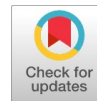


# A Quality by Design (QbD) Approach for the Development and Validation of RP-HPLC Method for the Quantification of Silymarin Tablet

Sanit J. Revankar, Shweta M. Pandare, M. S. Palled, Shailendra S. Suryawanshi



**Abstract:** This study emphasizes the crucial role of Quality by Design (QbD) in developing pharmaceutical procedures, particularly in risk assessment. It demonstrates how QbD principles were applied to create a precise and effective HPLC method for Silymarin Tablets, ensuring consistent quality within specified criteria. The optimized method, developed using a Design of Experiment approach, employs a C18 column (150 mm x 4.6 mm, 5µm) with isocratic elution using a 95:25 ratio of acetonitrile to orthophosphoric acid buffer (pH 3). Peaks were detected using a PDA detector calibrated at 287 nm, with a flow rate of 1.0 mL/min. The column oven temperature was maintained at 25°C, and a 10 µL injection volume was used. Thorough validation, adhering to USP <1225> and ICH Q2 (R1) standards, ensures the method's reliability. Key factors such as accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ) were comprehensively assessed. The method exhibits exceptional sensitivity, selectivity, efficiency, precision, accuracy, and cost-effectiveness, making it ideal for pharmaceutical analysis of Silymarin tablets. It has been validated to effectively differentiate between marketed products, including those closely resembling the original. This method is intended for routine quality control analysis in the pharmaceutical industry, highlighting its suitability and reliability for ongoing use.

**Keywords:** AQbD, Quality by design, RP-HPLC, Silymarin, DoE, Quantification, Pharmaceutical formulations, Method development, Method validation, ICH, USP Method validation.

## I. INTRODUCTION

In the past, developing analytical methods required a laborious process of fine-tuning one system characteristic at a time, which led to multiple experimental runs and additional refinement.

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The pharmaceutical industry's struggles with issues like out-of-spec (OOS) and out-of-trend (OOT) results have exposed flaws in the current system. Information about regulatory agencies sending out QC warnings emphasizes how urgently a novel strategy is required. The US FDA, EMA, and other ICH countries have all embraced Quality by Design (QbD), which is a proactive approach to resolving problems with analytical methods and associated systems in the pharmaceutical industry. [1,2].

This journal study focuses on applying QbD concepts to the development and validation of analytical procedures for Silymarin, with a focus on accuracy and dependability. By employing a rigorous and risk-based approach, QbD makes it easier to comprehend key method qualities, identify potential dangers, and design an extensive control strategy. [3,4]. By incorporating these concepts into the lifetime of an analytical method, researchers can maximize efficiency, minimize unpredictability, and guarantee adherence to regulatory standards. Silymarin, a flavonoid complex from milk thistle (Silybum marianum), is renowned for its potent antioxidant, anti-inflammatory, and hepatoprotective properties. It aids liver detoxification, protects against toxin-induced damage, and supports overall liver function. Silymarin's mechanisms of action include scavenging free radicals, inhibiting lipid peroxidation, and enhancing protein synthesis in liver cells. It is also explored for its benefits in managing conditions like hepatitis, cirrhosis, and fatty liver disease, making it a valuable natural supplement for liver health. [5,6].

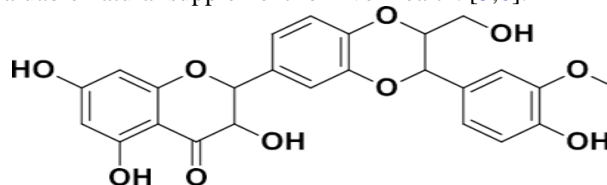


Figure 1. Chemical Structure of Silymarin

Using the concepts of AQbD, a chromatographic separation technique for silymarin was created in order to meet this demand. A Design of Experiment (DOE) methodology was used to optimize the procedure, taking into account important variables like flow rate and acetonitrile concentration and evaluating responses like retention time and theoretical plate. An optimal strategy was developed using statistical analysis, such as interaction and counterplots. [7,8]. USP <1225> and ICH Q2 (R1)4 criteria were met by the validated technique, which also included evaluations of traits like robustness, accuracy, precision, and LOD and LOQ.



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Excellent sensitivity, selectivity, speed, accuracy, precision, and affordability were all displayed by it, making it very suitable for usage in the pharmaceutical sector to test Silymarin tablets.

## II. EXPERIMENTAL

### A. Chemicals, Reagents and Samples

For the intended study, silymarin was procured from Caranio Private Limited, Hyderabad. The reagents used were of analytical grade and were procured from Merck Pvt. Ltd..

### B. Equipment

The HPLC used was a Waters e2695 separation module. Software used was Empower QS, Gradient Pump with integrated Degasser, Column Chemsil ODS-C18 column (250 mm × 4.6 mm, 5.0 μm particle size), and Detector 2998 PDA Detector.

### C. HPLC Method Development through AQbD Framework

The HPLC method for silymarin was developed following an AQbD methodology structured in eight systematic steps. [9-11]. The goal profile of the analytical process was first qualitatively established using a fishbone diagram, which took into account variables including the composition of the mobile phase, column specifications, buffer pH, injection volume, flow rate, and other elements. In order to get data on the molecular structure, molecular weight, pKa, functional groups, presence of chromophores, partition coefficient, solubility, and possible analytical techniques applicable to the analyte of interest, a thorough literature search was conducted in the second phase. Method scouting, the third step, involved defining the method parameters while considering the suitability of the stationary phase, the physiological chemical characteristics of the analyte, and the mobile phase. The fourth phase involved identifying projected important technique factors both qualitatively and quantitatively so as to make a distinction between components that were expected and those that were not. Using DoE for a multidimensional interaction analysis and identifying essential process parameters over a range were the tasks of the fifth phase.

After the sixth stage of screening and optimization, a scientific understanding of the relationship between input factors and output responses was created, coupled with an efficient design space. The final functioning technique was outlined in the seventh step, which also included method validation and stability-indicating experiments to evaluate the solution's resilience, accuracy, precision, and stability [12,13]. In the last phase of lifecycle management and monitoring, market products and the reference listed drug (RLD) were evaluated, identifying necessary adjustments for enhancement. The entire process ensured that HPLC method development adhered to thorough and organized principles, emphasizing Quality by Design (QbD) principles throughout.

## III. ASSESSING EXPERIMENTAL OUTCOMES AND DETERMINING THE BEST APPROACH PARAMETERS

Examining the impacts and combinations of two factors—mobile phase ratio and flow rate—on numerous responses—specifically, retention time and theoretical plate—was the main goal. The experimental design shown in Table 1 was

created to take into account the range of each variable in order to achieve this.

Much research would normally be required to determine the true influence of two factors on the two target answers. A DoE was created using Design Expert version 13 in order to expedite this procedure and hasten the identification of interaction patterns in a way that adheres to scientific rigor. The Department of Education implemented a thorough central composite design that included three (03) center points in every block [13,14].

Table 2 lists the eleven unique experimental combinations that were found based on the DoE. Different organic solvent types and flow rates were used in these combinations to produce different mobile phases. With a 10 μL injection volume and a C18 column (150 mm x 4.6 mm, 5 μm particle size), the goal was to optimize the HPLC method for evaluating silymarin. The HPLC system, which had a column oven, auto-injector, and PDA detector, ran at a wavelength of 287 nm [15,16].

## IV. EVALUATION OF EXPERIMENTAL RESULTS AND IDENTIFICATION OF OPTIMAL METHOD CONDITIONS

Contour plots, 3D response surface plots, overlay plots, and other statistical models were used to carefully analyze the reactions to perturbations (Table 3) in each of the different components (Figure 2 a-2e). Gaining a comprehensive comprehension of the actual influence of the two factors on the responses was made possible by these analyses.

In conclusion, 3D response surface plots were used to identify the kinds of interactions that occurred between the different variables and responses in the Silymarin experiment. The acetonitrile concentration and the mobile phase flow rate were shown to be inversely related in Silymarin theoretical plates. The retention period was directly correlated with acetonitrile content and flow rate. Counter plots, which represented response surfaces for retention time and theoretical plate with regard to different parameters, were used to demonstrate the results of the Design of Experiments (DoE). The theoretical plates shrank at low acetonitrile concentrations and flow rates. Retention durations were shorter at high flow rates and low acetonitrile concentrations. Among the many parameters, the concentration of acetonitrile significantly impacted the retention period; the flow rate had a significant impact on theoretical plates; and the combination of acetonitrile concentration and flow rate had a significant impact on Silymarin's asymmetry.

To get the best composite desirability, a response optimization was performed using Design Expert version 13, accounting for these effects on replies and standardized value determination. These elements were taken into account when creating the ultimate chromatographic method for silymarin in order to ensure method validation. Gradient HPLC equipment from multiple manufacturers, including Waters, USA, Dionex, USA, and Agilent, USA, was used throughout the process. These systems included an auto-injector, column ovens, and PDA detectors. The HPLC column used was a 150 mm x 4.6 mm C18 optimized column with a 5μM particle size.



A pH 3 OPA buffer and 95:5 acetonitrile, which had been tuned for maximum efficiency, made up the mobile phase. A UV detector calibrated to 287 nm was used to identify peaks, and a flow rate of 1.0 mL/min was found to be optimal.

**Table 1: Chosen Factors and their Levels for Design of Experiments (DoE) Investigations**

S.N.	Molecules Factors	% Acetonitrile		Flow rate (mL/min)		Response
		Min.	Max.	Min.	Max.	
1	Silymarin	90	95	0.8	1.2	Theoretical plates

**Table 2: Experimental Design for Preliminary Assessment in Silymarin Pre-Method Development**

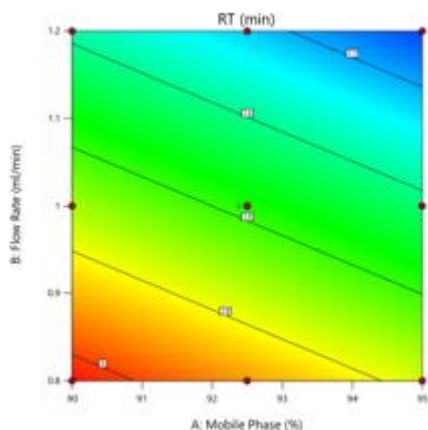
S.N.	Method parameters	
	% Acetonitrile	Flow rate (mL/min)
1	92.5	1
2	95	0.8
3	95	1.2
4	92.5	1
5	90	0.8
6	90	1
7	92.5	1.2
8	95	1
9	92.5	0.8
10	90	1.2
11	92.5	1

**Table 3: Responses to DoE Central Composite Design by Design Expert for Silymarin**

S.N.	Method Parameters		Response	
	% Acetonitrile	Flow rate (mL/min)	Retention time (min)	Theoretical plates
1	92.5	1	2.421	8615
2	95	0.8	3.066	5513
3	95	1.2	2.039	4635
4	92.5	1	2.421	8615
5	90	0.8	3	7335
6	90	1	2.408	6753
7	92.5	1.2	2.024	7550
8	95	1	2.457	5788
9	92.5	0.8	3.025	10299
10	90	1.2	3.004	8762
11	92.5	1	2.421	8615

Factor Coding: Actual

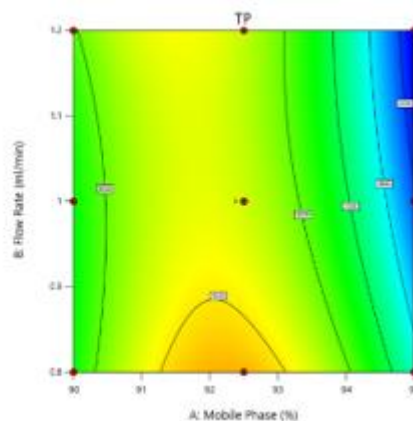
RT (min)  
● Design Points  
2.024 3.066  
X1 = A  
X2 = B



a

Factor Coding: Actual

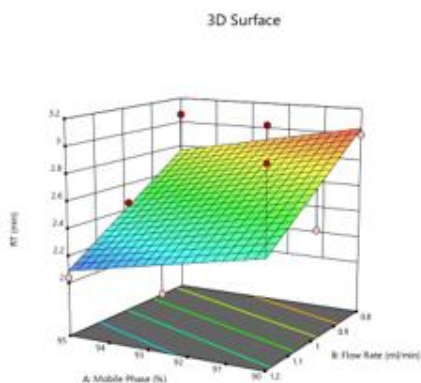
TP  
● Design Points  
4635 10299  
X1 = A  
X2 = B



b

Factor Coding: Actual

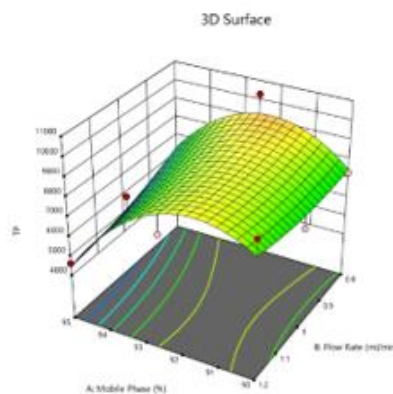
RT (min)  
● Above Surface  
○ Below Surface  
2.024 3.066  
X1 = A  
X2 = B



c

Factor Coding: Actual

TP  
● Above Surface  
○ Below Surface  
4635 10299  
X1 = A  
X2 = B



d



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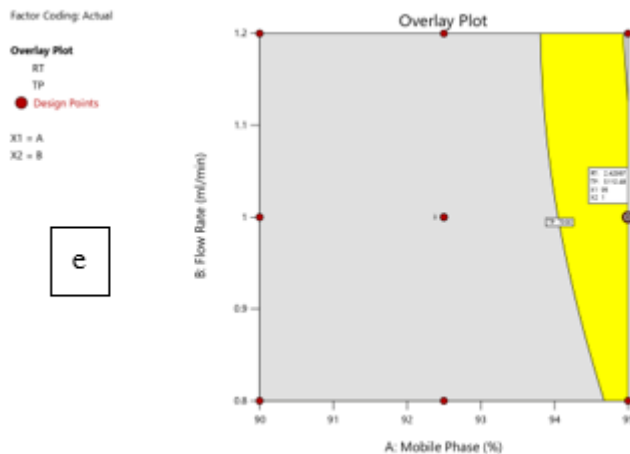


Figure 2: A: Contour Plot for Rt B: Contour Plot for Tp C: 3d Response Surface Plot for Rt D: 3d Response Surface Plot for Tp E: Overlay Plot

## V. METHOD VALIDATION

### 5.1. System suitability

A representative chromatogram (Figure 3) was used in a thorough system suitability test to check multiple parameters. According to the study, there were 5850 theoretical plates, the area and retention time for each of the six replicate injections had percentage RSDs (relative standard deviation) of 1.06% and 0.05%, respectively, and the peak asymmetry was 0.94.

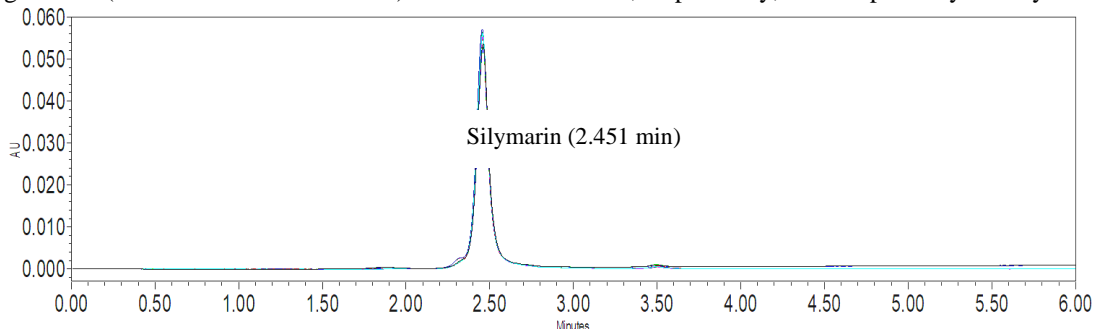


Figure 3: Representative Chromatogram of Silymarin

### 5.2. Specificity

Specificity was done by injecting blank, and there was no interfering peak found, hence the method was found to be specific.

### 5.3. Linearity

Table 4 displays a linear calibration curve for concentration over a concentration range of 10–50 µg/ml. When the graph was plotted with area versus concentration, the regression equation for the calibration curve was found to be  $y = 9378.2x + 3640.8$  with a 0.998 correlation coefficient (Fig. 4).

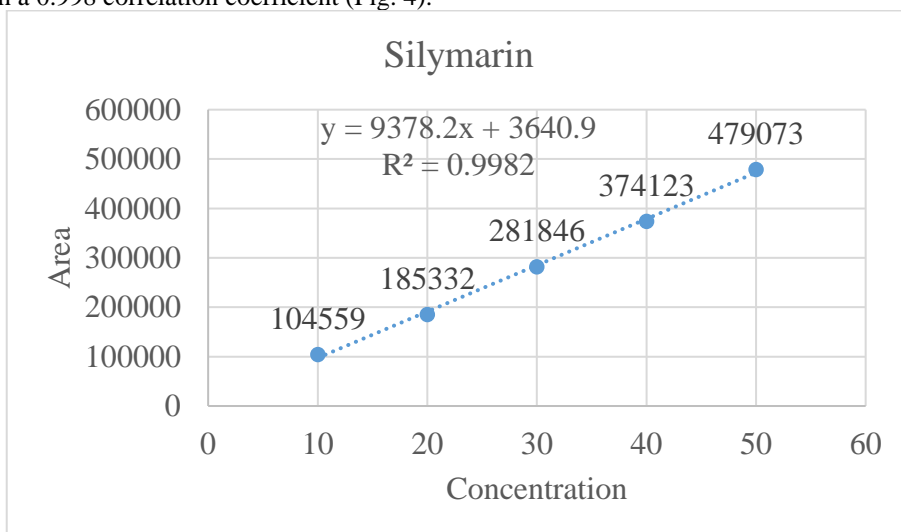


Figure 4: Linearity Study of Silymarin



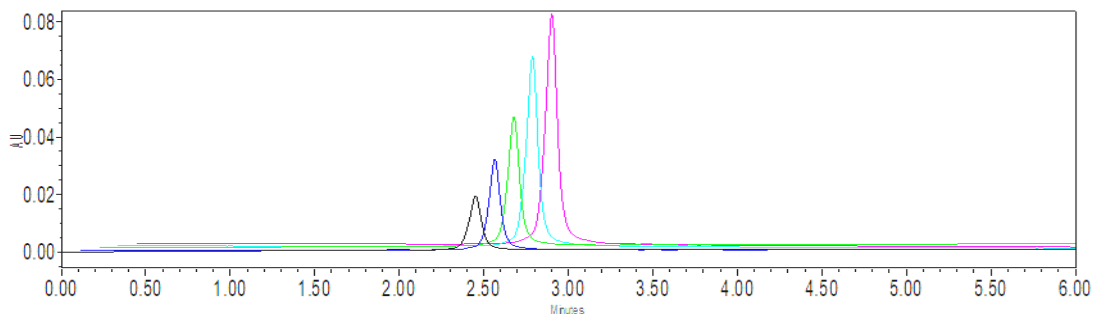


Figure 5: Overlay Spectra for Silymarin

Table 4: Summary Results for Linearity Study of Silymarin

Concentration (µg/ml)	10	20	30	40	50	Correlation Coefficient
Silymarin	104559	185332	281846	374123	479073	0.998

5.4. Precision

Less than 2% was discovered to be the RSD for silymarin repeatability, based on 3 measurements of the same concentration (10, 20, and 30 µg/ml). The developed approach was found to be exact when the RSD value was less than 2.

5.5. Accuracy

Recovery studies were used to ensure accuracy. Three different amounts of spiking were used to prepare sample solutions: 80%, 100%, and 120%. Table 5 displays the percentage recovery statistics that were acquired using the suggested HPLC procedure. According to ICH Q2 (R1) requirements, the developed method was deemed accurate based on a recovery percentage of 98–102%.

Table 5: Summary results of accuracy study

Level	80%	100%	120%	Mean	%RSD
Silymarin	99.35	100.1	101.2	100.2	1.61

5.6. Robustness and Ruggedness Studies

Using sample concentrations of 30 µg/mL, the effects of modifications in the mobile phase ratio (Organic Phase: Buffer), detector wavelength, and mobile phase flow rate on the peak area of the primary analyte were examined. The methods appear to be resilient when the %RSD is less than 2%, as seen by the lack of substantial differences in results even after these approach parameters were changed. The LOD and LOQ for silymarin were ascertained using the Dionex HPLC Chromeleon software. According to the findings, the corresponding values were 2.56 µg/ml and 7.77 µg/ml.

5.7. Assay content of marketed products

The established techniques proved to be efficacious in evaluating the identification and assay value of commercially available silymarin. The exceptionally clear chromatographic separations demonstrated that the excipients had not interfered. As shown in Table 6, the product under test complies within the intended acceptance range (98% to 102%).

Table 6: Data for assay

Name of molecule	% Assay Value
	Silybon-70
Silymarin	102.8%

VI. DISCUSSION

HPLC quality-by-design technology has been painstakingly developed to guarantee accurate silymarin measurement in

pharmaceutical formulations [18][19][20]. Critical parameters for the silymarin analysis by HPLC, which include theoretical plates and retention time, were identified by the analytical target product profile using a risk assessment technique. The target product profile is impacted by critical quality aspects, including the composition and flow velocity of the mobile phase. Eleven independent runs were carried out using a full factorial design with Design Expert version 13, which included two factors and two replies with three center points per block. Carefully regulated variations were seen in the injection volume, instrument design, and column selection. A quality-by-design methodology was employed to effectively develop an HPLC procedure for silymarin using a C18 column (150 mm x 4.6 mm, 5 µm particle size). The optimal mobile phase was a 95:5 ratio of CAN to OPA acid buffer (pH 3). The flow rate was 1.0 mL/min, with detection using a PDA detector at 287 nm. The injection volume was 10 µL.

The method validation yielded satisfactory results across various parameters, including specificity, linearity, LOD, LOQ, robustness, accuracy, precision, and system suitability. This validated method was applied to test Silymarin tablets marketed under the Silybon-70 brand in India, and the findings were positive.

VII. CONCLUSION

To create an HPLC technique for silymarin, the AQbD principles were closely followed. A thorough multivariate analysis was carried out, looking at crucial process variables such as different combinations of flow rate and the composition of the mobile phase. This comprehensive analysis was carried out on several levels in order to determine the ideal system, which in turn defined the final design space. The utilization of Design of Experiment Software in Design Expert version 13 enabled this investigative procedure, which improved the comprehension of variables impacting chromatographic separation. This ensured that the techniques attained their goals to a satisfactory extent and cleared the path for the advancement of chromatographic optimization for further uses.



# A Quality by Design (QBD) Approach for the Development and Validation of RP-HPLC Method for the Quantification of Silymarin Tablet

The validated method for determining silymarin consistently met all confirmed metrics, adhering to acceptable criteria. It demonstrated robustness, ruggedness, linearity, accuracy, precision, and specificity among its key characteristics. The QbD strategy minimized problems during method validation and adoption by providing a comprehensive grasp of method variables. The automated approach to creating QbD methods with Minitab software not only sped up the method but also resulted in a more dependable way than manual production. A statistical study demonstrated the robustness, accuracy, selectivity, and reproducibility of the procedure. With its capacity to distinguish marketed products, especially those that are similar to the innovation product, this approach is ideally positioned for broader implementation in routine quality control analysis within the pharmaceutical industry.

## DECLARATION STATEMENT

Funding	No, I did not receive.
Conflicts of Interest	No conflicts of interest to the best of our knowledge.
Ethical Approval and Consent to Participate	No, the article does not require ethical approval and consent to participate with evidence.
Availability of Data and Material	Not relevant.
Authors Contributions	All authors have equal participation in this article.

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**Shweta M. Pandare** M. Pharm Shweta M. Pandare, have completed a Bachelor of Pharmacy (B.Pharm) and a Master of Pharmacy (M.Pharm) in Pharmaceutical Chemistry from KLE College of Pharmacy, Belagavi. Throughout my academic journey, I have gained a robust foundation in pharmaceutical sciences, with a specific focus on pharmaceutical chemistry. My education has provided me with comprehensive knowledge and practical skills essential for excelling in the pharmaceutical industry. I am equipped to contribute to research and development, ensuring the creation of safe and effective pharmaceutical products. My training has prepared me to meet the challenges of the ever-evolving field of pharmacy.



**Dr. M. S. Palled** M. pharm, PhD, having completed my M.Pharm and Ph.D. Currently, I am a professor at KLE College of Pharmacy, where I teach in the Department of Pharmaceutical Chemistry. My extensive academic background and research experience have equipped me with in-depth knowledge and expertise in pharmaceutical sciences. As a professor, I am dedicated to educating and mentoring the next generation of pharmacists and researchers. My work involves not only teaching but also contributing to innovative research in pharmaceutical chemistry, aiming to advance the field and improve therapeutic outcomes.





**Dr. Shailendra S. Suryawanshi** M. pharm, PhD, having completed my M.Pharm and Ph.D. Currently, I am working as an Assistant Professor at KLE College of Pharmacy in the Department of Pharmaceutical Chemistry. My advanced academic training and research experience have provided me with a deep understanding of pharmaceutical sciences. In my role as an assistant

professor, I am committed to imparting knowledge and fostering the skills of students, preparing them for successful careers in the pharmaceutical industry. Additionally, I am actively involved in research initiatives aimed at advancing the field of pharmaceutical chemistry and developing innovative therapeutic solutions.

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