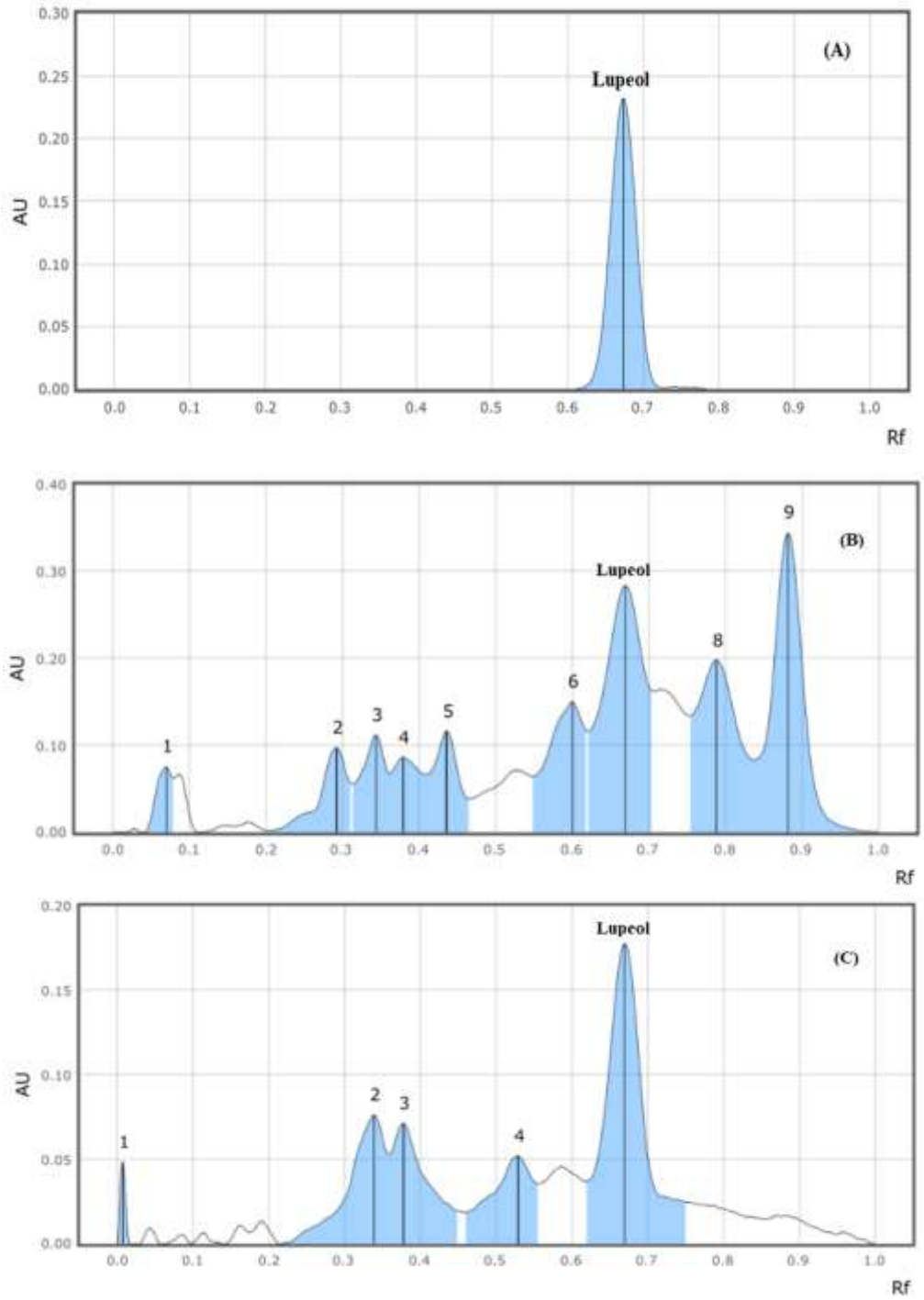


Fig. 2: HPTLC chromatogram of lupeol standard (A), methanol extract (B) and hydro-methanol extract (C) after derivatization (540 nm).

Linearity curve

A calibration curve was plotted by peak area against concentration. Linearity was evaluated with different concentrations for lupeol. The linear regression of standard curve was determined with $r^2 \pm SD = 0.99319 \pm 0.05$. The linear regression line is $Y = 1.514\times10^{-8}x + 1.47\times10^{-3}$. With comparison to lupeol peak area of samples, a regression data for

concentration range of 100-800 ng/spot of standard lupeol, has shown better linear relationship (Table 2 and Fig. 3).

Table 2: Linear regression data for the calibration curve of Lupeol and R_f.

Parameters	Lupeol
Linearity range (ng/spot)	100-800 ng/spot
Regression equation	$Y = 1.514\times10^{-8}x + 1.47\times10^{-3}$
Correlation coefficient (r^2)	0.99319± 0.05
Coefficient of variation (CV)	4.912 %
R_f	0.67 ± 0.0012
LOD	9.34 ng/band
LOQ	28.31 ng/band

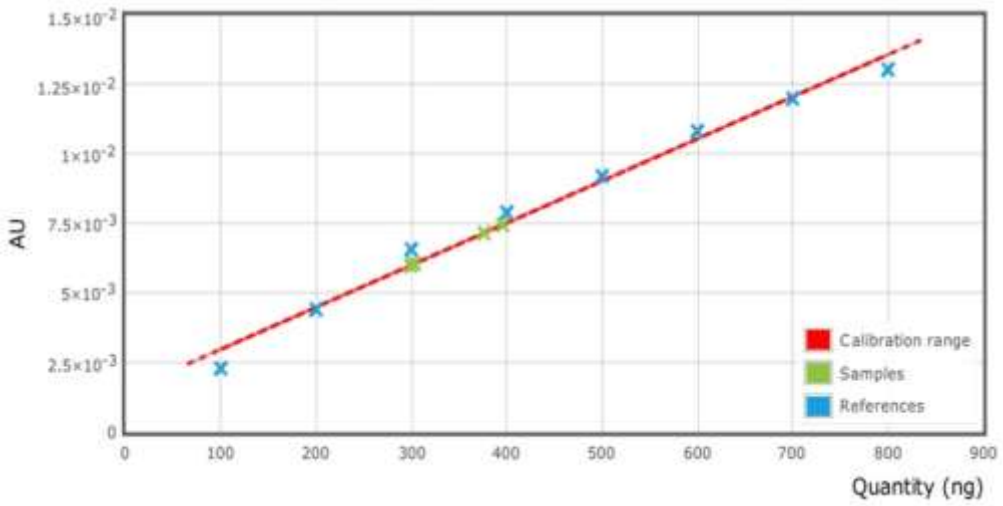


Fig. 3: Calibration plot of lupeol using concentration versus peak area.

Analysis of sample

The samples were spotted in triplicates along with standard lupeol on TLC plate, developed and derivatized. Quantification of lupeol present in herbal extract was determined with the help of linearity curve. Lupeol content in methanol extract consists of 77.20 µg/ml with % RSD = 3.578 while hydro- methanol extract consists of 60.27 µg/ml with % RSD = 0.982 (% RSD ≤ 5). No interference was observed in the samples with adjacent constituents. Resolution between peaks was also good (Table 3 and Fig. 4).

Table 3: Quantification of lupeol in plant extract of *E. scaber* after derivatization (540 nm).

Extract	Concentration (µg/ml)	% RSD	Concentration (µg/mg)
Methanol	77.20	3.578	386.0
Hydro-methanol	60.27	0.982	301.3

RSD – Relative standard deviation

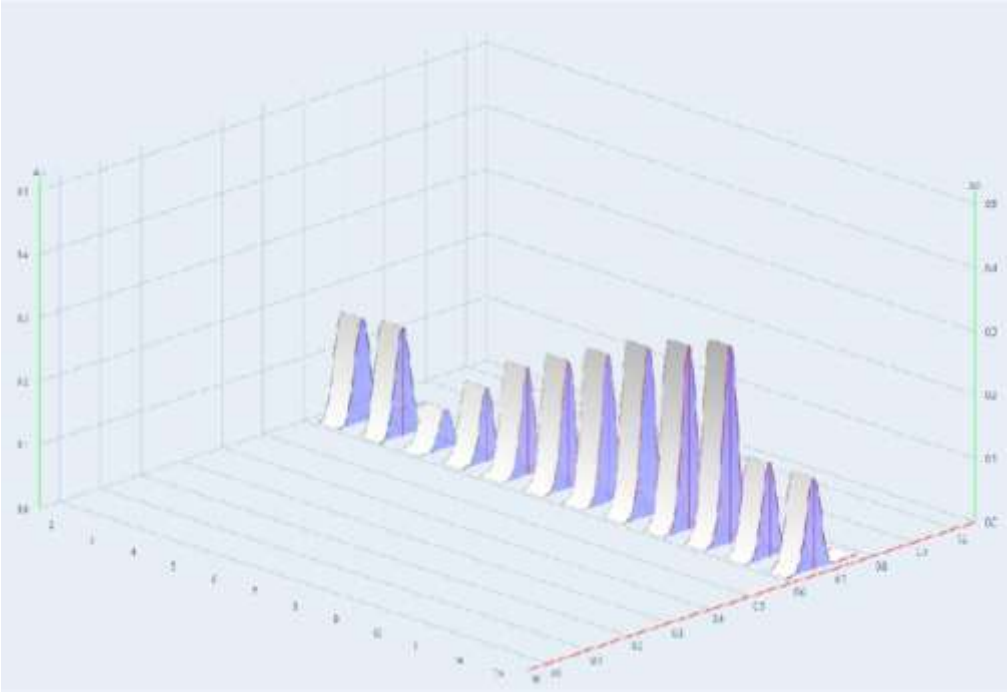


Fig. 4: 3-D chromatogram for quantification of lupeol in plant extracts after derivatization (540 nm).

Lupeol was identified in n-hexane and methanolic extract of root, stem and leaves of *E. scaber* using GC-MS. It was reported that 9 hours extraction time using methanol, stem shows the highest area percentage (69.502%) of lupeol (Ahmad et al., 2009). According to Beeran et al. (2015), lupeol was found in the dichloromethane fraction of ethanolic extract of *E. scaber* at R_f 0.60 with 10.37% of the fraction. The mobile phase used by the authors was toluene: ethyl acetate: formic acid (6:3:1) for HPTLC fingerprinting.

Method Validation LOD and LOQ

For the estimation of LOD and LOQ, different concentrations of standard lupeol were applied along with methanol as blank and determined on the basis of signal to noise ratio. LOD was calculated at an S/N of 3:1 and LOQ at 10:1. This was experimentally verified by diluting the known concentration of standard lupeol until the average responses were 3 and 10 times the SD of the responses. The LOD and LOQ were found to be 9.34 ng/band and 28.31 ng/band, respectively (Table 1).

Specificity/Selectivity

In specificity, impurity method was used for validation. This method was ascertained by analyzing standard lupeol and extract. Bands of hydro-methanol extract, methanol, mobile phase, 60% methanol solvent and standard lupeol was applied on 20×10 TLC plate in

triplicates. Plate was developed and derivatized. The spot for lupeol in the sample was confirmed by comparing with the Rf (0.67) of the spot with that of standard. While no other tracks showed any corresponding spots. Thus, this method found to be highly selective (Fig. 5).

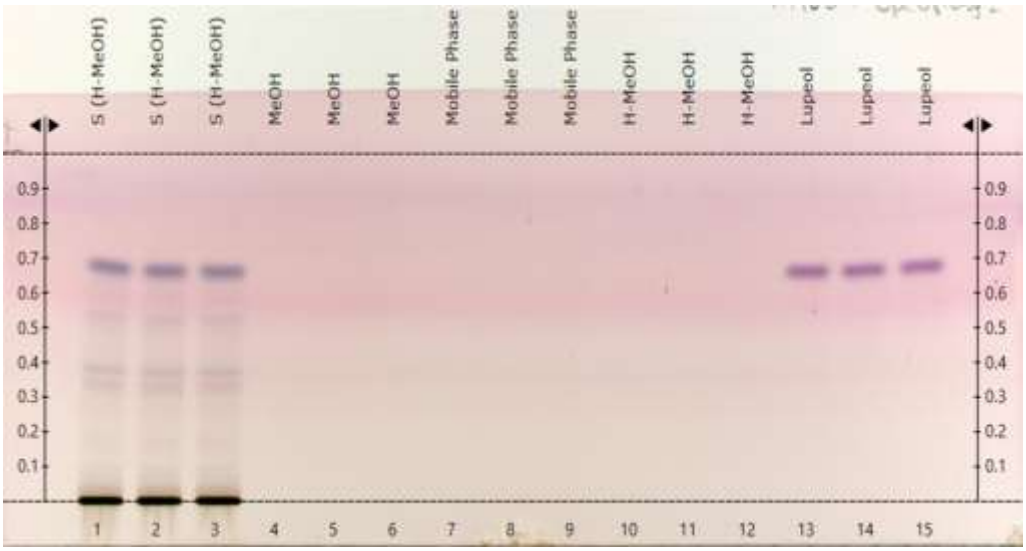


Fig. 5: HP-TLC chromatoplate for specificity after derivatization (540 nm).

Track 1-3 is hydro-methanol extract (5µl/spot); Track 4-7 is methanol solvent (5µl/spot); Track 7-9 is mobile phase (5µl/spot); Track 10-12 is 60% methanol solvent (5µl/spot) and Track 13-15 is standard lupeol (5µl/spot).

Accuracy

The hydro-methanol sample was spiked with 80%, 100% and 120% of the standard lupeol and the mixture was analyzed by using the proposed method. The experiment was conducted in triplicates. The content of the lupeol in the sample was quantified and percentage recovery was calculated. This was done to check the recovery of the drug at different concentration levels in the sample, which should be greater than 85% in herbal samples. Thus, standard recovery of lupeol for 80%, 100% and 120% spiked are 90.18%, 86.78% and 86.92%, respectively which gives average of 87.96% recovery. The method is said to be accurate when % RSD $\leq$ 2 (Table 4).

Table 4: Accuracy as recovery data of lupeol (n = 3).

% standard spiked to the sample	Volume (µl)		Concentration spotted (ng)		Theoretical Conc. (sample + std.)	Total Conc. obtained (area ± SD)	% RSD	% Standard Recovery
	Std.	Sample	Std.	Sample				
80	4	5	355	431	786	711.66 ± 7.09	0.99	90.46
100	5	5	450	431	881	773.00 ± 8.88	1.14	87.74
120	6	5	523	431	954	831.00 ± 6.00	0.72	87.00
% Average recovery								88.40

SD – Standard deviation; RSD – Relative standard deviation.

Precision

The precision is a method to check extent to which the individual test results of multiple injections of a standard agree. Intra-day precision and inter-day precision of the method were determined. The mean, standard deviation (SD) and percent relative standard deviation (% RSD) were calculated for peak area. Intra-day precision was evaluated by analysis of replicate application of freshly prepared standard lupeol solution of same concentration (500 ng/spot) six time on the same day. While, inter-day precision was calculated for three consecutive days. The method is said to be precised when the value of % RSD ≤ 2 (Table 5). The stability of the compound lupeol was found to be for 48 hrs from the time of preparation of stock solution. Henceforth, the compound had started degrading by giving % RSD > 2.

Table 5: Intra-day and inter-day precision of HPTLC method (n = 6).

Volume of standard (µl)	Intra-day Precision		Inter-day Precision		Inter-system Precision	
	Mean conc. (ng) ± SD	% RSD	Mean conc. (ng) ± SD	% RSD	Mean conc. (ng) ± SD	% RSD
5	511.88 ± 4.32	0.84	529.43 ± 6.70	1.27	537.83 ± 7.07	1.31

SD – Standard deviation; RSD – Relative standard deviation.

CONCLUSION

The quantity of active principle present, always have an effect on the activity of plant extract. So it is essential to develop a standardized method for optimization of bio-markers in herbal products. Thus, a rapid, specific, accurate and precise HPTLC method for quantitative estimation of lupeol present in dried leaf powder of *Elephantopus scaber* has been developed and validated. When compared with other constituents of the plant material, the method in this work resulted in good R_f, peak area and resolution of the marker. As per the HPTLC images, all the sample constituents were clearly separated without any diffusion and tailing (Fig. 1 and 2). Lupeol was found to be present in methanol and hydro-methanol leaf extract of about 38.6% w/w and 30.13% w/w, respectively. Thus, the comparative study of this bio-

marker has showed that it is present in higher concentration in methanol extract than the hydro-methanol extract.

In summary, the proposed HPTLC method for the lupeol analysis from leaf extract is very simple, sensitive and suitable method for routine quality control analysis and quantification of lupeol in *E. scaber*. This method may also be useful for other herbal formulations and standardization purposes.

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CONFLICT OF INTEREST

Conflict of interest declared none.

REFERENCES

1. Ahmad A, Alkarkhi AF, Hena S and Khim LH. Extraction, separation and identification of chemical ingredients of *E. scaber* L. using factorial design of experiment. Int. J. Chem., 2009; 1: 36-49.
2. Beeran AA, Maliyakkal N, Rao CM and Udupa N. The enriched fraction of *Elephantopus scaber* triggers apoptosis and inhibits multi-drug resistance transporters in human epithelial cancer cells. Phcog. Mag., 2015; 11(42): 257-268.
3. Chang CL, Shen CC, Ni CL and Chen CC. A new sesquiterpene from *E. scaber*. Hiromitsu J., 2012; 65: 49-56.
4. Farha A.K. and Remani P. Phytopharmacological profile of *Elephantopus scaber*. Pharmacologia, 2014; 5(8): 272-285.
5. Gong F, Wang BT, Chau FT, Liang YZ. Data preprocessing for chromatographic fingerprint of herbal medicine with chemometric approaches. Analytic Letters, 2005; 38: 2475–2492.
6. Huang CC, Lo CP, Chiu CY and Shyur LF. Deoxyelephantopin, a novel multifunctional agent, suppresses mammary tumour growth and lung metastasis and doubles survival time in mice. Br. J. Pharmacol., 2010; 159: 856-871.
7. ICH. Validation of analytical procedures: Text and Methodology (Q2AR1), 2005.

8. ICH. Guidance on analytical method validation. In: Proceedings of International Convention on Quality for the Pharmaceutical Industry, Toronto, Canada, 2002.
9. ICH Q2A. Text on validation of analytical procedures. In: Proceedings of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. October 27, 1994. http://www.bcg-usa.com/regulatory/docs/ich/ICHQ2A.pdf.
10. Mors WB, Nascimento MC, Pereira BMR and Pereira NA. Plant natural products active against snake bite: The molecular approach. Phytochemistry, 2000; 55: 627-642.
11. Pitchai D, Roy A and Ignatius C. In vitro evaluation of anticancer potentials of lupeol isolated from *Elephantopus scaber* L. on MCF-7 cell line. J. Adv. Pharm. Technol. Res., 2014; 5(4): 179-184.
12. Ragasa, CY, Sy J, Agbayani V, Infante R, Abaya M and Coll JC. Antimutagens from *E. scaber*. Kimika, 1995; 11: 25-28.
13. Sankaranarayanan S, Bama P, Ramachandra J, Jayasimman R and Kalaichelvan PT et al. In vitro platelet aggregation inhibitory effect of triterpenoid compound from the leaf of *E. scaber* linn. Int. J. Pharm. Pharm. Sci., 1995; 2: 49-51.