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RESEARCH ARTICLE

Stability Indicating HPTLC Method for Estimation of Cubebin in Crude Drug and Marketed Formulations

Mansi Patel[1], Tanvi Dodiya[2*] and Disha Prajapati[2]

[1]Department of 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; pateltanvi1982@gmail.com

Abstract

Background: Cubebin is a naturally occurring lactone lignan isolated from the plant species *Piper cubeba*. It exhibits a range of biological activities, including anti-inflammatory, antimicrobial, and anticancer properties. This compound has garnered significant interest for its potential therapeutic applications in traditional and modern medicine. **Aim:** This study aimed to develop a novel stability-indicating High-Performance Thin Layer Chromatography (HPTLC) technique that is simple yet precise in estimating cubebin in crude drug and marketed formulations. **Method:** Cubebin was estimated using precoated TLC silica gel 60 F_{254}plate as a stationary phase and n-hexane: ethyl acetate: formic acid: methanol (6:3.5:0.5:0.3 v/v/v/v) as a mobile phase with the chamber saturation time of 20 mins. **Key Findings:** Cubebin was detected and quantified at the wavelength of 283nm. The R_f value of cubebin in standard solution, *Piper cubeba* extract, *Dhanwantari Vati*, and *Khadiradi Vati* was found to be 0.494±0.0015, 0.492±0.002, 0.490±0.0020, 0.499±0.001 respectively. The calibration curve exhibited linearity within the range of 3000-13000 ng per band with a correlation coefficient (r^2) of Cubebin was 0.9968. The method developed was utilized for the quantitation of cubebin in *Piper cubeba* and both the marketed formulations. **Conclusion:** A novel HPTLC method was developed and validated in accordance to ICH guidelines for the analysis of cubebin in crude drug and marketed formulations. The method was found to be simple, precise, accurate, and specific. This method is suitable for quality control and stability testing of cubebin-containing formulations.

Keywords: Cubebin, *Dhanwantari Vati, Khadiradi Vati, Piper cubeba*

Abbreviations: AR - Analytical Research Grade; cm – centimetre; GC - gas chromatography; HPLC - High Performance Liquid Chromatography; H_2O_2 - hydrogen peroxide; HCl - hydrochloric acid; HPTLC - High-Performance Thin Layer Chromatography; ICH - International Council for Harmonisation; LOD - Limit of detection; LOQ - limit of quantitation; mm – millimetre; ng – nanograms; nm – Nanometer; R_f - retardation factor; RSD - relative standard deviation; TLC - Thin-layer chromatography; UV – ultraviolet; µg – micrograms; µL – microliters; µg/ml – micrograms per milliliter

1. Introduction

Herbal medications utilize plants in various forms for their therapeutic benefits. Plants contain diverse chemical compounds that interact with the body to prevent or treat illnesses and enhance overall health. Over recent decades, the use of herbal remedies has surged globally, leading to expanding markets for these products both internationally and within individual countries. This trend underscores the increasing popularity and acceptance of herbal drugs as viable alternatives for promoting wellness and managing health conditions worldwide[1].

Cubebin is chemically identified as 2,3-bis(3,4-methylenedioxybenzyl)-butyrolactol[2]. *Piper cubeba* and *Piper nigrum* are the most common sources of Cubebin[3]. The main natural reservoir of cubebin is

*Author for correspondence

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Piper cubeba L., a climbing plant known by various names such as java pepper or kabab chini, belonging to the Piperaceae family. This plant is cultivated primarily for its fruits and essential oil[4].

Stability studies involve evaluating how a substance or product retains its chemical, physical, and microbiological properties over a defined period under specific storage conditions. These studies are essential to determine the shelf life, storage recommendations, and regulatory compliance of pharmaceuticals, food items, cosmetics, and other goods. According to the ICH guideline, stress testing aims for the identification of potential degradation products, aiding in the assessment of a molecule's inherent stability and the establishment of degradation pathways. Additionally, it serves to validate the procedures used for indicating stability[5].

The stability-indicating assay is a crucial technique used in the pharmaceutical industry to analyze stability-related samples. With the introduction of ICH guidelines, there is a clear mandate for establishing this assay method. These guidelines outline the requirement for carrying out forced degradation studies under the influence of different conditions such as pH, light exposure, oxidation, and dry heat, to isolate the drug from its degradation by products. This method enables the analysis of individual degradation products[6].

High-performance thin-layer chromatography is an advanced analytical method that maximizes the potential of thin-layer chromatography. Through features like automation, scanning, comprehensive optimization, selective detection principles, minimal sample preparation, and hyphenation, HPTLC emerges as a potent analytical technique for obtaining chromatographic data from intricate mixtures of inorganic, organic, and biomolecules[7]. HPTLC is suitable for creating chromatographic fingerprinting techniques to analyze and characterize herbal extracts, similar to HPLC and GC. Additionally, HPTLC offers the advantage of producing visually informative images. In contrast to GC and HPLC, HPTLC allows for the simultaneous estimation of multiple samples on a single plate[8].

2. Materials and Methods

2.1 Materials

Cubebin was procured from yucca enterprises, Mumbai, India. *Piper cubeba* seeds and both marketed formulations were purchased from the local market in Surat. Methanol, n-hexane, ethyl acetate, HCl, NaOH, and H_2O_2 of AR grade were procured from Sulab, Vadodara, and formic acid was purchased from Oxford Lab Fine Chem LLP.

2.2 Chromatographic Conditions

The stationary phase used was precoated silica gel 60 F_{254}aluminum sheets 10*10 cm^2. TLC plates were activated by prewashing with methanol. The optimized mobile phase was n-hexane: ethyl acetate: formic acid: methanol (6:3.5:0.5:0.3 v/v/v/v). The chamber saturation time was 20 minutes and the length of the chromatographic run was 80mm.

2.3 HPTLC Instrumentation

The samples were spotted as bands onto a pre-coated silica gel 60F_{254} by using a CAMAG Linomat V instrument, utilizing a CAMAG-LINOMATE Syringe (Hamilton syringe). A slit dimension of 6 x 0.45 mm was utilized, and a scanning speed of 100 mm/s was employed. Development was conducted in a twin-trough glass chamber saturated with the mobile phase, following a linear ascending pattern. The densitometric scanning was conducted by CAMAG-TLC scanner 4 under the absorbance mode, with a wavelength of 283nm. A deuterium lamp was employed as the radiation source.

2.4 Preparation of Solutions

2.4.1 Preparation of Cubebin Standard Solution

Accurately weighed 1mg of cubebin standard was transferred into 1ml methanol in an eppendorf tube, to make 1000µg/ml.

2.4.2 Preparation of Solution for Calibration Curve

The Standard solution of Cubebin, ranging in volume from 3, 5, 7, 9, 11, and 13 µl, was applied using a Hamilton syringe and a CAMAG Linomat V applicator to a TLC plate with a concentration of 1000–13000 ng/band to create a calibration curve.

2.4.3 Preparation of Sample Solution from Piper cubeba Extract

About 5gm of *Piper cubeba* seeds were powdered and kept for maceration with 10ml of methanol for about 1

hour. The solution was filtered and the filtrate obtained was completely dried in a water bath. From this about 1mg of extract was dissolved in methanol.

2.4.4 Preparation of Sample Solution from Marketed Formulations

About 5gm of marketed formulations (*Khadiradi vati* and *Dhanwantari vati)* were powdered and kept for maceration with 10ml of methanol for about 1 hour. The solution was filtered and the filtrate obtained was completely dried in a water bath. From this about 1mg of extract was dissolved in methanol.

2.4.5 Forced Degradation Studies

Stress degradation studies were carried out under acidic, alkaline, oxidative, thermal, and photolytic conditions according to ICH Q1A (R2) guidelines.

2.4.5.1 Acidic Degradation

1 ml of 1N HCl was added to 1 ml of cubebin standard solution, and then the solution was kept for 1 hour at room temperature and then cooled down. 5µl of the solution was then spotted onto a TLC plate, developed using optimized chromatographic conditions, and then scanned for analysis.

2.4.5.2 Alkaline Degradation

1 ml of 1N NaOH was added to 1 ml of cubebin standard solution, and then the degradation reaction time was incrementally increased from 1 to 6 hours until the desired degradation was achieved. 5µl of the solution was then spotted onto a TLC plate, developed using optimized chromatographic conditions, and then scanned for analysis.

2.4.5.3 Oxidative degradation

1 ml of 3% H_2O_2 was added to 1 ml of cubebin standard solution, and the solution was kept for 6 hours at room temperature. 5µl of the solution was then spotted onto a TLC plate, developed using optimized chromatographic conditions, and then scanned for analysis.

2.4.5.4 Thermal Degradation

1 mg of Cubebin was weighed accurately, kept at 100°C for 3 hours, and transferred into 1ml methanol in a volumetric flask. 5µl of the solution was then spotted onto a TLC plate, developed using optimized chromatographic conditions, and then scanned for analysis.

2.4.5.5 Photolytic Degradation

1 ml of Cubebin standard solution was kept under UV light for 3 hours till the desired degradation was achieved. 5µl of the solution was then spotted onto a TLC plate, developed using optimized chromatographic conditions, and then scanned for analysis.

2.4.6 Method Validation

2.4.6.1 System Suitability

System Suitability testing was conducted to evaluate the suitability of the developed method. Cubebin standard solution was spotted six times using the same volume of 5µl/spot, with a concentration of 1000µg/ml. This analysis was conducted under the established chromatographic conditions, and the spots were then examined for retardation factor and area.

2.4.6.2 Specificity

Specificity was evaluated by analyzing both standard and test samples using the developed mobile phase. The identification of the standard in samples was verified based on the retardation factor values and UV spectrum of the distinct bands corresponding to the standard peaks.

2.4.6.3 Linearity

1 to 13µl from the cubebin standard solution (1000µg/ml) was applied on the TLC plate to achieve concentrations of 1000, 3000, 5000, 7000, 9000, 11000, and 13000 ng/band. The plot of peak area against concentration was generated to create the calibration curve.

2.4.7 Precision

2.4.7.1 Method Repeatability

To evaluate the method's precision, 5µl of cubebin standard solution was repeatedly applied six times on a single plate, following the development of plate and measuring the peak area of each of the six bands.

2.4.7.2 Intraday Precision

The method's intraday precision was established by analyzing triplicate samples of the solution of cubebin in 3 different concentrations (5000, 7000, 9000 ng/band) on the same day and comparing their respective responses.

2.4.7.3 Interday Precision

The method's interday precision was assessed by analyzing triplicate samples of the solution of cubebin in

3 different concentrations (5000, 7000, 9000 ng/band) over three consecutive days within a week. The results, expressed as % RSD, were reported by comparing the corresponding responses.

2.4.8 Accuracy

Percent recovery was calculated to evaluate the accuracy of the developed analytical method. A known amount of standard spiked to a pre-quantified standard sample solution. The method's accuracy was evaluated through a recovery study from sample solution at three standard addition levels (80%, 100%, and 120%), and %RSD was calculated.

3. LOD and LOQ

The linearity data was utilised to establish the Limits of Detection (LOD) and Limit of Quantification (LOQ) for the method. This shows the smallest detectable and quantifiable amounts of cubebin that the method can accurately measure. It was calculated by the following formula of standard deviation and slope method.

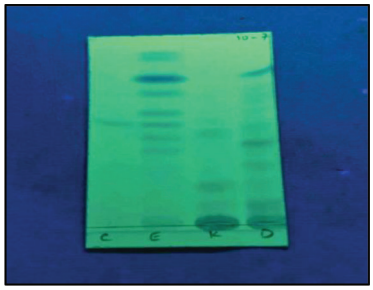

Figure 1. TLC-developed plate.

LOD= 3.3*$\boldsymbol{\sigma/s}$ and LOQ= 10*$\boldsymbol{\sigma/s}$

Where,

σ = Standard deviation of the response

S = Slope of the calibration curve

4. Results and Discussion

4.1 Optimization of Mobile Phase

The optimized mobile phase was achieved through experimental trial and error using various solvent systems such as toluene, n-hexane, ethyl acetate, formic acid, and methanol to determine the most effective ratio for achieving the optimal solvent system in HPTLC. It was observed that n-hexane: ethyl acetate: formic acid: methanol (6:3.5:0.5:0.3 v/v/v/v) showed proper separation on the TLC plate as shown in Figure 1 and so this mobile phase was finalized. Further, this mobile phase was developed on HPTLC and the developed plate is shown in Figure 2. The HPTLC chromatogram

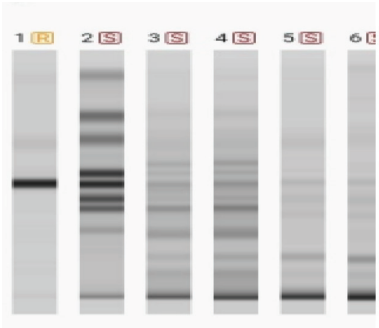

Figure 2. HPTLC plate. Track 1- Cubebin, Track 2- *Piper Cubeba* extract, Track 3- *Dhanwantari vati,* Track 4- *Dhanwantari vati,* Track 5- *Khadiradi vati.*

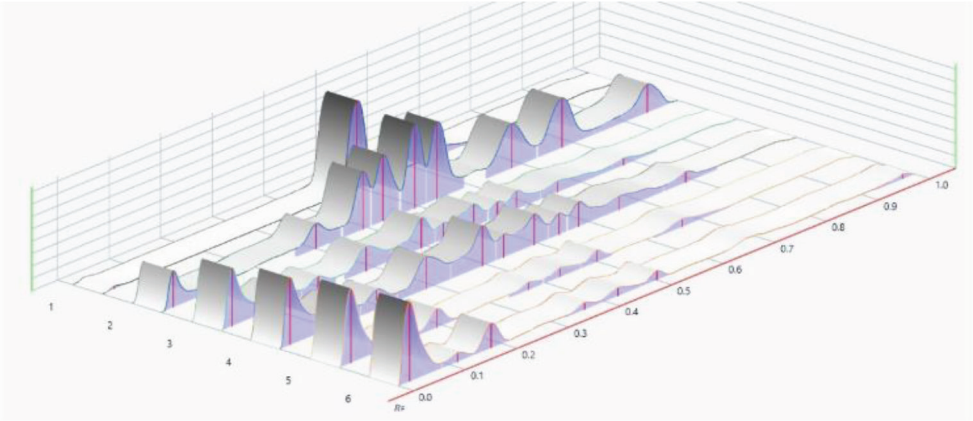

Figure 3. HPTLC 3D Densi to gram for Track 1- Cubebin, Track 2- *Piper cubeba* extract, Track 3 - *Dhanwantari vati,* Track 4 - *Dhanwantari vati,* Track 5- *Khadiradi vati.*

of cubebin standard, *P. cubeba* extract, and marketed formulations are shown in Figure 3. Quantification of the sample was done at a wavelength of 283 nm and the R_f values of each spot are mentioned in Table 1.

The mobile phase comprising of n-hexane: ethyl acetate: formic acid: methanol (6:3.5:0.5:0.3, v/v/v/v), allowed effective detection of cubebin in standard, crude drug and formulation samples. To ensure proper detection and quantification scanning was done at a wavelength of 283 nm.

3.2 Forced Degradation Study

In this study, the degradation analysis of cubebin was conducted to determine its stability in marketed formulations on its standard form rather than in marketed formulations, to avoid the complexity of identifying cubebin and its degradation products amid the presence of multiple other constituents. The results of the degradation study of cubebin are mentioned in Table 2.

Table 1. R_f values of cubebin

Sr. No.	Spot	R_f value of Cubebin
1	Cubebin standard	0.494±0.0015
2	*Piper cubeba* extract	0.492±0.002
3	*Dhanwantari Vati*	0.490±0.0020
4	*Khadiradi Vati*	0.499±0.001

Table 2. Results of degradation of Cubebin

Sr. No.	Stress conditions	%degradation
1	Acid (1N HCl)	48.77%
2	Alkaline (1N NaOH)	11.39%
3	Oxidative (3%H_2O_2)	35.59%
4	Thermal (100°C)	13.63%
5	Photolytic (UV light)	27.89%

The forced degradation study showed that the acidic condition showed the highest degradation of Cubebin, while it remains relatively stable under alkaline condition.

3.3 Method Validation

3.3.1 System Suitability

System suitability test was conducted by running six replicates of the same concentration under the established chromatographic conditions. The retardation factor and peak areas of the observed standards are presented in Table 3 and the standard deviation and % RSD were calculated.

3.3.2 Specificity

Specificity was determined by analyzing cubebin standard and test samples using the developed mobile phase. Figure 4 depicts the overlay spectra of cubebin, *P. cubeba* extract, and marketed formulations (*Dhanwantari vati* and *Khadiradi vati*).

3.3.3 Linearity

Linearity was determined by spotting cubebin solution on a 10*10 cm TLC plate in volumes ranging from 3, 5, 7, 9, 11, and 13 µl. The plot of peak area against concentration was generated to create the calibration curve as shown in Figure 5. The 3D HPTLC densitogram of the linearity of cubebin is shown in Figure 6.

Cubebin showed linear response with a concentration ranging from 3000-13000 ng/band. The r^2 obtained was 0.996.

3.3.4 Precision

3.3.4.1 Method Repeatability

Repeatability was evaluated through six determinations at a concentration of 5000 ng/band, SD and % RSD was calculated as shown in Table 4.

Table 3. System Suitability results

Concentration (ng/band)	R_f value	Area	Mean	SD	%RSD
5000	0.522	0.02347	0.023103	0.000456	1.973
	0.511	0.02302			
	0.501	0.02308			
	0.496	0.02261			
	0.492	0.02266			
	0.489	0.02378			

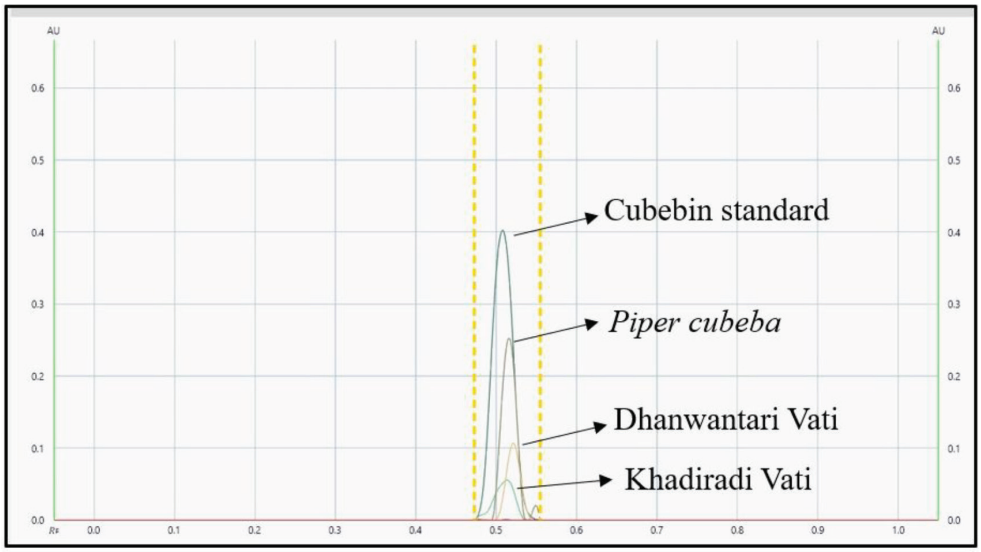

Figure 4. Overlay of cubebin with *Piper cubeba* extract and formulations (*Dhanwantari Vati* and *Khadiradi Vati*).

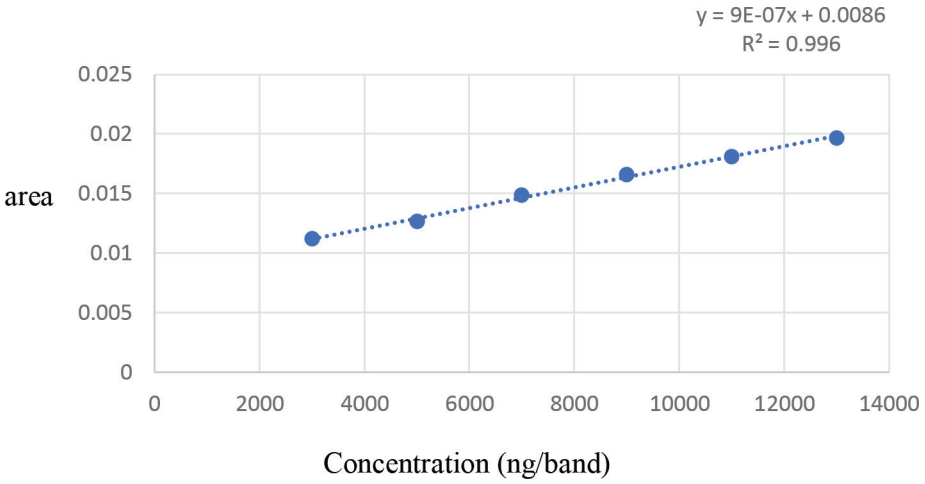

Figure 5. Calibration curve of Cubebin.

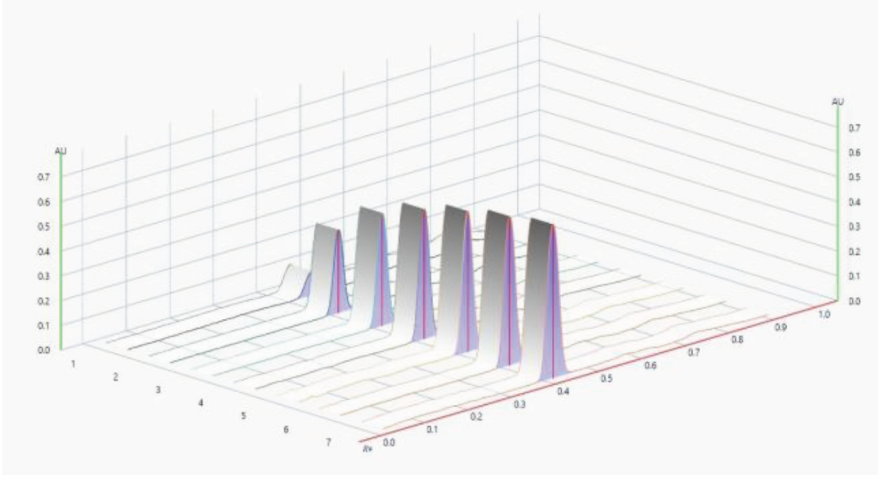

Figure 6. 3D HPTLC density to gram of linearity of cubebin.

3.3.4.2 Intraday precision

Intra-day precision was conducted using three determinations at concentrations of 5000, 7000, and 9000 ng/band, at three different hours (1hr, 2hr, 3hr), SD and % RSD were calculated as shown in Table 5.

Table 4. Repeatability

Sr. No.	Concentration (ng/band)	Area	Mean	%RSD
1	5000	0.01256	0.01245	0.76
2		0.01240		
3		0.01229		
4		0.01249		
5		0.01250		
6		0.01248		

3.3.4.3 Interday Precision

Interday precision was conducted using three determinations at concentrations of 5000, 7000, and 9000 ng/band, at three different days (day 1, day 2, and day 3), SD and % RSD was calculated as shown in Table 6.

3.3.5 Accuracy

Accuracy was evaluated using the standard recovery method across the three concentrations (80 %, 100%, 120%). The accuracy test results for cubebin in *P. cubeba* extract, *Dhanwantari vati*, and *Khadiradi vati* are summarized in Table 7, 8, and 9 respectively.

3.3.6 Limit of Detection and Limit of Quantification

The LOD and LOQ were determined from the residual standard deviation of response and the slope of the calibration curve using the following formula:

Table 5. Intraday precision results

Concentration	Hour 1	Hour 2	Hour 3	Mean	SD	%RSD
5000	0.01239	0.01254	0.01257	0.01250	0.0000964	0.77
7000	0.01483	0.01492	0.01467	0.01480	0.0001266	0.85
9000	0.01660	0.01690	0.01702	0.01684	0.000216	1.2

Table 6. Interday precision results

Concentration	Day 1	Day 2	Day 3	Mean	SD	%RSD
5000	0.01250	0.01263	0.01272	0.01261	0.0001106	0.87
7000	0.01490	0.01451	0.01455	0.01465	0.0002145	1.4
9000	0.0168	0.0163	0.0165	0.01653	0.000252	1.5

The result indicates that the %RSD values for cubebin are within the acceptable criteria set by ICH, being less than 2%. Hence, it can be concluded that the method proposed for the estimation of cubebin is precise.

Table 7. Accuracy results of *P. cubeba* extract

% spiked	Sample amount (ng/band)	Spiked amount (ng/band)	Total conc. (ng/band)	Total amount recovered	% recovery	%RSD
80	5000	4000	9000	9106	101.04±0.73	0.73
100	5000	5000	10000	9958	100.05±0.41	0.41
120	5000	6000	11000	11021	99.99±0.17	0.17

Table 8. Accuracy results of *Dhanwantari vati*

% spiked	Sample amount (ng/band)	Spiked amount (ng/band)	Total conc. (ng/band)	Total amount recovered	% recovery	%RSD
80	5000	4000	9000	9031	100.37±0.81	0.80
100	5000	5000	10000	10072	100.76±0.37	0.37
120	5000	6000	11000	11105	100.56±0.62	0.62

Table 9. Accuracy results of *Khadiradi Vati*

% spiked	Sample amount (ng/band)	Spiked amount (ng/band)	Total conc. (ng/band)	Total amount recovered	% recovery	%RSD
80	5000	4000	9000	9103	100.36±0.75	0.74
100	5000	5000	10000	10106	100.79±0.24	0.23
120	5000	6000	11000	11052	100.2±0.30	0.30

The result of accuracy reveals that % recovery of cubebin is within acceptance criteria 98-102 %.

LOD = 3.3* SD/Slope

LOQ = 10* SD/Slope

The LOD and LOQ values of Cubebin are mentioned in Table 10.

Table 10. LOD and LOQ of cubebin

Biomarker	LOD	LOQ
Cubebin	2133.29	6464.54

3.3.7 Quantification

Quantification of Cubebin in *P. cubeba* and marketed formulations was done by the developed and validated HPTLC method from the linear equation y = mx+c. The amount of cubebin present in *P. cubeba, Dhanwantari Vati,* and *Khadiradi Vati* is mentioned in Table 11.

Table 11. Quantification of cubebin

Sample	Amount of Cubebin (ng/band)
Piper cubeba extract	16377.7
Dhanwantari Vati	4600
Khadiradi Vati	4177.7

Table 12. Summary

PARAMETERS		RESULTS	
R_f value		0.494	
Wavelength (nm)		283	
Acid degradation (%)		48.77%	
Alkaline degradation (%)		11.39%	
Oxidative degradation (%)		35.59%	
Thermal degradation (%)		13.63%	
Photolytic degradation (%)		27.89%	
Linearity range (ng/band)		3000-13000	
r^2		0.996	
Repeatability (%RSD)		0.76	
Intraday precision		**Concentration (ng/band)**	**%RSD**
		5000	0.77
		7000	0.85
		9000	1.2
Interday precision		**Concentration (ng/band)**	**%RSD**
		5000	0.87
		7000	1.4
		9000	1.5
Accuracy	***Piper cubeba* extract**	**% spiked**	**%RSD**
		80	0.73
		100	0.41
		120	0.17
	Dhanwantari vati	**% spiked**	**%RSD**
		80	0.80

Table 12. Continued...

PARAMETERS		RESULTS	
		100	0.37
		120	0.62
	Khadiradi Vati	**% spiked**	**% RSD**
		80	0.74
		100	0.23
		120	0.30
LOD (ng)		2133.29	
LOQ (ng)		6464.54	
Quantification (ng/band)	***Piper cubeba* extract**	16377.7	
	Dhanwantari Vati	4600	
	Khadiradi Vati	4177.7	

5. Summary

The HPTLC technique was created and validated to determine cubebin in crude drug and marketed formulations. The outcomes for each validation parameter confirmed the method's linearity, accuracy, precision, and specificity, aligning with ICH guidelines. The method exhibited strong linearity across the chosen range. Table 12 presents a summary of this developed analytical method.

6. Conclusion

The method developed is validated in accordance with ICH guideline Q2 (R1). Within a concentration range of 3000-13000 ng/band, the method demonstrates linearity, with a r^2 reaching 0.996 at 283nm. Results from the analysis indicate high reproducibility and reliability. Consequently, the devised HPTLC method emerges as simple, accurate, and precise, suitable for estimating cubebin in crude drug and marketed formulations. Furthermore, it fulfils the requirement of conducting degradation studies outlined in ICH guidelines. With its ability to detect cubebin degradation, this method presents an asset for quality control purposes, aligning well with established practices outlined in ICH guidelines.

7. References

1. Bhowmik D, Kumar KS, Tripathi P, Chiranjib B. Traditional herbal medicines: An overview. Archives of Applied Science Research. 2009; 1(2):165-77.
2. Mulik MB, Laddha KS. Isolation, characterization, and quantification of bioactive dibenzylbutyrolactone lignan (-)-cubebin from fruits of *Piper cubeba* L. Indian Journal of Natural Products and Research. 2015;6(3):189-193. http://nopr.niscpr.res.in/handle/123456789/32673
3. Godoy de Lima R, Barros MT, da Silva Laurentiz R. Medicinal attributes of Lignans extracted from *Piper cubeba*: Current developments. Chemistry Open. 2018; 7(2):180-91. https://doi.org/10.1002/open.201700182 PMid:29435403 PMCid:PMC5795757
4. Kumar P. A review on medicinal plant *Piper cubeba* L. and its pharmaceutical properties. International Journal of the Science of Food and Agriculture. 2021; 5(1):174-179. https://doi.org/10.26855/ijfsa.2021.03.022
5. ICH guidelines, Q1A (R2): Stability testing of new drug substances and products (revision 2), International Conference on Harmonization. Available from: (http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm128204.pdf), 2003.
6. Bakshi M, Singh S. Development of validated stability-indicating assay methods-critical review. Journal of Pharmaceutical and Biomedical Analysis. 2002; 28(6):1011-40. https://doi.org/10.1016/S0731-7085(02)00047-X PMid:12049968
7. Srivastava M, editor. High-Performance Thin-Layer Chromatography (HPTLC). Springer Science and Business Media. 2010. p. 15. https://doi.org/10.1007/978-3-642-14025-9
8. Kamboj A, Saluja AK. Development of validated HPTLC method for quantification of stigmasterol from leaf and stem of *Bryophyllum pinnatum*. Arabian Journal of Chemistry. 2010; 10:S2644-50. https://doi.org/10.1016/j.arabjc.2013.10.006