JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2024/43889
RESEARCH ARTICLE

Development and Validation of High-Performance Thin Layer Chromatography Method for Simultaneous Estimation of Catechin, Bergenin and Diosgenin in *Varunadi Kvatha Churna*

Keshavnam Chitte[1], Disha Prajapati[2*], Tanvi Dodiya[2] and Janvi Patel[1]

[1]Department of Pharmaceutical Quality Assurance, Parul Institute of Pharmacy and Research, Parul University Limda, Vadodara - 391760, Gujarat, India

[2]Department of Pharmacognosy, Parul Institute of Pharmacy and Research, Parul University Limda, Vadodara - 391760, Gujarat, India; dishaprajapati52@gmail.com

Abstract

Background: *Varunadi Kvatha Churna*, an *Ayurvedic* formulation, is renowned for its efficacy in managing urinary disorders due to the synergistic actions of herbs like *Pashanbhed, Varuna*, and *Gokshura*. **Aim:** This study aims to develop and validate a High-Performance Thin-Layer Chromatography (HPTLC) method for the simultaneous estimation of Catechin, Bergenin, and Diosgenin in *Varunadi Kvatha Churna.* **Methods:** The study utilized analytical grade organic solvents and standards of Catechin, Bergenin, and Diosgenin. A marketed formulation of *Varunadi Kvatha Churna* was procured, and sample solutions were prepared. HPTLC analysis was conducted using a CAMAG system with specific chromatographic conditions and vision CAT Software. **Result:** The optimal mobile phase was determined to be toluene: ethyl acetate: glacial acetic acid: methanol (7:4:0.5:1v/v/v/v) and Silica gel $G^{60}F_{254}$ in the stationary phase. Using the absorbance reflectance mode, quantification was done at 494 nm for Catechin, 277 nm for Bergenin, and 449 nm for Diosgenin. It found that the R_f values of Catechin, Bergenin, and Diosgenin were, respectively, 0.31, 0.18, and 0.73. The method exhibited good linearity in the range of 2000–10,000 ng/band for concentration. The LOD was found to be 168, 219.1, and 63.5 ng/band of Catechin, Bergenin, and Diosgenin. Catechin, Bergenin, and Diosgenin were found to have LOQs of 509.1, 654.6, and 192.4 ng/band, respectively. The method exhibited good precision, accuracy, system suitability, and specificity. The robust study confirmed the method's stability under varying conditions. **Conclusion:** The developed HPTLC method for simultaneous estimation of Catechin, Bergenin, and Diosgenin was found to be simple, linear, accurate and repeatable. The method can be used further for the estimation of biomarkers in crude drugs, herbal and *Ayurvedic* formulations.

Keywords: Bergenin, Catechin, Diosgenin, Factorial Design, HPTLC, *Varunadi Kvatha Churna*

1. Introduction

Varunadi Kwatha Churna is a classical *Ayurvedic* formulation renowned for its efficacy in managing various urinary disorders. Comprised of herbs like *Pashanbhed*, *Varuna*, and *Gokshura*, this polyherbal remedy has been traditionally used to alleviate symptoms associated with conditions such as urinary tract infections, kidney stones, and urinary retention. Its therapeutic potential contributed to the synergistic actions of its constituent herbs, which possess diuretic, lithotriptic, and anti-inflammatory properties, making it a promising subject for further scientific exploration and clinical validation[1]. Catechin is a flavonoid compound found in various plants, particularly tea, cocoa, and fruits like berries. The *Capparidaceae* family's *Crataeva nurvala*, a key medicinal plant used in famous diuretic *Ayurvedic* medicine, contains higher amounts

*Author for correspondence

Article Received on: 06.05.2024 Revised on: 18.11.2024 Accepted on: 22.11.2024

of Catechin[2]. Their potential health advantages, including their anti-inflammatory, neuroprotective, and antioxidant properties, have been thoroughly investigated[3,4]. Bergenin is a naturally occurring compound found in certain medicinal plants, like *Bergenia species*. The *Saxifragaceae* family's *Bergenia ligulata*, a key medicinal plant used in traditional medicine, contains higher amounts of Bergenin, a potent secondary metabolite, than any other identified species[5]. It possesses antioxidant, anti-inflammatory, and hepatoprotective properties. Bergenin has been investigated for its potential therapeutic applications in various disorders, including diabetes, inflammation, and liver diseases[6,7]. The *Zygophyllaceae* family's *Tribulus terrestris*, a key medicinal plant used in traditional medicine, contains higher amounts of Diosgenin, a steroidal sapogenin, than any other identified species[8]. This bioactive phytochemical is a precursor for the synthesis of steroidal drugs and has shown promising effects in the treatment of cancer, hypercholesterolemia, inflammation, and infections[9]. HPTLC is a sophisticated instrument for a developed analytical technique used for rapid separation and quantification of complex mixtures, offering enhanced resolution and sensitivity compared to traditional TLC methods[10,11]. Hence, this technique is utilized for the simultaneous quantitative estimation of Catechin, Bergenin, and Diosgenin in the *Varunadi Kvatha Churna.*

2. Materials and Methods

2.1 Standards and Chemicals

The analytical grade of the organic solvents that were purchased from Sulab, Vadodara, India. Catechin, Bergenin, and Diosgenin were obtained from Yucca Enterprises, Mumbai.

2.2 Marketed Formulation

The marketed formulation of *varunadi kvatha* was procured from the online website.

2.3 Preparation of Reference Standard Solution (Catechin, Bergenin and Diosgenin)

Each standard compound of Catechin, Bergenin, and Diosgenin was accurately weighed to 1 mg and dissolved by sonication in 1 ml methanol to get 1000 µg/ml of reference standard solution.

2.4 Preparation of Sample Solution

Varunadi Kvatha Churna (10 gm) was precisely weighed and transferred to 250 ml in a conical flask. Methanol (100 ml) was added to a conical flask and refluxed for 30 min.

To prepare a sample solution, one milligram of the extract was dissolved in one millilitre of methanol to yield a 1000 µg/ml sample solution.

2.5 Instrumentation

The specification of the instruments utilized in the research comprised of CAMAG, Linomat 5 sample applicator, a Hamilton microliter syringe, and HPTLC scanner 4 that was operated by vision CATS software, UV chamber, twin trough chamber (20 × 10 cm), precoated silica gel $G^{60}F_{254}$ aluminium plate (20 × 20cm, 100µm thickness; E. Merck, Darmstadt, Germany), and an electronic balance (Model: BL-220H Shimadzu Corp., Japan) were utilized. Design Expert 13 software was used for all data calculations.

2.6 Chromatographic Conditions

The optimized chromatographic condition for the HPTLC included the use of precoated silica gel G_{60} F_{254} plates as a stationary phase. The mobile phase used was toluene: ethyl acetate: glacial acetic acid: methanol (7:4:0.5:1 v/v/v/v). Linomat 5 applicators were employed for spotting standards and sample solution on the plate. The development of the plate was done in a twin trough chamber (20×10 cm) with a chamber saturation time of 20 minutes at 27±2 °C. The HPTLC scanners were used for scanning in absorbance reflectance mode using a deuterium lamp and tungsten lamp as the source of radiation. The optimized scanning wavelength for Bergenin was 277 nm, and Catechin and Diosgenin were 494,449 nm respectively. After derivatization with vanillin sulphuric acid reagent. After densitometric scanning, the R_f values were found to be 0.31, 0.18 and 0.73 for Catechin, Bergenin, and Diosgenin respectively.

2.7 Method Validation

ICH Q2 (R2) guidelines were followed in the validation of the developed HPTLC method with parameters

such as linearity, system suitability, precision, accuracy, sensitivity, specificity, and robustness.

2.7.1 Linearity

A mixture of all three standards in different volumes (6 to 30 µl) of working standard solution (1000µg/mL) of Catechin, Bergenin, and Diosgenin on the HPTLC plate was applied. To assess if there was a linear relationship between peak area and concentration of these three standards, 5 replication measurements were done in the concentration range of 2000–10000 ng/band. The results were expressed in ng/band. Calibration curves were constructed by plotting the area of the band's peak against standard concentrations. After that, a typical linear regression analysis was performed on these curves.

2.7.2 Sensitivity

The sensitivity of the developed method was evaluated using

$$\text{Limit of detection - } 3.3 \times \frac{\text{Standard deviation}}{\text{Slope}}$$

$$\text{Limit of Quantitation - } 10\times \frac{\text{Standard deviation}}{\text{Slope}}$$

Where, "S" is the calibration curve's slope of the regression lines' y-intercepts, and "Sd" is the standard deviation.

The LOD and LOQ were calculated based on the result standard deviation and the drug linearity calibration curve slope

2.7.3 Precision

Using repeatability, interday precision, and intraday precision, the developed method's precision was examined. The measurements were in triplicates to assess the reproducibility. On HPTLC plates, the different solutions were used to create bands containing 2000, 4000, and 6000 ng/band of Catechin, Bergenin, and Diosgenin, respectively.

2.7.4 Accuracy

The method's accuracy was assessed in triplicates for three different concentration levels (80%, 100%, and 120%). Catechin, Bergenin, and Diosgenin standards were spiked at unknown amounts of formulation, and the recovery and percentage RSD were calculated.

2.7.5 System Suitability

The suitability system of the developed method the standards at the same concentration (2000 ng/band) were spotted as 5 replicates and developed under the defined chromatographic conditions. The % RSD of the area under the curve is determined to establish system suitability.

2.7.6 Specificity

By comparing the spectrum of the Catechin, Bergenin, and Diosgenin standard, mixture, and combination with formulation, the specificity of the procedure was determined. A comparison of the R_f and overlay peak purity spectrum with the standard was used to confirm the peak for Catechin, Bergenin, and Diosgenin in the formulation.

2.7.7 Robustness

An analytical procedure is said to be robust if it can withstand both intentional and inadvertent changes to its method parameters. At a given level, where factors are symmetrically spaced at each variable axis, three factors are needed for a factorial design with 2^3 runs. Three factors the saturation time (A), the volume of ethyl acetate (B), and the solvent front (C) were chosen for this study based on the trial runs' criticality and the knowledge gathered from method development. Table 1 provides the variables chosen from the experimental domain.

The R_f value of three biomarkers was taken into consideration as the response, and the ranges that were investigated were slight deviations from the procedure settings. In this case, the robustness of the formulation was assessed using a two-level factorial design and a three-factor design. This involved running a three-factor factorial design with eight runs at high and low levels and recording the results. Table 2 reports on the experimental plan of the chosen factors. To reduce the biased effects of uncontrolled factors, all trials were conducted in a randomized manner.

2.8 Quantification of Catechin, Bergenin and Diosgenin

To prepare the test solution for HPTLC analysis, one milligram of test extract was dissolved in one millilitre of methanol. Samples and 1 µL (1000 ng/band) of test sample solutions were applied in duplicate on an

Table 1. 2^3 factorial design factors and levels

Independent factors	Low level	High level
Saturation time (A)	3.8	4.2
Volume of ethyl acetate (B)	18	22
Solvent front (C)	78	82
Dependent factors	R1: R_f value of Catechin R2: R_f value of Bergenin R3: R_f value of Diosgenin	

Table 2. Effect on various factors on R_f value of Catechin, Bergenin and Diosgenin

Std	Run	Factor 1	Factor 2	Factor 3
		A: Saturation Time (min)	B: Volume of Ethyl Acetate (ml)	C: Solvent Front (mm)
1	6	18	3.8	78
2	3	22	3.8	78
3	7	18	4.2	78
4	2	22	4.2	78
5	8	18	3.8	82
6	1	22	3.8	82
7	5	18	4.2	82
8	4	22	4.2	82

HPTLC plate. The conditions were followed to develop the plate, and it was scanned at 494, 277, and 449 nm (the maximum values of Catechin, Bergenin, and Diosgenin, respectively). Peak areas were noted, and the calibration plot was used to quantify the samples' contents of Diosgenin, Bergenin, and Catechin.

3. Results

3.1 Optimized Chromatographic Conditions

To develop an HPTLC method for estimating Catechin, Bergenin, and Diosgenin concurrently. Early in the process of developing the approach, several mobile phases were tested. A mobile phase that yields a denser band of Catechin, Bergenin, and Diosgenin was required, and this was determined by polarity. Toluene: ethyl acetate: glacial acetic acid: methanol (7:4:0.5:1% v/v/v) was the optimal mobile phase, where Catechin, Bergenin, and Diosgenin produced good peak shapes. The R_f values were found to be 0.31, 0.18 and 0.73 for Catechin, Bergenin, and Diosgenin respectively. The chromatogram of standards, a mixture of standards and the formulation is described in Figure 1.

3.2 Selection of Detection Wavelength

In the UV chamber, only Bergenin can be seen, and others cannot be seen at a conventional wavelength; hence, to derivatize and see the plate, a spraying reagent must be used. All the phytoconstituents in the Camag TLC Scanner IV had their UV spectra taken before and after derivatization to determine the proper wavelength. Figure 2 revealed that the wavelengths of Catechin, Bergenin, and Diosgenin were 494, 277, and 449 nm, respectively.

3.3 Linearity

In the indicated 2000–10,000 ng/band concentration range for Catechin, Bergenin, and Diosgenin, respectively, the three marker compounds demonstrated a strong correlation coefficient (r^2 = 0.9993, 0.9995, and 0.9995). An overlay HPTLC chromatogram for the linearity of Bergenin, Catechin, and Diosgenin at 277, 494, and 449 nm is shown in Figures 3 and 4.

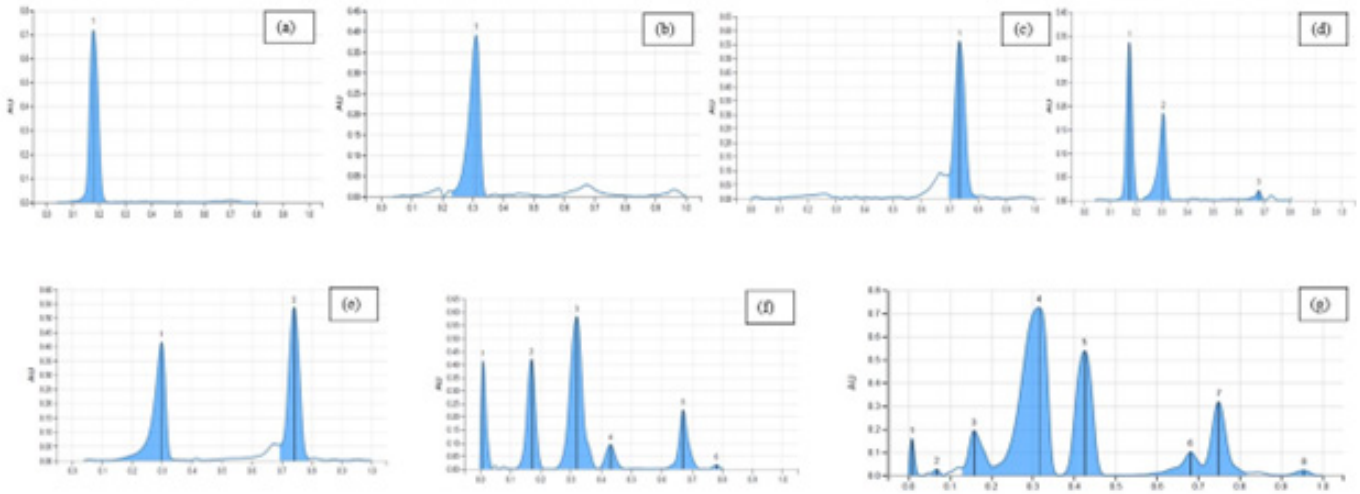


Figure 1. HPTLC chromatogram of standards, a mixture of standards and formulation. (HPTLC chromatogram of standard **(a).** Bergenin, **(b).** Catechin, **(c).** Diosgenin, **(d).** mixture (Bergenin) before derivatization, **(e).** mixture Catechin and Diosgenin after derivatization, **(f).** *Varunadi Kvatha Churna* before derivatization, **(g).** *Varunadi Kvatha Churna* after derivatization).

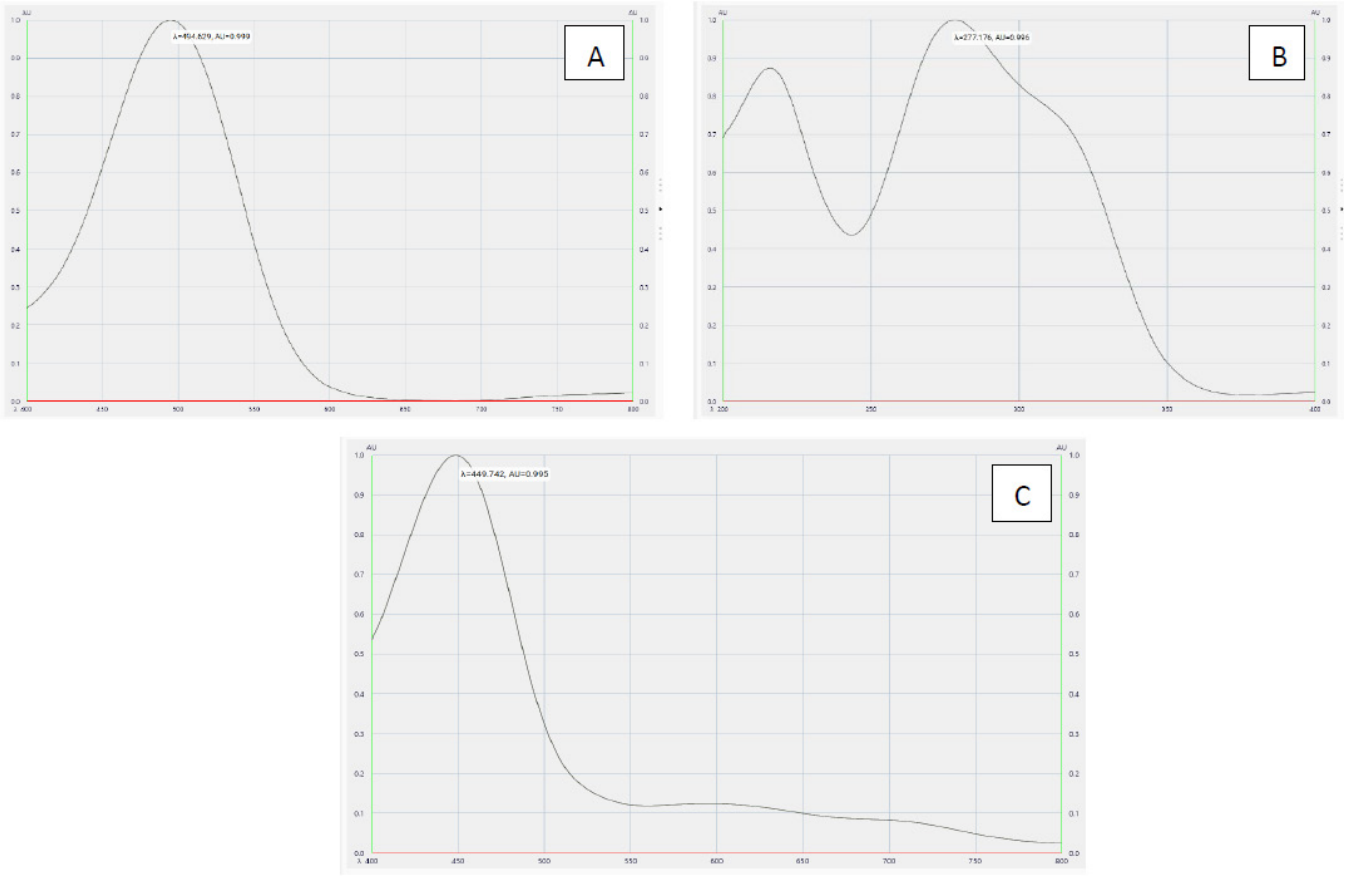


Figure 2. Selection of detection wavelength **(A)**. catechin, **(B)**. bergenin and **(C)**. diosgenin.

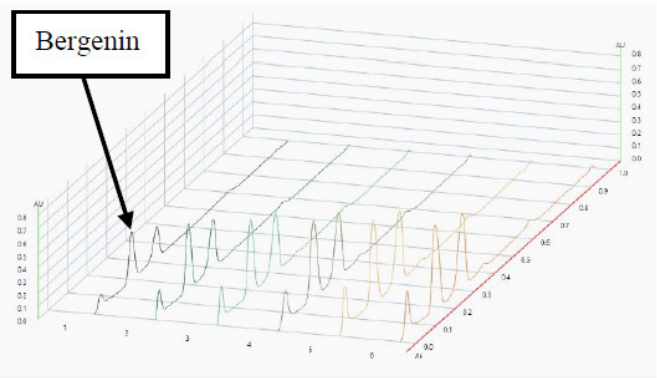


Figure 3. HPTLC overlay of standard Bergenin (before derivatization).

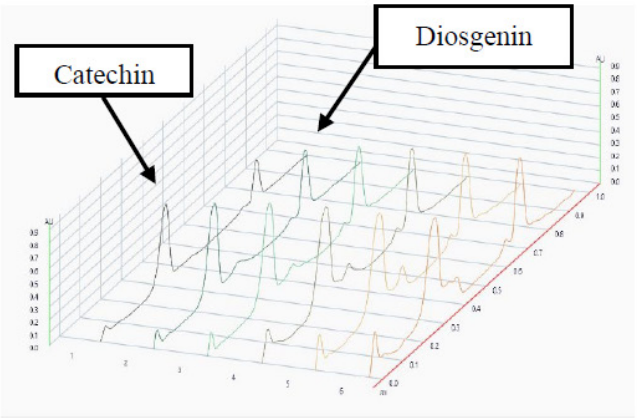


Figure 4. HPTLC overlay of standards Catechin, Diosgenin (after derivatization).

3.4 Sensitivity

LOD for Catechin, Bergenin, and Diosgenin was found to be 168, 219.1, and 63.5 ng/band. However, LOQ was discovered to be 509.1, 654.6, and 192.4 ng/band, and repeatability (%RSD) was discovered to be 1.1267, 1.1025, and 0.7335, hence demonstrating the method's sensitivity as reported in Table 3. Provided three concentrations of six times-spotted %RSD estimated for repeatability. The experiment was also conducted three times with intra-day and inter-day precision, and the Catechin, Bergenin, and Diosgenin peak areas' average %RSD values were calculated. The outcomes of repeatability, intra-day, and inter-day precision—all expressed in % RSD ≤ 2—confirm the procedure's precision, as reported in Table 3.

3.5 System Suitability

System suitability was performed by taking five replicates of the same concentration on the developed chromatographic condition. The retardation factor and area of all the standards were observed, and the calculated % RSD and standard deviation were less than 2%, which shows the method is suitable as reported in Table 3.

3.6 Accuracy

When implementing the suggested methodology for evaluating recovery post-standard spiking in the formulation at concentration levels (80%, 100%, and 120%), the recovery percentages were 101.44% for Catechin, 99.814% for Bergenin, and 100.20% for Diosgenin, as illustrated in Table 3.

3.7 Specificity

The three drugs' peak purity in the formulation and standard assess specificity in the presence of additional interference by comparing the spectra of Bergenin, Catechin, and Diosgenin. At the beginning, peak finish, and peak R_f, see Figure 5. The peak purity results are displayed in Table 4.

3.8 Robustness Study by Experimental Design

The expert trial version 13 was designed to compute the experimental outcomes. Table 5 displays the factorial models for response R_f for three standards.

The model was validated using an analysis of variance and the software Design Expert 13. (Table 6) The factor's response adequate precision is better than the required four. A significant effect is implied if the model P value is less than 0.05, while a non-significant effect is indicated if the model P value is greater than 0.05 regarding the response variables. In conclusion, all three biomarker models are non-significant.

3.8.1 Perturbation Plot Showing Effect of Various Factors on Responses of Catechin, Bergenin and Diosgenin

According to the design suggested, models are displayed as perturbations, and the plot response of Catechin and Diosgenin is that Figure 6 shows factor B is extremely sensitive, moderately sensitive in the response of the C factor, and less sensitive in factor A and the perturbation plot response of Bergenin

Table 3. Summary of validation parameters Catechin, Bergenin and Diosgenin

Parameters	Catechin	Bergenin	Diosgenin
Regression equation	y = 3E-06x + 0.0071	y = 3E-06x + 0.0277	y = 3E-06x + 0.0217
Correlation coefficient	0.9993	0.9995	0.9995
Calibration range (ng/band)	2000-10000	2000-10000	2000-10000
The standard deviation of the intercept	0.0071	0.0277	0.0217
The standard deviation of the slope	3E-06	3E-06	3E-06
Limit of detection (ng/band)	168	219.1	63.5
Limit of quantification(ng/band)	509.1	654.6	192.4
Repeatability (%RSD) (n-6)	1.1267	1.1025	0.7335
Inter-day Precision (%RSD) (n-3)	0.7962	1.2973	0.8220
Intra-day Precision (%RSD) (n-3)	0.4305	0.6942	0.6988
Accuracy (% Recovery)	101.4468	99.8134	100.208
System Suitability Test (%RSD)	1.164685	1.213183	0.812705
Specificity	Specific	Specific	Specific

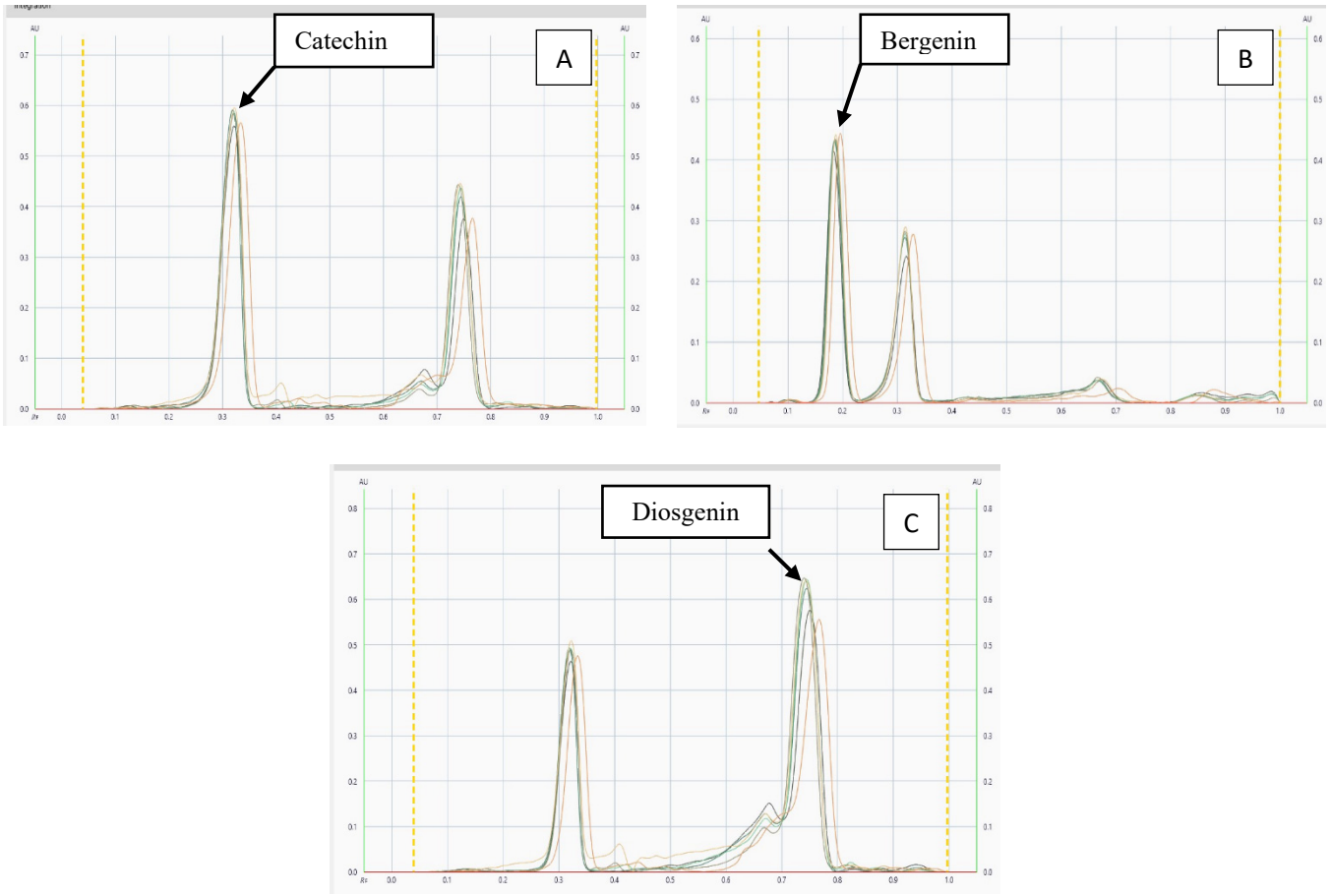


Figure 5. Overlay chromatogram of **(A).** Catechin, **(B).** Bergenin and **(C).** Diosgenin, mixture and formulation.

Table 4. Peak purity

Formulation				Standard		
Peak purity	Catechin	Bergenin	Diosgenin	Catechin	Bergenin	Diosgenin
r (s,m)	0.999312	0.999149	0.999619	0.999385	0.999838	0.999956
r (e,m)	0.999693	0.998910	0.999741	0.968233	0.998881	0.999627

Table 5. Responses of 2^3 factorial designs for robustness

Std.	Run	Factor 1	Factor 2	Factor 3	1 Response	2 Response	3 Response
		A: Saturation Time (min)	B: Volume of Ethyl Acetate (ml)	C: Solvent Front (mm)	Catechin	Bergenin	Diosgenin
1	6	18	3.8	78	0.294	0.175	0.719
2	3	22	3.8	78	0.285	0.171	0.723
3	7	18	4.2	78	0.308	0.182	0.733
4	2	22	4.2	78	0.314	0.239	0.738
5	8	18	3.8	82	0.282	0.159	0.704
6	1	22	3.8	82	0.278	0.170	0.710
7	5	18	4.2	82	0.387	0.185	0.804
8	4	22	4.2	82	0.384	0.247	0.796

Table 6. Estimated response models and statistical variables derived from ANOVA

Factors Response (R*f* value)	Catechin	Bergenin	Diosgenin
PRESS	0.0016	0.0008	0.0018
Model type	2FI	2FI	2FI
Model p-value	0.0790	0.0756	0.0983
% CV	1.56	1.85	0.7158
R-Squared	0.9982	0.9984	0.9972
Adj. R-Squared	0.9876	0.9886	0.9807
Adequate precision	23.6498	26.6087	19.4022
Pred. R-Squared	0.8862	0.8958	0.8236
Polynomial equation model for Y	+0.3165-0.0013A +0.0317B+0.0162C+0.0020AB-0.0005AC+0.0210 BC	+0.1910+0.0157A+0.0222B-0.0008C+0.0140AB+0.0025AC +0.0035BC	+0.7409+0.0009A+0.0269B+ 0.0126C-0.0016AB+0.0014AC +0.0196BC

is factor B extremely sensitive, factor C moderately sensitive, and factor C less sensitive. The response to the saturation time (A) factor, the volume of ethyl acetate (B), and the solvent front (C).

3.8.2 3D Response Plots

The responses of Catechin and Diosgenin are displayed in the 3D response surface plot. The ethyl acetate volume increases along with the R_f value in the AB graph, but the solvent front increases and the Rf value increases silently in the AC graph. In the response of Bergenin, both ethyl acetate volume and saturation time increase along with the high change in R_f value in the AB graph, but the saturation time increases and the R_f value increases silently in the AC graph. All responses are reported in Figure 7.

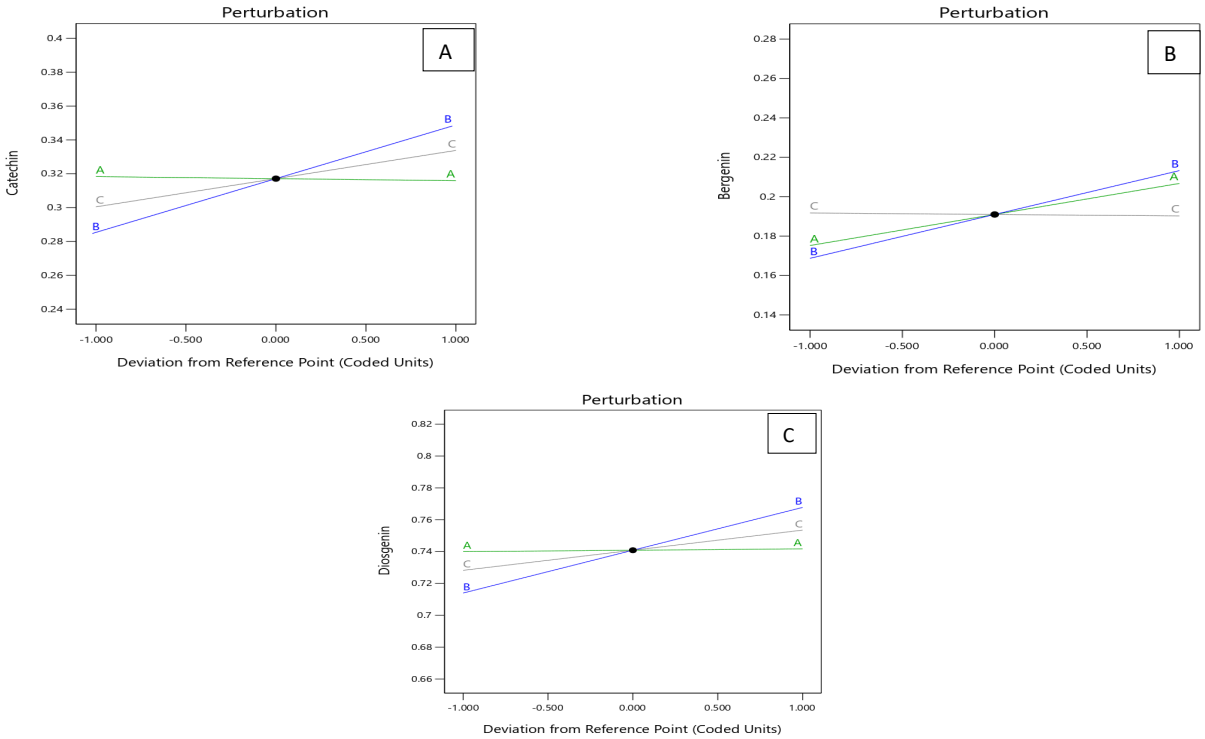


Figure 6. A perturbation graph illustrating the impact of factors A, B, and C on **(A)**. Catechin, **(B)**. Bergenin and **(C)**. Catechin.

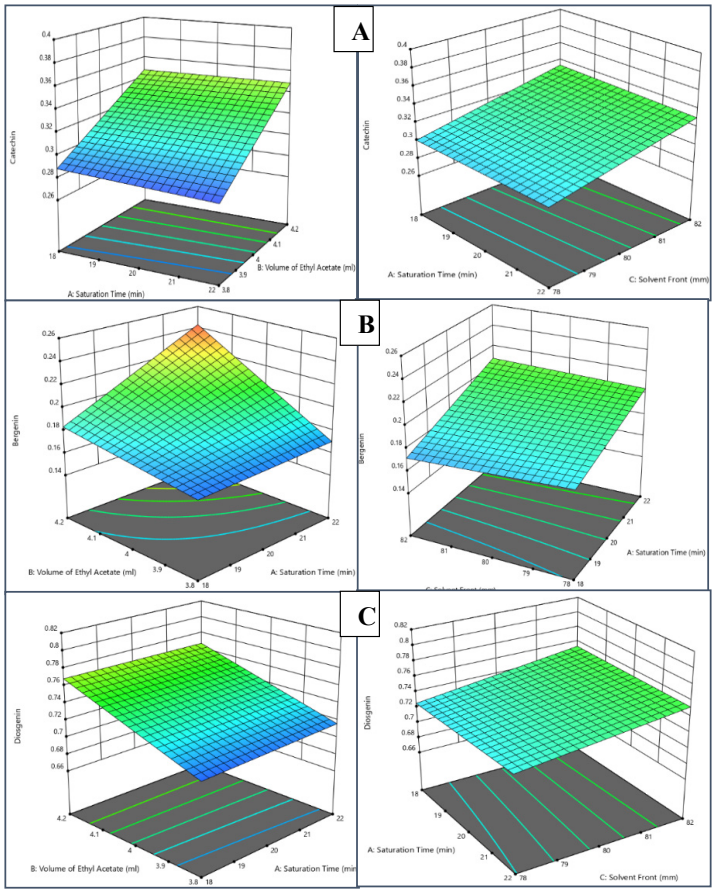


Figure 7. 3D Plot of the RSM for responses (Rf of Catechin-A), (Rf of Bergenin-B) and (Rf of Diosgenin-C).

Table 7. Quantification of standards in *Varunadi Kvatha Churna*

Standards	Amount of present in a sample ng/spot (% w/w)
Bergenin	4035 (0.4035)
Catechin	2083.33 (0.2083)
Diosgenin	1240 (0.1240)

3.9 Qualification of Standards

All three standards are quantified by using a standard calibration curve equation and the results reported are as below in Table 7.

4. Conclusion

The developed and validated HPTLC method for the simultaneous estimation of Catechin, Bergenin and Diosgenin can be used in the future for the analysis of crude drugs, and herbal and *Ayurvedic* formulations.

5. Authors Contribution

Keshavnam Chitte carried out the experimental work and drafted the manuscript. Disha Prajapati provided guidance in the overall study design, supervised the experimental work, and reviewed the manuscript for critical insights and accuracy. Tanvi Dodiya assisted in data interpretation, ensured the scientific rigor of the research, and Janvi Patel supported the manuscript writing process.

6. References

1. Thasineku S, Singh BK, Bharkher DL, Shrestha S, Shah RK, Gautam S. The effectiveness of *Goksuradi Guggulu* and *Varunadi Kwath* with *Goksuradi Guggulu* in the management of Mutrasmari wsr to urolithiasis. Int J Res AYUSH Pharm Sci. 2020; 4(11):463-9. https://doi.org/10.47070/ijraps.v4i11.98
2. Bhardwaj A. A brief review on healing properties of *Varuna-Crataeva nurvala*. Think India J. 2019; 22(17):57-65.
3. Kumar D, Sharma S, Kumar S. Botanical description, phytochemistry, traditional uses, and pharmacology of *Crataeva nurvala Buch*. Ham.: an updated review. Fut J Pharm Sci. 2020; 6(113):1-10. https://doi.org/10.1186/s43094-020-00106-1
4. Dharmender R, Madhavi T, Reena A, Sheetal A. Simultaneous quantification of bergenin, (+)-catechin, gallicin, and gallic acid; and quantification of β-sitosterol using HPTLC from *Bergenia ciliata* (Haw.) Sternb. *Forma ligulata Yeo* (Pasanbheda). Pharm Anal Acta. 2011; 1:104. https://doi.org/10.4172/2153-2435.1000104
5. Dhalwal K, Shinde VM, Biradar YS, Mahadik KR. Simultaneous quantification of bergenin, catechin, and gallic acid from *Bergenia ciliata* and *Bergenia ligulata* by using thin-layer chromatography. J Food Compos Anal. 2008; 21(6):496-500. https://doi.org/10.1016/j.jfca.2008.02.008
6. Khan MS, Khan W, Ahmad W, Singh M, Ahmad S. Bergenin determination in different extracts by high-performance thin-layer chromatographic densitometry. J Pharm Bioallied Sci. 2015; 7(4):272-4. https://doi.org/10.4103/0975-7406.168024. PMID: 26681881; PMCID: PMC4678973.
7. Patel DK, Patel K, Kumar R, Gadewar M, Tahilyani V. Pharmacological and analytical aspects of bergenin: a concise report. Asian Pac J Trop Dis. 2012; 2(2):163-7. https://doi.org/10.1016/S2222-1808(12)60037-1
8. Kshirsagar VB, Deokate UA, Bharkad VB, Khadabadi SS. HPTLC method development and validation for the simultaneous estimation of diosgenin and levodopa in marketed formulation. Asian J Res Chem. 2008; 1(1):36-9.
9. Jesus M, Martins AP, Gallardo E, Silvestre S. Diosgenin: recent highlights on pharmacology and analytical methodology. J Anal Methods Chem. 2016; (2016):1-16. https://doi.org/10.1155/2016/4156293. PMID: 28116217; PMCID: PMC5225340.
10. Sonia K, Lakshmi KS. HPTLC method development and validation: An overview. J Pharm Sci Res. 2017; 9(5):652-7.
11. Shewiyo DH, Kaale EA, Risha PG, Dejaegher B, Smeyers-Verbeke J, Vander Heyden Y. HPTLC methods to assay active ingredients in pharmaceutical formulations: A review of the method development and validation steps. J Pharm Biomed Anal. 2012; 66:11-23. https://doi.org/10.1016/j.jpba.2012.03.034. PMID: 22494517.