

# A Novel Rapid Approach for the Estimation of Ketoconazole Using Reverse Phase Ultra Performance Liquid Chromatography in Bulk and Tablet Dosage from

Sumanta Mondal<sup>1,\*</sup>, Chanchala Alekya<sup>1</sup>, Prasenjit Mondal<sup>2</sup>

<sup>1</sup>Department of Pharmaceutical Chemistry, GITAM Institute of Pharmacy, GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, INDIA.

<sup>2</sup>Department of Pharmaceutical Chemistry, Vaageswari Institute of Pharmaceutical Science, Ramakrishna Colony, Karimnagar, Telangana, INDIA.

## ABSTRACT

**Objectives:** To Develop a novel, rapid, validated ultra-performance liquid chromatographic (UPLC) method for estimation of Ketoconazole in a bulk and tablet dosage form according to ICH Guidelines. **Methods:** The chromatographic separation was achieved using Endoversil (50 x 2.1mm, 1.7µm) UPLC column. The mobile phase used was a mixture of Phosphate buffer pH 5.5: Methanol (40:60) at isocratic mode and eluents were monitored at 265 nm using PDA detector. Force degradation study was conducted for, acidic, alkaline, thermal, peroxide and photolytic conditions. **Results:** By this method Ketoconazole was eluted with retention time of 0.520 min. The method was continued and validated accordance with ICH guidelines. Validation revealed the method is rapid, specific, accurate, precise, reliable and reproducible. Calibration curve plots were linear over the concentration ranges 8

to 32 µg/mL for Ketoconazole. Limit of detection (LOD) was 0.02µg/ml and limit of quantification (LOQ) was 0.06µg/mL for Ketoconazole. **Conclusion:** The developed method was found suitable for the assay of ketoconazole in bulk and tablet dosage form.

**Key words:** Ketoconazole, UPLC, Method Development, Method Validation, ICH Guidelines, UPLC estimation of ketoconazole.

### Correspondence

**Dr. Sumanta Mondal**

Associate Professor, GITAM Institute of Pharmacy, GITAM (Deemed to be University), Visakhapatnam-530045, Andhra Pradesh, INDIA.

Phone no: +91 9703615761

E-mail: mondalresearch@gmail.com

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## INTRODUCTION

Ketoconazole (C<sub>26</sub>H<sub>28</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>4</sub>) is a synthetic, imidazole antifungal medication used primarily to treat fungal infections<sup>1,2</sup> Ketoconazole is sold commercially as a tablet for oral administration<sup>3-5</sup> (although this use has been discontinued in a number of countries) and in a variety of formulations for topical administration, such as creams (used to treat tinea; cutaneous candidiasis, including candidal paronychia; and pityriasis versicolor) and shampoos (used primarily to treat dandruff, seborrheic dermatitis of the scalp).<sup>6-8</sup> The less toxic and generally more effective triazole antifungal agents fluconazole and itraconazole are usually preferred for systemic use. The European Medicines Agency's Committee on Medicinal Products for Human Use (CHMP) has recommended that a ban be imposed on the use of ketoconazole for systemic use in humans throughout the European Union, after concluding that the risk of serious liver injury from systemic ketoconazole outweighs its benefits.<sup>9</sup> In Australia, the oral formulation of ketoconazole has already been discontinued.<sup>10</sup> The IUPAC name of ketoconazole is 1-[4-(4-{[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)piperazin-1-yl]ethan-1-one. The chemical structure of the ketoconazole was shown in Figure 1.

A survey of literature reveals that there is a few HPLC method<sup>11-13</sup> available for the estimation of ketoconazole alone. Reported articles has several disadvantages, eg, either long retention time, low sensitivity, ambiguity in sample preparation and several non-reliable aspects. Few UPLC methods<sup>14</sup> has been reported for the bioanalytical study but no UPLC method has been reported for the ketoconazole alone. The present research work tried to minimize the mentioned disadvantages of the earlier reported methods and to develop a novel, reliable, simple validated ultra-performance liquid chromatographic method for the estimation of ketoconazole alone and in tablet dosage form, in accordance with ICHQ2B guidelines.<sup>15</sup>

## MATERIALS AND METHODS

### Chemicals and Reagents

Pharmaceutical grade working standards ketoconazole was obtained from Pharmatrain Laboratories, Hyderabad, India as gift sample. The tablets of Ketoconazole, KETOCIP (200 mg tablets) from Cipla Limited. Were collected from local Market. All chemicals and reagents were required for the method development and validation and Stability Studies were purchased from final chemical Ltd. Fisher Scientific and Merck, Mumbai.

### Instrumentation Conditions

The analysis was performed using Ultra performance liquid chromatography (UPLC) Acquity Waters, PDA detector. Software: Empower 2 equipped with Auto Sampler. Analytical balance 0.1mg Sensitivity (Afcoset ER-200A), pH meter (Adwa – AD 1020), Ultra Sonicator. The column used is Endoversil (50 x 2.1mm, 1.7µm) UPLC column.

### Preparation of Phosphate buffer

To prepare Phosphate buffer pH 5.5 solution 6.8gm of Potassium di hydrogen ortho phosphate was included in 1000 ml distilled water. Solution to pH 5.5 was adjusted by using sodium hydroxide.

### Preparation of mobile phase

Prepared phosphate buffer 400 ml (40%) and 600 ml (60%) Methanol HPLC grade was mixed and degas in ultrasonic water bath for 5 min. Filter through 4.5 µm filter under vacuum filtration. The prepared mobile phase also used as a diluent.

### Preparation of standard stock solution and working solution

Accurately weighed 20 mg of ketoconazole and transferred into a 25ml clean dry volumetric flask. Diluent was added and sonicated to dissolve

it completely and make volume up to the mark with the diluent. Further pipette 0.3 ml aliquote of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent to obtained 24 µg/mL of ketoconazole. This solution is considered as working solution.

## Assay of ketoconazole in Pharmaceutical Dosage Form

Ten ketoconazole tablets (each tablets containing 200 mg) were taken and average weight was determined. Tablets were finally powdered and triturated well. A quantity of powder equivalent to 20 mg of ketoconazole were transferred to 25 ml volumetric flask and 15 ml of mobile phase/ diluent was added to dissolve and sonicated for 15 min. The volume was made up to 25 ml with mobile phase. Then 0.3 ml of the aliquot was diluted to 10 ml with mobile phase. The solution was filtered using a membrane filter (0.45 µm). The solution prepared was injected in to the UPLC system, chromatogram was recorded and data was analysed for the content of ketoconazole.

## Method validation

ICH guidelines were followed to carry out the validation study of ketoconazole and same was followed for the force degradation study of the ketoconazole.

## Accuracy

The accuracy of the proposed method has been conducted by recovery studies that was performed by preparing different levels at 80%, 100% and 120% of pure drug of ketoconazole were taken and added to the pre-analysed formulation of concentration.

## Precision

Precision study was performed by conducting repeatability and intermediate precision of the ketoconazole sample in accordance with ICH guidelines.

## Repeatability

This study was conducted by injected the six replicate of the sample. The peak areas and retention times obtained by actual determination of six replicates of a fixed amount of drug ketoconazole (API) has been considered. The percent relative standard deviation was calculated for ketoconazole

## Intermediate precision

The intermediate precision (within-laboratory variation) study was conducted by two analysts, using two UPLC systems on different days and the relative percent purity data was evaluated. The percentage relative standard deviation was calculated.

## Linearity

Calibration standards solutions were prepared by appropriately mixed and further diluted standard stock solutions in the concentration ranges from 8-32 µg/mL for ketoconazole. Samples injections were performed for each prepared concentration. Peak areas were plotted against the corresponding concentration to obtain the linearity graphs. Chromatograms of each solution were recorded

## LOD and LOQ

The LOD and LOQ of ketoconazole were determined by using signal to noise approach as defined in ICH guidelines. The LOD and LOQ were assessed at signals to noise ratio of 3:1 and 10:1 respectively by injecting dilute solution of drug was injected into the chromatograph and signal

to noise (S/N) ratio was calculated.

## Robustness

It is the measure for the capacity of the developed method to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness was performed by injecting the ketoconazole standard solution in to the UPLC system by altering the detection wavelength, flow rate and also by changing the composition of the organic solvent from the normal chromatographic conditions.

## Degradation studies

Stress study<sup>16</sup> was carried out in a environmental test chamber (by Acamus Technologies, India) at 60°C and relative humidity was maintained 75%, as per ICH prescribed stress condition such as acidic, basic, thermal, oxidative and photolytic stresses.

Acid degradation study was conducted in environmental test chamber (Acamus Technologies, India) at 60°C and 75% relative humidity with 1M HCL. 1 ml of stock solution was transferred in 10 ml of volumetric flask, 1 ml of 1 M HCL was added to the flask, kept in environmental test chamber for 16 hr. After the suitable stress period, solution was neutralized using 1M NaOH and make up the volume with mobile phase.

Base degradation study was conducted at 60°C and 58% relative humidity using same environmental chamber. 1 ml of stock solution was transferred in 10 ml volumetric flask mixed with 1 ml of 1M NaOH and kept for 16 hr. After the suitable stress period the solution was neutralized with 1 M HCL and the volume was made with mobile phase.

Oxidative degradation study was performed in versatile environmental chamber at 40°C, 75% relative humidity using 6% H<sub>2</sub>O<sub>2</sub>. For this purpose 1 ml of stock solution was taken in 10 ml volumetric flask and 1 ml of 6% H<sub>2</sub>O<sub>2</sub> was added in to flask and kept at 60°C for 16 hr, finally make up the volume up to mark with mobile phase. Thermal degradation study also has been carried out using environmental chamber at 40°C, 75% relative humidity in oven at 105°C, 1 ml of stock solution was taken in 10 ml volumetric flask and kept in chamber for 144 hr. and for dry heat thermolysis, 1 mg of dry drug in solid form was placed in oven at 110°C for 2 days.

Photolytic degradation study was carried out in sunlight (60000- 70000 lux) during day time and in U.V light at 254 nm for the period of 48 hr. 1 ml of stock solution was taken in 10 ml volumetric flask and make the volume up to mark with mobile phase was used for the study.

## RESULTS

### Method Development

Different chromatographic conditions were investigated to achieve a novel UPLC method for ketoconazole. Several parameters such as mobile phase composition, column, detection wavelength, pH of mobile phase and diluents were optimized. Various proportions of solvents, buffer, were evaluated in order to obtain suitable composition of the mobile phase. Finally ketoconazole eluted with good peak shape and very low retention time using the mobile phase phosphate buffer, pH 5.5 and methanol as a mobile phase (60:40) with a flow rate of 0.25 mL/min. The retention time obtained for ketoconazole is 0.520 min. Quantification was achieved with PDA detection at 265 nm. The method was validated as per ICH guidelines. The optimised chromatogram was shown in Figure 2.

### Method validation

The developed method was successfully applied for the subsequent validation studies. Both precision and accuracy were determined with

standard quality control samples. The results of accuracy as a mean % recovery was found 100.52 which is within the acceptable limit and the % RSD was not more than 2 %, shown in Table 1. The repeatability study results was found 1.02 as a %RSD. For the intermediate precision the % amount found was calculated and %RSD was found 0.8, results of precision were shown in Table 2. In the assay of marketed dosage form of ketoconazole the average percentage assay was found 98.35 %, The assay chromatogram was depicted in Figure 3 and result was shown in Table 3. The linearity was determined for six concentrations and the correlation coefficient was found to be 0.999 for ketoconazole which is within the specified limits and the obtained linearity range was 8-32 µg/mL as shown in the Figure 4 and in Table 2. The limit of detection and limit of quantitation were found to be 0.02 µg/ml and 0.06 µg/ml respectively. Robustness study of the method was carried out by changing three parameters from the chromatographic conditions such as changes in mobile phase composition ( $\pm 5\%$ ), changes in flow rate ( $\pm 0.1$  ml/min) and detection wavelength ( $\pm 2$  nm) and the % RSD of the tailing factor was calculated found to be less than 2.0 as shown in Table 4. Degradation studies of ketoconazole were performed under the influence of acid, alkali, oxidation, thermal, photolytic. Degradation was found for all stressed condition. Acidic stressed condition shows 5.13%, alkaline shows 4.80%, peroxide condition shows 3.95%, The thermal and photo degradation shows 6.56% and 4.54% degradation respectively, The detail results were shown in Table 5 and chromatograms shown in Figure 5.

**Table 1: Accuracy data of the developed method for ketoconazole.**

% Level	Amount of sample taken (µg/mL)	Amount recovered	%recovery	Average
50%_01	125	123.5645	98.15	98.93
50%_02	125	124.6532	99.72	
50%_03	125	123.6845	98.94	
100%_01	250	248.3602	99.45	99.50
100%_02	250	249.1056	99.64	
100%_03	250	249.0564	99.62	
150%_01	375	372.8681	99.43	98.75
150%_02	375	368.8649	98.36	
150%_03	375	369.3201	98.48	

**Table 2: Summary of validation parameters.**

Parameters	Results
Beer's law limit in mg/ml	8-32
Co-relation co-efficient	0.999
LOD µg/ml	0.02
LOQ µg/ml	0.06
Precision	0.06
(% RSD)	0.06

**Table 3: Assay of marketed formulation.**

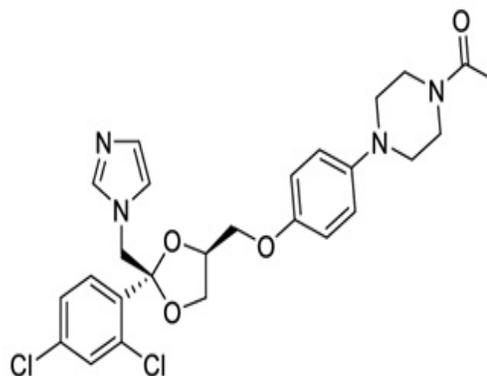
Brand	Labelled amount of Drug (mg)	Mean ( $\pm$ SD) amount (mg) found by the proposed method ( $n=6$ )	Assay % ( $\pm$ SD)
Ketocip (Ketoconazole 200 mg) Cipla Ltd	200mg	196.46 ( $\pm$ 0.476)	98.35 ( $\pm$ 0.531)

**Table 4: Robustness study.**

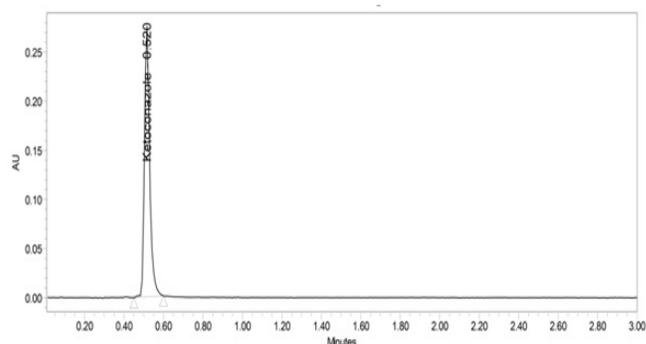
Change in parameter	% RSD ( $n=3$ )
Flow (1.1 ml/min)	0.45
Flow (0.9 ml/min)	0.96
Less Organic	0.96
More Organic	1.87
Wavelength of Detection (267 nm)	1.35
Wavelength of detection (263nm)	0.97

**Table 5: Stress degradation study.**

Stress condition	Time	Assay of active substance	% degradation	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	4Hr.	94.16	5.13	100.0
Basic Hydrolysis (0.1 M NaOH)	4Hr.	95.20	4.80	100.0
Thermal Degradation (60°C)	6 Hr.	93.44	6.56	100.0
Photolytic	24Hr.	96.44	3.56	100.0
3 % Hydrogen peroxide	24Hr.	96.05	3.95	100.0



**Figure 1:** Chemical structure of Ketoconazole.



**Figure 2:** Optimised chromatogram of ketoconazole.

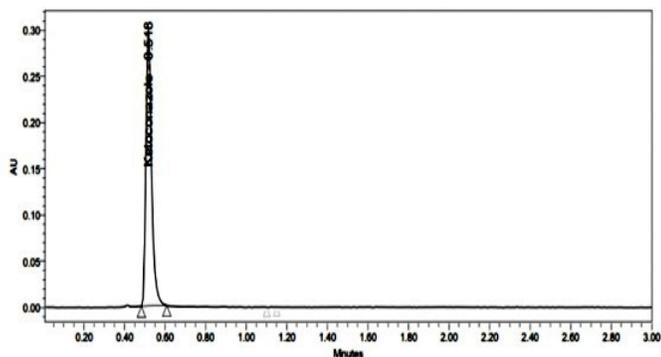


Figure 3: Assay of marketed dosage form.

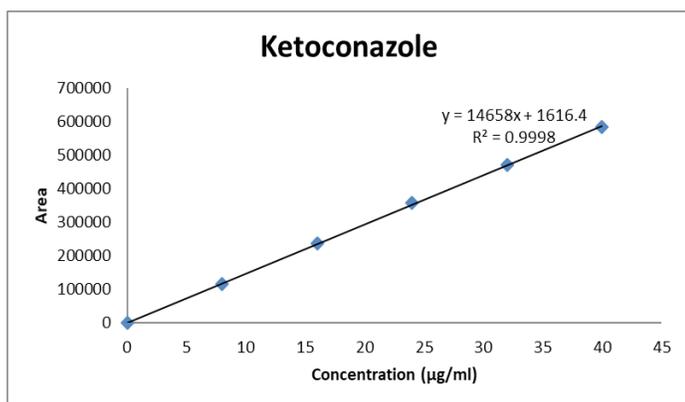


Figure 4: Linearity of the developed method.

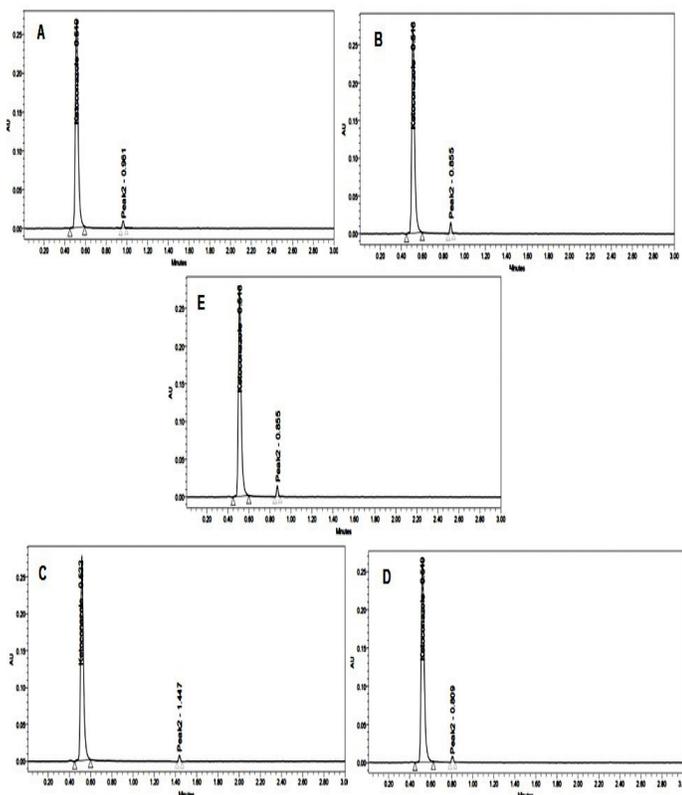


Figure 5: Degradation chromatograms of the ketoconazole at various stressed condition. Acidic(A), Alkaline(B), Oxidative (C), Photolytic (D), Thermal (E).

## DISCUSSION

The present research work the optimised chromatographic conditions was confirmed after several trials. Using the developed optimised condition ketoconazole eluted with good peak shape, very short retention time using a mixture of phosphate buffer and methanol in the volume ratio of 60:40 with a flow rate of 0.25 mL/min. The retention time obtained for ketoconazole is 0.520 min. Precision and accuracy were determined as per the guidelines and the % recovery was found within the acceptable limit i.e. not more than 2.0%, indicated the accuracy of the developed method. In repeatability study the amount found was calculated and %RSD was found satisfactory and within the limit. The results of precision study indicated that the developed method was found precise. The average percentage assay 98.35 % was considered within the limit and found suitable to analyse using developed method for the estimation of ketoconazole in dosage form. In the linearity study of the method the correlation coefficient was found near to 0.999 for ketoconazole which indicates its specified linearity. The least squares method was used to establish the regression line and the curves were linear. The limit of detection and quantitation values proves the effectiveness and sensitivity of the developed method. In the specificity study no excipients peaks were found at the retention time of the analyte and indicates the specificity of the method. In robustness study the tailing factor was considered and the % RSD of the tailing factor was found less than 2.0 which proved the robustness of the developed method, because no such significant changes were found on deliberate changes in the optimised parameters. Degradation studies results of ketoconazole indicated that acidic and thermal stressed condition leads to little more degradation in compare to other stressed condition, but in every stressed condition the ketoconazole chromatogram was found very specific.

## CONCLUSION

Empirical evidences of the validation study results strongly clammed about the novelty of the developed method in compared to reported methods. The developed method, which is found 'rapid' because it significantly reduced the total analysis time less than 1 min which is the lowest analysis time required. The method justifies "easy", because the proposed method does not involved use of dual wavelength, gradient techniques. The present method is "stability indicating" as this has been shown less degradation pattern in stressed conditions and good separation of ketoconazole among the other degraded peaks. All the results of validation parameters were found within the limits as per the ICH Q2B guidelines. Hence the present developed method can be considered as a fast, reliable, validated and can be utilized for the routine analytical and quality control study of the ketoconazole in the bulk and tablet dosage form.

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## CONFLICT OF INTEREST

The authors declared no conflict of interest" in the manuscript

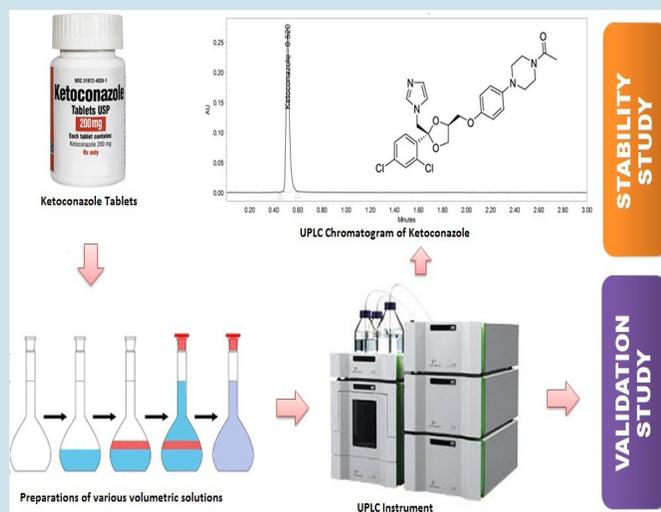
## ABBREVIATIONS

UPLC: Ultra performance liquid chromatography; ICH: International conference on harmonization; PDA: Photo diode array; LOD: Limit of detection; LOQ: Limit of quantitation; SD: Standard deviation; RSD: Relative standard deviation.

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## PICTORIAL ABSTRACT



## SUMMARY

The present work has been designed to develop a UPLC method for the estimation of Ketoconazole. The chromatographic Separation was achieved with an Endoversil (50 x 2.1mm, 1.7 $\mu$ m) UPLC column. The mobile phase used was a mixture of Phosphate buffer pH 5.5: Methanol (40:60) The developed method was validated as per ICH guidelines. The result (%RSD) of the each validated parameters were within the limit. Force degradation studies show various degradation patterns of Ketoconazole. The developed method was applied successfully for the determination of Ketoconazole in tablet dosage form.

## ABOUT AUTHORS



**Dr. Prasenjit Mondal** is an Associate Professor And HOD at the Vaageswari Institute of Pharmaceutical sciences, Ramakrishna Colony Karimnagar. His doctoral work focused on the Bioanalytical method development and validation in human plasma using LC-MS/MS and pharmacokinetic study. He has published more than 45 research articles in various high impact international and national journals. He guided 27 M.Pharm students and published two academic books.



**Dr. Sumanta Mondal:** [Associate Professor & NSS Programme Officer of GITAM Institute of Pharmacy, GITAM (Deemed to be University), Andhra Pradesh, India]. His research involves bioactivity and phytochemical studies of various medicinal plant species. He has published one Book and more than 88 research articles in various international & national journals. He has guided more than 22 M. Pharm students and presently 5 students are pursuing PhD & two are awarded under his guidance.



**Ms. Chanchala Alekya,** student of M.Pharmacy (Pharmaceutical Analysis), GITAM Institute of Pharmacy, GITAM (Deemed to be university), Visakhapatnam, pursuing project work under the guidance of Dr. Sumanta Mondal