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RESEARCH ARTICLE

Method Development and Validation of Simultaneous Estimation of Lupeol, Diosgenin, Oleanolic Acid and Caffeic Acid in *Ayurvedic* Formulation by High-Performance Thin Layer Chromatography

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Abstract

Background: Herbal remedies are widely used for wellness due to their reliability, perceived safety and cultural acceptance. Presently, HPTLC is used widely in quantifying phytoconstituents due to its affordability, efficiency, reduced mobile phase consumption and minimal sample clean-up. **Aim:** The goal of the current research was to develop and validate a fast, accurate HPTLC technique capable of concurrently estimating lupeol, diosgenin, oleanolic acid and caffeic acid in an *Ayurvedic* formulation. **Method:** The HPTLC method employed a silica gel G 60F_{254} coated plate and a mobile phase of n-Hexane: Ethyl acetate: Methanol: Formic acid in 7.2:2:1:0.1, v/v/v/v. Diosgenin, lupeol and oleanolic acid, were derivatised with vanillin-sulphuric acid to enable detection at 449nm, 604nm and 540nm, respectively while caffeic acid was detected at 322nm. The method was validated through ICH Q2 (R2) standards, covering system suitability, linearity, accuracy, precision and robustness. Robustness was evaluated using a Fractional Factorial Design (FFD) of 2^{4-1}, investigating both high-level (+2) and low-level (-2) factors. **Results:** Linearity for lupeol, diosgenin, oleanolic acid and caffeic acid linearity ranged from 1000-5000 ng/band, with correlation coefficients (r^2) of 0.9964, 0.9945, 0.9989 and 0.9997, respectively. Limits of both detection and quantification were determined for lupeol (95.26 and 288.68 ng/band), diosgenin (95.27 and 288.67 ng/band), Oleanolic Acid (OA) (91.66 and 277.77 ng/band), and Caffeic Acid (CA) (82.50 and 250 ng/band). Accuracy was evaluated using recovery methods, demonstrating satisfactory outcomes for lupeol 99.54–101.50 %, diosgenin 100.06–101.09 %, OA 99.89–101.09 % and CA 100.40–100.58 %. Precision study % RSD values were < 2%. The Rf values for lupeol, diosgenin, OA and CA were found to be 0.702, 0.525, 0.453 and 0.186, respectively. An FFD tested the HPTLC method's robustness, showing no impact on lupeol and diosgenin retention factor, but n-Hexane volume significantly influenced OA and CA which is managed carefully. **Conclusion:** The proposed HPTLC techniques were discovered to be precise, accurate and reproducible. The statistical study demonstrates their consistent repeatability, making them equally appropriate for regular assessment of marketed formulations without prior separation.

Keywords: Caffeic Acid, Diosgenin, DoE, HPTLC, Lupeol, Oleanolic Acid

1. Introduction

Herbal remedies are increasingly being used for wellness purposes due to their reliability, perceived safety and cultural acceptance. To ensure the pureness and consistency of pharmaceutical products, spectroscopic and chromatographic techniques are used. In recent times, HPTLC has gained popularity for quantifying

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phytoconstituents due to its affordability, shorter analysis duration, reduced mobile phase consumption per sample and minimal sample clean-up requirements[1-7].

Panviraladi Bhasma Tablets, an *Ayurvedic* formulation, contains *Panavirala* (*Borassus flabellifer*), *Katalati* (*Achyranthes aspera*), *Kokilaksa* (*Acantha longifolia*) and *Kadali* (*Musa paradisiaca*). It is prescribed for conditions such as oedema, abdominal swelling, abdominal lump and ascites. *Panavirala*, the Sanskrit name for *Borassus flabellifer*, is a member of the Arecaceae family. This tree contains diosgenin, a phyto steroid sapogenin. *Achyranthes aspera*, also known as *katalati*, belongs to the Amaranthaceae family and contains OA, a pentacyclic triterpenoid. *Kokilaksa,* the Sanskrit name for *Asteracantha longifolia*, is a part of the Acanthaceae family and contains lupeol, another pentacyclic triterpenoid. *Kadali* or *Musa paradisiaca*, belongs to the Musaceae family and contains CA, a hydroxycinnamic acid[8-13].

The Design of Experiments (DoE) emerges as the most efficient and widely recognised approach in the development and validation of HPTLC techniques. It involves utilising experimental designs, conducting ANOVA analysis to generate mathematical models and creating graphical representations to illustrate the correlation between factors and responses. Additionally, DoE provides a scientifically rigorous way of understanding the multifactorial effects of chromatographic variables on responses, facilitating the identification and determination of optimal chromatographic conditions. In this study, a two-level fractional factorial design was utilised in the robustness assessment to assure the reliability of the developed HPTLC technique for quantitatively assessing lupeol, diosgenin, oleanolic acid and CA[14,15].

2. Materials and Methods

2.1 Standards and Chemicals

The standard biomarkers diosgenin, lupeol, OA and CA were purchased from Yucca Enterprises, Mumbai, India. All analytical grade (AR-grade) solvents for HPTLC were purchased from Sulab (Vadodara, India) and Oxford Lab (Maharashtra, India).

2.2 Marketed Formulation

Panaviraladi Bhasma tablets were procured from a local market in Vadodara.

2.3 Instruments and Software

A CAMAG HPTLC system from Switzerland, fitted with both aLinomat 5 automatic sample applicator along with TLC Scanner 4 through Vison CAT software, was utilised. Design Expert version 13 was used for both experimental design and optimisation analysis.

2.4 Preparation of Standard Reference Solution

Weigh accurately about 1mg of each standard (Diosgenin, Lupeol, OA and CA) and dissolve in 1ml of methanol in separate 10ml volumetric flasks. The volume was filled up to mark by methanol to achieve 100µg/ml solutions for each standard.

2.5 Preparation of Sample Solution

About 10 tablets were accurately taken and made powered in a mortar pestle. Then 10gm of powder was refluxed with methanol for 30 minutes. After that, the extract was filtered, dried and stored in a desiccator for future use. For the sample solution, the extract was dissolved in methanol to obtain a 1000 µg/ml of solution.

2.6 Optimisation of Chromatographic Condition

Based on the literature, multiple trials and employing Silica G60 F254, the mobile phase was optimised as n-hexane: ethyl acetate: methanol: formic acid (7:2:1:0.1, v/v/v/v). Before each run, the chamber was pre-saturated by the mobile phase within 20 minutes.

2.7 Method Validation[16]

The method was validated per ICH guidelines Q2 (R2), covering system suitability, specificity, linearity, precision, accuracy, Limit of Detection (LOD), Limit of Quantification (LOQ) and robustness.

2.7.1 System Suitability

System suitability testing proves the method's suitability. A standard solution of diosgenin, lupeol, OA and CA was spotted five times at 2000 ng/band. The retardation factor and peak area were measured using specified chromatographic conditions.

2.7.2 Specificity

When analysing a polyherbal formulation with multiple markers, it is crucial to compare our selected standards against other biomarkers in the sample. Each reference spot in a sample was confirmed by comparing its distinctive peak R_f values.

2.7.3 Linearity and Range

The linearity of the calibration curves for lupeol, diosgenin, OA and CA was established throughout the concentration range of 1000-5000 ng/band, using spot volumes of 4, 8, 12, 16 and 20 µl. The calibration plot was created by displaying the area against concentration, a regression coefficient was computed.

2.7.4 Precision

Precision was evaluated through repeatability and intra and inter-day measurements. Repeatability involved six measurements of the same concentration (2000 ng/band) within a day, computing mean standard deviation and % RSD. Intraday precision comprised three measurements each of three concentrations (2000, 3000, 4000 ng/band) at three distinct hours, also calculating mean standard deviation and % RSD. Inter-day precision involved three measurements of the three distinct concentrations (2000, 3000, 4000 ng/band) over three distinct days and mean standard deviation and % RSD were computed.

2.7.5 Accuracy

The percentage recoveries of all four phytomarkers were determined at three concentration levels: 80%, 100% and 120%.

2.7.6 LOD and LOQ

Detection and quantification were determined through the calibration curve method. The standard deviation of y-intercepts of regression lines is utilised as the standard deviation. It was calculated as per ICH guidelines questions as described below.

$(LOD) = 3.3 \times \sigma/S$

$(LOQ) = 10 \times \sigma/S$

"σ" stands for the standard deviation of regression y-intercepts, while "S" is the slope of a calibration curve.

2.7.7 Robustness Study Using Full Factorial Design

Robustness was assessed using FFD, which varied four critical factors: ethyl acetate volume (A), n-hexane volume (B), chamber saturation time (C), and solvent front (D). Changes in these factors affected the retardation factor values, deliberately adjusted from the optimum settings to analyze the deviation of the response (R_f) for all biomarkers. To minimise bias, trials were randomised. Eight experimental runs with coded values for factor levels were conducted using FFD.

2.7.8 Quantification of the Formulation

Adequate quantification of standards in formulation was crucial, determined using formulation areas and regression equations.

3. Results and Discussion

Four compounds were simultaneously evaluated using a mobile phase of n-hexane: ethyl acetate: methanol: formic acid (7.2:1:0.1, v/v/v/v). CA was visualized at 322nm before derivatisation, while diosgenin, OA and lupeol were detected at 449nm, 540nm and 604nm, respectively (Figure 1), after derivatisation with vanillin sulfuric acid reagent. R_f values for CA, OA, diosgenin and lupeol were 0.186, 0.453, 0.525 and 0.702, respectively. The optimised chromatographic condition is mentioned in Table 1.

3.1 Validation of the Developed HPTLC Method

3.1.1 System Suitability

System suitability parameters like retardation factor and peak, area are reported in Table 2.

Table 1. Optimised chromatographic condition

HPTLC	Optimised HPTLC Condition
Mobile Phase	n-Hexane: Ethyl acetate: Methanol: Formic acid (7:2:1:0.1 v/v/v/v)
Stationary Phase	Silica gel G60 F_{254} HPTLC plates
Solvent	Methanol
Chamber	Twin Trough Chamber (10× 20cm)
Saturation Time	20 minutes
Wavelength	Caffeic Acid: 322nm Diosgenin: 449nm, Oleanolic Acid: 540nm, Lupeol: 604nm
Visualisation in UV	UV chamber was used for the Caffeic Acid at 254nm.

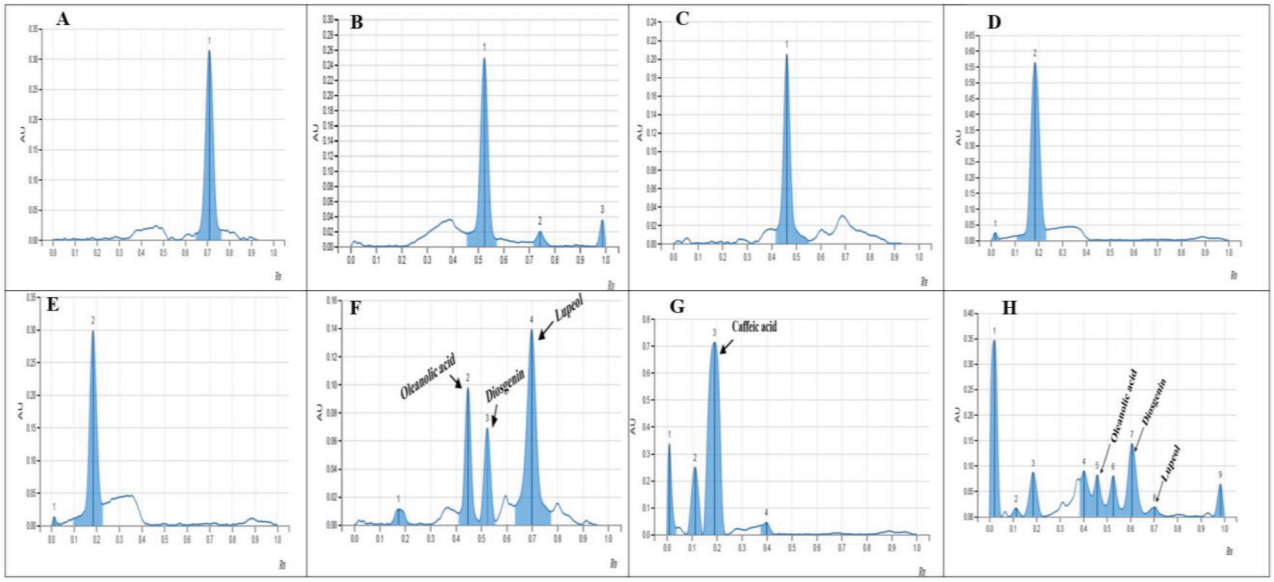


Figure 1. Chromatogram of lupeol, diosgenin, OA and CA in standards, mixture and formulation.

A, B, C, D: chromatograms of standards lupeol, diosgenin, OA and CA; **E:** CA in the mixture before derivatisation; **F:** a mixture of OA, diosgenin and lupeol after derivatisation; **G:** CA in the formulation before derivatisation; **H:** OA, diosgenin and lupeol in formulation after derivatisation.

Table 2. System suitability of diosgenin, lupeol, oleanolic acid and caffeic acid

Biomarkers	Conc.(ng/band)	Mean $R_{f\pm}$ SD	%RSD	Mean Area $_{\pm}$ SD	% RSD
Lupeol	2000	0.706 ± 0.00985	1.39	0.00776 ± 0.00011	1.44
Diosgenin		0.523±0.00563	1.06	0.00566 ± 0.00008	1.57
OA		0.45594 ±0.00136	0.29	0.00617 ±0.00008	1.34
CA		0.1878 ± 0.00327	1.74	0.02536 ± 0.00024	0.94

*n=6, SD= Standard deviation, % RSD (Relative standard deviation)

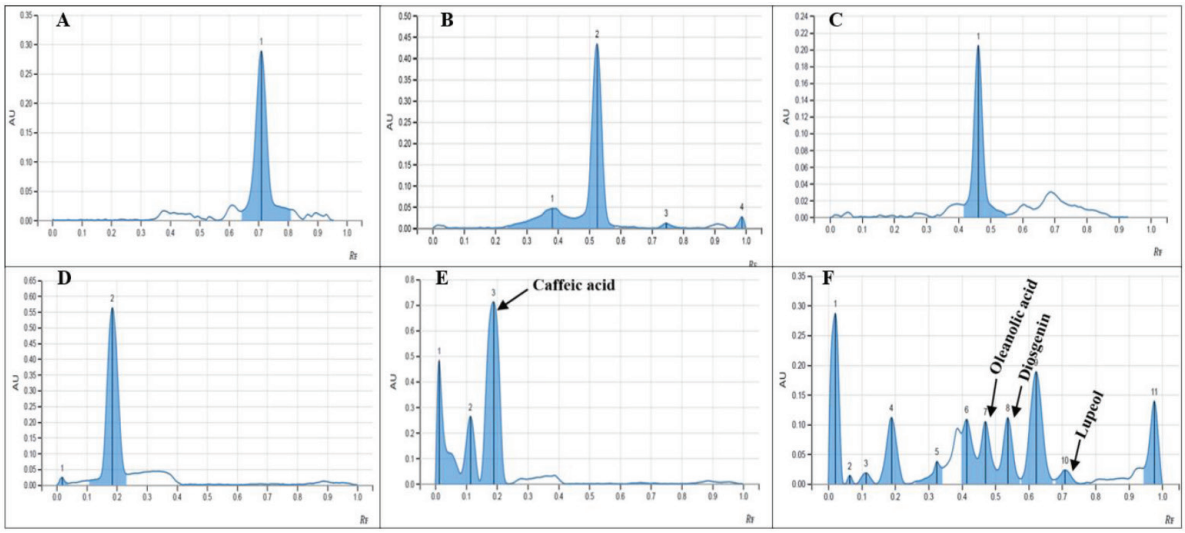


Figure 2. Lupeol, diosgenin, OA and CA peaks in standards and formulation**.**

A, B, C and D: standard peaks for lupeol, diosgenin, OA and CA; E: CA in the formulation before derivatisation; F: OA, diosgenin and lupeol after derivatisation.

The method's suitability was confirmed as the % RSD in repeatability was found to be below 2%.

3.1.2 Specificity

All standards and formulations (peak profiles R_f) are compared and presented in Figure 2. Standards and formulation peak purity are given in Table 3. The corresponding peak purity of all compounds was more than 0.998 indicating the specificity of the method.

3.1.3 Linearity

Linearity was evaluated across five concentrations, and calibration curves were constructed by correlating peak area with concentration. Table 4 summarises the linearity parameters. Individual calibration curves were generated for Lupeol (Figure 3), Diosgenin (Figure 4), OA (Figure 5), and CA (Figure 6). Moreover, Figure 7 presents an overlay of CA and Figure 8 displays an overlay of Lupeol and Diosgenin and OA.

Table 3. Specificity study data

Assigned	R_f	r(s,m)	R(m,e)	Purity
Lupeol	0.702	0.999922	0.999571	OK
formulation	0.704	0.999631	0.997150	OK
Diosgenin	0.523	0.999975	0.999822	OK
Formulation	0.526	0.999894	0.998754	OK
OA	0.453	0.998402	0.992669	OK
Formulation	0.405	0.999033	0.998251	OK
CA	0.189	0.999791	0.998777	OK
Formulation	0.192	0.999384	0.993960	OK

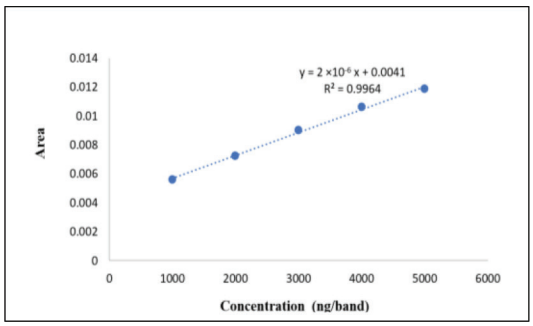


Figure 3. Calibration curve of lupeol.

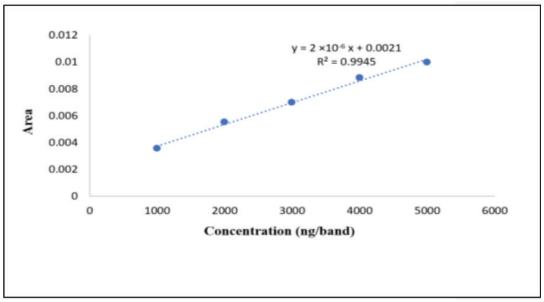


Figure 4. Calibration curve of diosgenin.

3.1.4 Accuracy

The standards' accuracy was evaluated using the standard recovery method across three concentrations (80%, 100% and 120%). The accuracy data is presented in Table 5. All four compounds achieved a recovery percentage within 98-102% and % RSD was < 2 complying with the accuracy standards outlined in ICH guidelines.

3.1.5 Precision

Repeatability was determined through six determinations at a 2000 ng/band concentration, it% RSD was found < 2indicating good precision (Table 6). Intra- and inter-day evaluations were conducted using three determinations at three concentrations (2000, 3000, 4000 ng/band), and the results and %

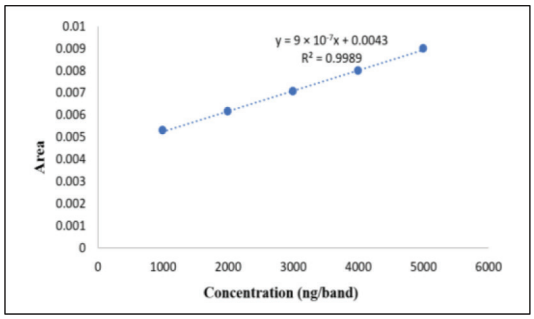


Figure 5. Calibration curve of OA.

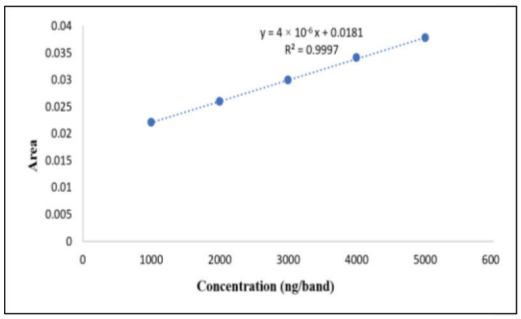


Figure 6. Calibration curve of CA.

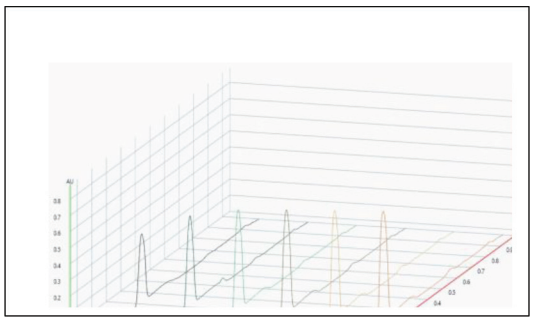


Figure 7. Overlay of CA.

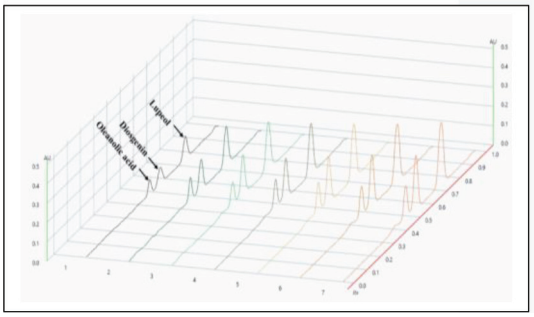


Figure 8. Overlay of lupeol, diosgenin and OA.

Table 4. Method linearity parameters

Parameters	Lupeol	Diosgenin	OA	CA
Linearity Range (ng/band)	1000-5000 ng/band			
Regression Equation	0.000002x+0.001	0.000002x + 0.0021	0.0000009x + 0.0043	0.000004x + 0.0181
R^2	0.9964	0.9945	0.9989	0.9997
Slope ± SD	0.000002±0.0000001	0.000002±0.00000001	0.000003 ±0.000002	0.000004±0.0000001
Intercept ± SD	0.00407± 0.000058	0.00213± 0.000057	0.0043 ±0.0001	0.0181 ±0.0001
LOD (ng/band)	95.26	95.27	91.66	82.50
LODQ (ng/band)	288.68	288.67	277.77	250

*n=3

Table 5. Accuracy study data of lupeol, diosgenin, oleanolic acid and caffeic acid

Standard	% Spiked	Amount Present (ng/band)	Amount Added (ng/band)	Amount Recovered (ng/band)	% Recovery	% RSD
Lupeol	80	1585	1268	2840	99.54	0.43
	100	1585	1585	3175	100.15	0.15
	120	1585	1902	101.51	101.51	0.60
Diosgenin	80	1695	1596	3293	100.06	0.68
	100	1695	1695	3396	100.17	0.32
	120	1695	2034	3785	101.50	0.66
OA	80	2111	1688	3795	99.89	0.42
	100	2111	2111	4260	100.90	0.89
	120	2111	2533	4691	101.09	0.50
CA	80	2260	1808	4092	100.58	0.63
	100	2260	2260	4570	100.40	0.45
	120	2260	2712	4999	100.50	0.50

*n=3, % RSD < 2

RSD were < 2 indicating the method was precise (Tables 7 and 8).

3.1.6 Robustness Study Employing Experimental Design

Experimental outcomes were computed using Design Expert Trial (Version 13), with responses detailed in Table 9. Equation models for various drugs are provided in Table 10. Model validation via ANOVA was conducted. Adequate precision values exceeding 4% (ratios of 19.30, 24.90, 36.05 and 20.32 for lupeol, diosgenin, OA and CA indicate sufficient signal-to-noise ratio for effective design space navigation. Desirable limits were met for a

coefficient of variation (CV) (<10%) ensuring model reliability. Overall, the model demonstrates great agreement among experimental and predicted values, effectively representing the studied phenomenon.

Table 6. Repeatability of diosgenin, lupeol, oleanolic acid and caffeic acid

Conc. (ng/spot)	Conc. (ng/band)	Mean (Area)	SD	% RSD
Lupeol	**2000**	0.007338	0.00008	1.19
Diosgenin		0.00559	0.00006	1.09
OA		0.00628	0.00005	0.95
CA		0.025797	0.000173	0.67

*n=6, % RSD< 2

Table 7. Intraday precision of diosgenin, lupeol, oleanolic acid and caffeic acid

Markers	Conc. (ng/band)	Mean Area	SD	% RSD
Lupeol	2000	0.00727	0.000075	1.03
	3000	0.00872	0.000070	0.80
	4000	0.01051	0.000095	0.9
Diosgenin	2000	0.00536	0.000051	0.95
	3000	0.00704	0.000066	0.96
	4000	0.00875	0.000085	0.97
OA	2000	0.00632	0.000055	0.87
	3000	0.00721	0.000060	0.83
	4000	0.00789	0.000070	0.88
CA	2000	0.02572	0.000080	0.31
	3000	0.02969	0.000155	0.50
	4000	0.03424	0.000120	0.35

*n=3

The Pareto charts of lupeol, diosgenin, OA and CA are shown in Figure 9 and the 3D surface plots of lupeol, diosgenin, OA and CA are shown in Figure 10.

A, B and C represent lupeol interactions (AB, BC, AC); D, E and F represent diosgenin interaction (AB, AD, AC); G, H, and I represent OA interactions (AB, BC, AC); and J, K, and L represent CA interactions (AB,AD,AC). A, D and G showed that ethyl acetate volume raised lupeol, diosgenin and OA, R_f increased, n-hexane volume increased and R_f was decreased. B, E and H indicate that R_f lupeol, diosgenin and OA were decreased when solvent front increased and R_f was increased when n-hexane volume increased. C, F and I show that ethyl acetate and saturation time increase

Table 8. Interday precision of diosgenin, lupeol, oleanolic acid and caffeic acid

Markers	Conc. (ng/band)	Mean Area	SD	% RSD
Lupeol	2000	0.00752	0.00008	1.14
	3000	0.00888	0.00009	1.01
	4000	0.01065	0.00011	1.07
Diosgenin	2000	0.00555	0.00008	1.43
	3000	0.00767	0.00008	1.05
	4000	0.00891	0.00010	1.12
OA	2000	0.00651	0.00011	1.7
	3000	0.00739	0.00008	1.15
	4000	0.00789	0.00007	1.0
CA	2000	0.02587	0.00019	0.74
	3000	0.02980	0.00016	0.55
	4000	0.03456	0.00018	0.53

*n=3

Table 9. Fractional factorial design obtained responses

A: Volume of ethyl acetate (ml)	B: Volume of n-hexane (ml)	C: Saturation time (min)	D: Solvent front (mm)	R_f of lupeol	R_f of diosgenin	R_f of OA	R_f of CA
2.2	6.8	22	78	0.823	0.633	0.535	0.253
1.8	7.2	22	78	0.741	0.547	0.419	0.199
1.8	6.8	18	78	0.74	0.556	0.439	0.266
1.8	6.8	22	82	0.751	0.566	0.468	0.229
2.2	7.2	22	82	0.75	0.493	0.403	0.15
1.8	7.2	18	82	0.74	0.499	0.38	0.145
2.2	6.8	18	82	0.799	0.545	0.45	0.206
2.2	7.2	18	78	0.749	0.540	0.433	0.198

Table 10. Predicted response model and statistical parameters

Response	Lupeol	Diosgenin	OA	CA
Types of Model	**2FI**			
Polynomial Equation	0.7616+0.0186A-0.0166B +0.0046C-0.0016D- 0.0141AB-0.0041AD	0.5474+0.0054A-0.0276B +0.0124C-0.0216D- 0.0086AB-0.0121AD	0.4409+0.0144A-0.0321B +0.0154C-0.0156D- 0.0051AB-0.0131AD	0.2057-0.0040A-0.0328B +0.0020C-0.0233D +0.0050AB-0.0005AD
Model P Value	0.1033	0.0982	0.0695	0.1032
% CV	0.60	1.10	1.04	3.09
Adequate Precision	19.30	24.90	36.05	20.32
R-Squared	0.9970	0.9972	0.9986	0.9970
Adj R-Squared	0.9787	0.9807	0.9904	0.9787
Pred R-Squared	0.8050	0.8236	0.9120	0.8052
PRESS	0.0014	0.0023	0.0014	0.0026

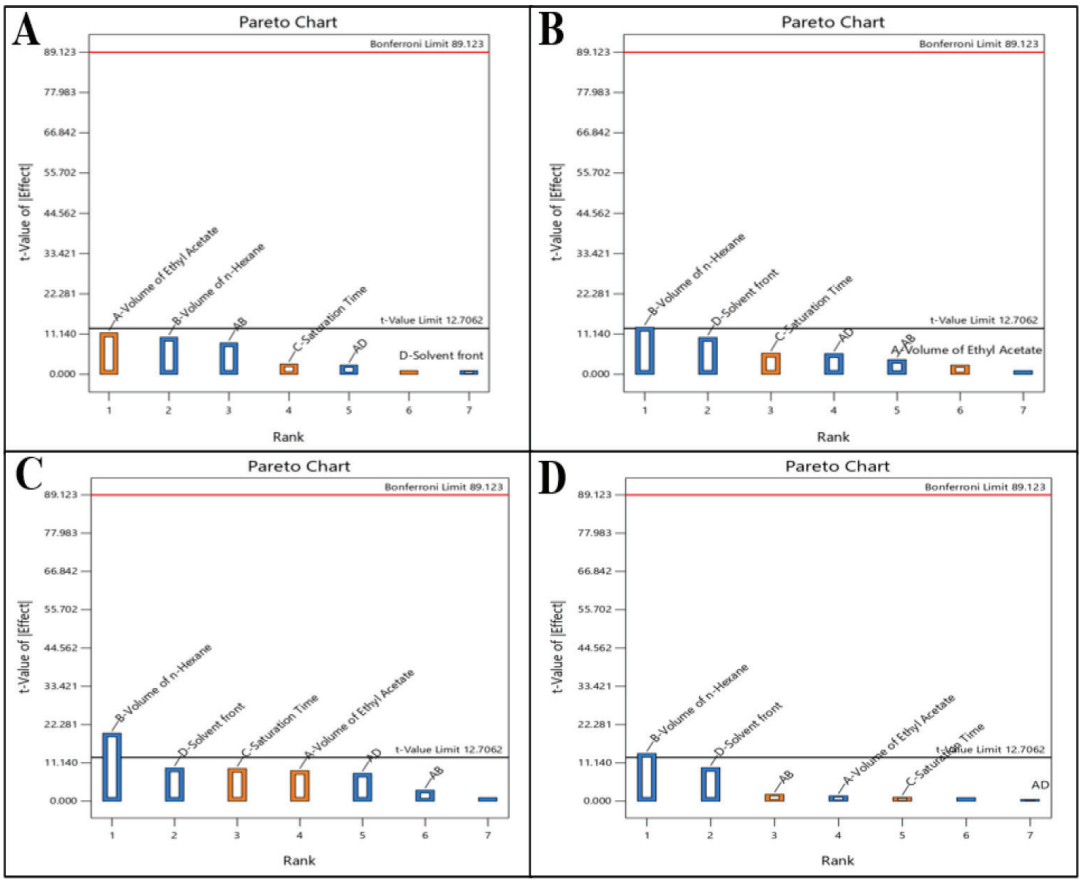


Figure 9. Pareto chart of lupeol, diosgenin, OA and CA.

A, B, C and D represent the Pareto chart for lupeol, diosgenin, OA and CA. Pareto chart showed that factors were not significant for lupeol and diosgenin, they had no significant impact on their Rf. However, n-hexane volume significantly influenced OA and CA, which is carefully controlled.

raised R_f for lupeol, diosgenin and OA. J showed that the R_f of CA was decreased with increases in ethyl acetate and n-hexane volumes. H shows that R_f of CA slightly decreases with increased ethyl acetate and solvent front. K shows that R_f of CA was increased as saturation time increased and R_f decreased as ethyl acetate volume rose.

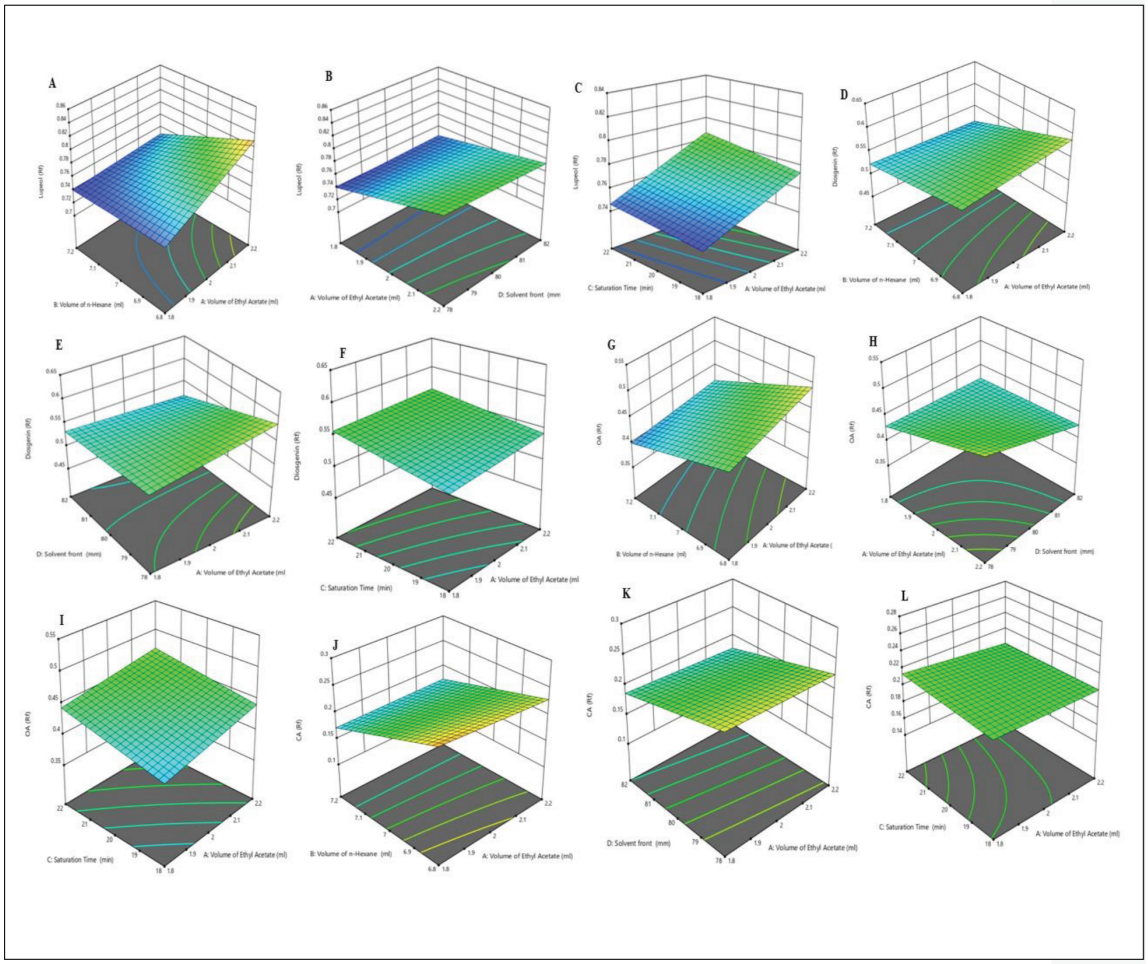


Figure 10. 3D Response surface plots of Lupeol, Diosgenin, OA and CA.

3.1.7 Quantification of Formulation

All four marker compounds are quantified by using a standard calibration curve equation and the outcomes are displayed in Table 11.

4. Conclusion

Analytical techniques to evaluate *Ayurvedic* medicines are developed and validated in this study. The findings indicate that the method is innovative, fast, specific and dependable for simultaneously estimating lupeol, diosgenin, OA and CA. The R_f values for lupeol, diosgenin, OA and CA were determined as 0.702, 0.525, 0.453 and 0.186, respectively. The precision and accuracy of signal measurements were verified across the 1000-5000 ng/band concentration range, with correlation coefficients of 0.9964 (Lupeol), 0.9945 (Diosgenin), 0.9989 (OA), and 0.9997 (CA). Recovery rates ranged from 99.54% to101.50% (Lupeol), from 100.06% to 101.09% (Diosgenin), from 99.89% to 101.09% (OA) and from 100.40% to 100.58% (CA). The levels of LOD (detection) and LOQ (limits of quantification) were as follows: Lupeol (95.26 ng/band, 288.68 ng/band), diosgenin (95.27 ng/band, 288.67 ng/band), OA (91.66 ng/band, 277.77 ng/band) and CA (91.66 ng/band, 277.77 ng/band). Precision studies indicated a relative standard deviation (%RSD) value under 2%.

Table 11. Quantification data of lupeol, diosgenin, oleanolic acid and caffeic acid

Biomarkers in formulation	The amount present in formulation (mg/gm)
Lupeol	0.1585
Diosgenin	0.1696
Oleanolic Acid	0.2111
Caffeic Acid	0.2260

FFD (2^{4-1}) demonstrated lupeol and diosgenin retention factors were not significantly affected by varying factors but n-hexane volume had a significant impact on OA and CA robustness, which is carefully managed. This method can be used for simultaneous quantification of the standards in various plant, herbal and *ayurvedic* formulations.

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