

# Development and Validation of a Spectrofluorimetric Method for the Estimation of Baicalein in Bulk and Formulation

<sup>1</sup>Dr.Sachin Shivling Bhusari, <sup>2</sup>Aarti Raju Popalghat, <sup>3</sup>Dr.Pravin.S.Wakte

<sup>1</sup>Assistant professor, <sup>2</sup>Student M.Tech (Drugs and Pharmaceutical), <sup>3</sup>HOD  
Pharmaceutical Technology Division  
department of chemical technology  
Dr. Babasaheb Ambedkar Marathwada University  
Aurangabad, Maharashtra 431001.

Corresponding Author: **Dr. Sachin Shivling Bhusari**

**Abstract-** A simple, accurate, precise, sensitive and cost-effective spectrofluorimetric method was developed and validated for the estimation of baicalein in bulk and formulation. The relative fluorescence intensity of baicalein was measured in pure methanol at an excitation wavelength of 371 nm and an emission wavelength 433 nm. Proposed method was found to be linear over the range of 50 to 800 ng/ml with correlation coefficient 0.999. Proposed method was validated using different analytical method validation parameters viz. Accuracy, precision, LOD, LOQ, robustness and ruggedness using QC standards as per the ICH guidelines. The percentage recovery was found to be 101.43% and percentage RSD values were found to be less than 2 for accuracy and precision studies. The detection and quantification limits for the proposed method were found to be 2.14ng/ml and 6.48ng/ml, respectively. A simple, accurate, precise, sensitive yet cost-effective spectrofluorimetric method was developed for the estimation of baicalein in bulk and formulation. The said spectrofluorimetric method was found to be economic as it comprises with methanol: water (40:60) as a solvent.

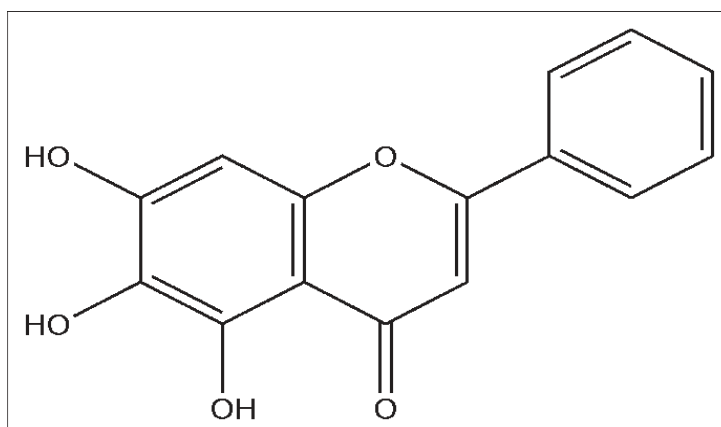
**Keywords-** Spectrofluorimetry, Baicalein, Validation, Excitation, Emission.

## INTRODUCTION

Baicalein is commonly known as Shyonaka and sonapath, belongs to family Bignoniaceae. Baicalein is present in root bark, seeds, and leaves of *Oroxylum indicum*. Originally we have isolated Baicalein from the leaves of *Oroxylum indicum*. Leaves are mainly consist of variety of chemicals with wide range of activities in that Baicalein has gained the attention of researchers working in natural products. Baicalein chemically is 5,6,7-trihydroxy-2-phenylchromen-4-one. Baicalein is the major representative of the flavonol subclass of flavonoids, derivative of flavone glycoside<sup>[1]</sup>. It is a non-specific protein kinase enzyme inhibitor<sup>[2-4]</sup>. It has also been reported to have estrogenic activities by activating estrogen receptors<sup>[5-6]</sup>. It is generally use for the treatments of pulmonary artery, Alzheimer disease, and Parkinson's disease. It has several pharmacological effects viz. Reducing oxidative stress, anti-inflammatory, inhibiting excitotoxicity and anti-oxidant, anti-viral, anti-bacterial, anticancer and anti-allergic therapies, anti -apoptosis effects. It is used in treatment of heart diseases, exercise induced respiratory problems, high cholesterol, diabetes, asthma, gout, cancers such as lung cancer, ovarian cancer and pancreatic cancer<sup>[7-8]</sup>. It is soluble in organic solvent like ethanol, methanol and water.

Nowadays abundant presence of baicalein in several foods and plants including imparts prominent dietary supplement activity to extracts therefore standardized *Oroxylum indicum* extracts are gaining commercial importance. Considering the above therapeutic importance of baicalein some of the analytical methods were reported from all over the globe but till today, there is no single spectrofluorimetric method available for the estimation of baicalein using cost effective, non-reactive and biocompatible solvents. Therefore, a simple, sensitive, accurate and precise spectrofluorimetric method was developed for the estimation of baicalein in bulk and its formulation.

**Fig. 1: Chemical structure of Baicalein**



## MATERIALS AND METHODS

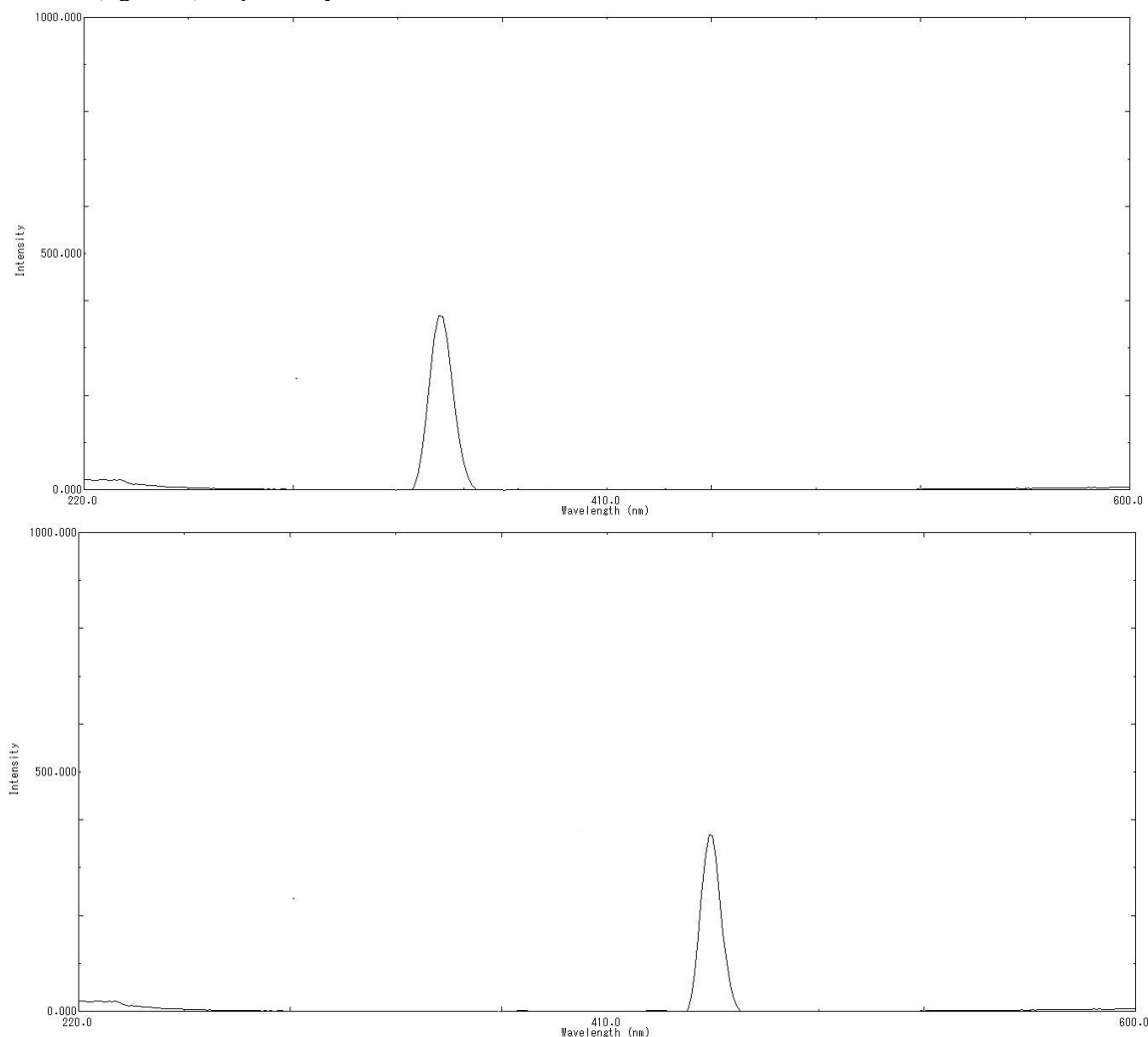
Baicalein was purchased from TCI chemicals (India) Pvt. Ltd. HPLC grade water, methanol, ethanol was used for study.

### Instruments used

The Spectrofluorimetric study was carried out with a ShimadzuRF-5301 Fluorimeter to determine levels of fluorescence in the baicalein. A xenon 150 w lamp was used as a light source. Quartz cells having 48 mm height, 10 mm path length with 0.5mm slit width were used for fluorescence measurement. Weighing balance (Vibra HT, Essae) with internal calibration mode was used for the weighing purpose.

### Preliminary analysis

A preliminary analysis was carried out to determine the excitation and emission wavelength of baicalein. Various solvents like distilled water, methanol, acetonitrile, ethanol and dimethyl formamide were used to determine appropriate media for baicalein. Baicalein showed maximum fluorescence intensity in Ethanol: Water (40:60 % v/v) as a media. Initially, baicalein solution of 100 ng/ml strength was prepared in ethanol. Prepared solution was scanned spectrofluorimetrically to obtain the excitation and emission wavelengths. The scanning was performed over 220nm to 600nm range and excitation and emission wavelength were found to be 444nm and 538nm (figure.2 ) respectively.



**Fig. 2: Excitation and Emission Spectra of Baicalein**

### Preparation of Standard Stock Solution

Accurately weighed 10 mg of baicalein was transferred into the calibrated volumetric flask and dissolved in 10 ml methanol to achieve a stock solution of 1000  $\mu\text{g/ml}$  (stock-I). Stock- I solution was suitably diluted with water to achieved further calibration standards.

### Construction of Calibration Curve

Calibration curve was prepared by diluting the stock-I (1000  $\mu\text{g/ml}$ ) solution to achieve the seven different calibration standards representing CAL STD 1 (50ng/ml), CAL STD 2 (100ng/ml), CAL STD 3 (200ng/ml), CAL STD 4 (300ng/ml), CAL STD 5 (400ng/ml), CAL STD 6 (600ng/ml), CAL STD 7 (800ng/ml) strength.. The fluorescence intensity was measured at pre-defined excitation and emission wavelengths of 371 nm and 433 nm respectively. The calibration curve representing concentration vs. Fluorescence intensity was plotted using excel program of Microsoft office 2013. Above mentioned procedure was repeated three times, so that reproducible results can be obtained.

### Spectrofluorimetric Method Validation

Validation is the process which provides a high degree of assurance, so as to produce a desired result and meeting its predetermined specifications and quality characteristics. Developed fluorimetric method for the estimation of baicalein was validated as per the ICH guidelines. Different validation parameters like linearity and range, accuracy, precision, robustness, ruggedness, limit of detection (LOD) and limit of quantitation (LOQ) were calculated using predefined calibration standards and or quality control standards as described below<sup>[9-10]</sup>.

#### Linearity and Range

Linearity of the proposed spectrofluorimetric method was calculated by using seven different calibration standards. After analysis of calibration standards, calibration curves in terms of concentration vs. Fluorescence intensity plots were developed and subjected to linear least square regression analysis. R<sup>2</sup> value was considered to be important factor for determining the linearity of the proposed method.

#### Accuracy

To determine the accuracy of the method, different quality control solutions were prepared independently from stock-I i.e., LQC : 60ng/ml, MQC : 350ng/ml and HQC : 750ng/ml and analyzed at level of 80%, 100% and 120% of its predefined concentrations .to the predefined concentrations, different amounts of baicalein were added (standard addition method) and the accuracy was calculated on the basis of percent recovery.

#### Precision

The precision of the method was checked by preparing different quality control solutions independently from stock-I i.e. LQC 60ng/ml, MQC 350ng/ml and HQC 750ng/ml at three different time intervals in a day. Same procedure was followed on three different consecutive days so as to obtain inter-day variation. The fluorescence intensities for baicalein were recorded and the results were expressed as % relative standard deviation (%RSD).

#### Robustness

Robustness of the proposed spectrofluorimetric method was established by changing composition of the ethanol by  $\pm 1.0$  %. MQC 350ng/ml samples of baicalein were prepared in ethanol with and analyzed at 371nm and 433nm (excitation-emission wavelength of baicalein). The results were calculated in terms of % RSD.

#### Ruggedness

Ruggedness of the proposed method was studied by analyst variation. MQC 350ng/ml samples of baicalein were prepared by three different analysts of the laboratory and were analyzed at 371nm and 433nm. The results were calculated in terms of % RSD.

#### Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and the LOQ of the drug were calculated by using the following equations as per ICH guidelines.

$$\text{LOD} = 3.3 \times \text{SD/S}$$

$$\text{LOQ} = 10 \times \text{SD/S}$$

Where, SD= standard deviation of lower most concentration of calibration curve

S= slope of calibration curve

#### Estimation of Baicalein in Oroxyllum indicum extract:

##### Drying treatment for preparation of Oroxyllum indicum Powder extracts

Estimation of Baicalein in Oroxyllum indicum extracts Oroxyllum indicum leaves were dried at 50°C using a Microtray drier (S.B. Panchal and company, Mumbai, India) and powdered using twin blade mixer (Bajaj electrical ltd., Mumbai, India). To select uniform particle size, powder was sifted in a sieve shaker (CIP Machineries, Ahmedabad, India) with sieves of different sizes (12, 24, 45, 85 and 120 mesh, Swastika electric and scientific works, Ambala, India) for a period of 15 min. Powder passed through 120 mesh sieve was collected and used for further extraction.

##### 1. Soxhlet assisted extraction (SAE) :

Soxhlet assisted extraction (SAE) technique was used for the extraction of Oroxyllum indicum leaves. 30 gm of powdered Oroxyllum indicum leaves was placed in a thimble (Borosil, Mumbai, India) which was inserted into a Soxhlet apparatus and extracted with 90 ml ethanol as an extraction solvent. SAE was performed for 5 hrs. After predefined extraction period, solvent was distilled off under reduced pressure using rotary vacuum evaporator (Heidolph instruments GmbH & co. Germany) to obtain the dry extract. Accurately weighed 1 mg of dry extract of Oroxyllum indicum was transferred in to the calibrated volumetric flask and dissolved using 1 ml of Methanol to achieve a stock solution of 1000  $\mu\text{g/ml}$  (Stock-I). Stock- II solution was suitably diluted with co-solvent system and analyzed for the baicalein content using proposed spectrofluorimetry method.

##### 2. Ultrasound Assisted Extraction (UAE) :

The extraction of Oroxyllum indicum was conducted using a tunable ultrasonic bath (PCi<sup>Tm</sup>Analytics, 230V AC, 50 Hz, Mumbai, Maharashtra, India). 10 gm of powder was weighed and placed in 100 ml of conical flask. The extraction of Oroxyllum indicum powder was carried out by placing the beaker in an ultrasonic bath with the fixed power of 150W. The conical flask was immersed in the ultrasonic bath and extracted for 20 min. After extraction process, extract was cooled to room temperature and kept for

centrifugation by using micro centrifuge at 25°C using 10,000 rpm for 10 min. Lastly, the supernatant was collected and filtered by using 0.45 µm fitted with syringe filter. The filtrate was suitably diluted with a co-solvent system and analyzed for the baicalein content using the proposed spectrofluorimetric method.

## Results and Discussion

### Construction of Calibration Curve

Quantification of baicalein samples by any instrumental method of analysis needs a reproducible calibration curve and a mathematical equation stating correlation between concentration and the response. As compare to graphical method, above stated method is widely accepted and reproducible in nature. To establish linearity of the proposed method, seven different calibration standards were prepared from the stock solution and analyzed at excitation wavelength 371nm and emission wavelength 433nm by spectrofluorimetric. Least square linear regression analysis was performed for the obtained spectrofluorimetric data using MS-excel 2013. Calibration curve was repeated five times for reproducibility. Various concentrations and their fluorescence intensities with mean ± standard deviation were reported (table 1).

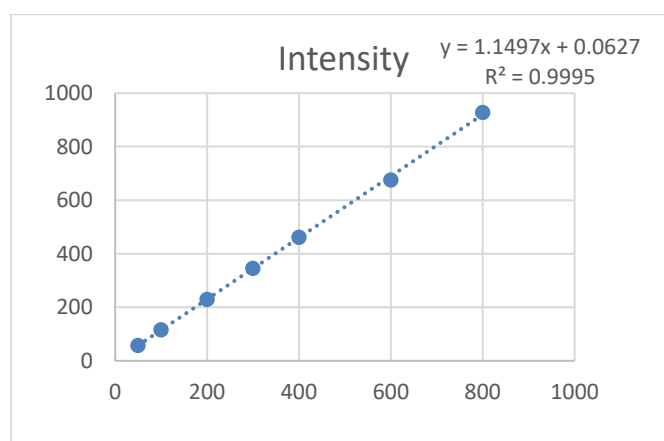
**Table 1: Calibration standard data for Baicalein**

S. No.	Concentration (ng/ml)	Fluorescence intensity
1	50	58.042±0.5344
2	100	116.071±0.3762
3	200	230.129±0.5464
4	300	346.123±0.4641
5	400	462.486±0.8805
6	600	676.161±0.4746
7	800	928.272±0.6183

### Spectrofluorimetric Method Validation

#### Linearity and Range

Linearity and range are the important parameters of analytical method that demonstrates the limit within which the intended method is to be used for its optimum performance. Considering the prime importance of linearity and the range, seven point calibration curve of baicalein was plotted covering a range of 50ng/ml to 800ng/ml. Different concentrations and the respective mean fluorescence intensities values are depicted in table 1. Calibration curve when subjected to least square regression analysis yielded an equation;  $y=1.149x + 0.062$  with correlation coefficient 0.999 (figure 3). From the linearity study, it was revealed that, developed method was linear over the concentration range of 50ng/ml to 800ng/ml.



**Fig. 3: Calibration Curve for Baicalein**

#### Accuracy

Accuracy is the closeness of test results to the true value obtained by method. The accuracy of an analytical method should be established over its calibration range so that at any point of determination, results obtained would be accurate. For baicalein, accuracy was determined using recovery studies. At 80, 100 and 120 % standard addition, mean recovery of baicalein was found in between 99.31 to 101.43 %. The relative standard deviation (% RSD) was found to be less than 2 as shown in table 2. From the results of accuracy studies, it was predicted that developed method is highly accurate.

**Table 2: Accuracy data of Spectrofluorimetric method for Baicalein**

Sr. no.	Concentration (%)	Origin level (ng/ml)	Amount added (ng/ml)	% recovery	Mean % recovery	% RSD
1	80	60	48	101.31	101.43	0.3091
2	80	60	48	101.20		
3	80	60	48	101.78		
4	100	350	500	99.43	99.31	0.1094
5	100	350	500	99.27		
6	100	350	500	99.22		
7	120	750	900	100.00	99.93	0.0979
8	120	750	900	99.98		
9	120	750	900	99.82		

### Precision

Precision is defined as closeness of agreement among the individual test result when the method is applied repeatedly to multiple sampling of homogeneous sample. Precise analytical method leads to accurate results. Intra- and inter-day precision of spectrofluorimetric method was established at LQC 60ng/ml, MQC 350ng/ml and HQC 750ng/ml levels of baicalein. The results expressed in terms of mean fluorescence intensity values, % assay and % RSD for the intra- and inter-day precision study are demonstrated in table 3 and table 4 respectively. Percent RSD values of intra-day precision study were found to be in between 0.101 and 0.622 whereas those of inter-day precision study were in between 0.087 and 0.341 overall, % RSD values of less than 2 demonstrated that developed spectrofluorimetric method is precise and reproducible.

**Table 3: Intra-day precision data of Spectrofluorimetric method for Baicalein**

S. No	Concentration Range (ng/ml)	Day 1			Day 2			Day 3		
		Amount (Mean)	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1	60	60.663	101.10	0.622	60.105	100.14	0.175	60.22	100.37	0.225
2	350	350.509	100.14	0.121	350.899	100.25	0.133	350.94	100.27	0.106
3	750	750.74	100.09	0.110	750.476	100.06	0.111	750.975	100.12	0.101

**Table 4: Inter-day precision data of Spectrofluorimetric method for Baicalein**

S. No	Concentration Range (ng/ml)	Day 1			Day 2			Day 3		
		Amount (Mean)	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1	60	60.332	100.55	0.341	60.541	100.90	0.335	60.743	101.23	0.134
2	350	350.786	100.22	0.120	350.611	100.17	0.139	351.029	100.29	0.087
3	750	750.724	100.09	0.107	750.4562	100.06	0.116	751.43	100.19	0.115

### Robustness

Robustness of an analytical method is the measure of its capacity to remain unaffected by small but deliberate change in method parameters. It is an important parameter of analytical method as a small, un-intentional change in method parameter like solvent composition. May occur during routine use and may hamper the performance of said method. It is expected that such change should not alter the performance of the method. Therefore, robust analytical method is preferred. Robustness of proposed spectrofluorimetric method was performed by changing the pH of water. After analysis, it was found that change in pH of water did not affect the performance of method. Percent RSD values were found to be in between 0.109 and 0.127 (table 5). Percent RSD values below 2 depicted that proposed spectrofluorimetric method is robust in nature.

**Table 5: Robustness data of Spectrofluorimetric method for Baicalein**

Sr. No.	Concentration (ng/ml)	Mobile phase composition (% v/v)	Amount	% RSD
1	350	41:59	403.493	0.127
2	350	40:60	402.858	0.130

3	350	39:61	403.175	0.109
---	-----	-------	---------	-------

### Ruggedness

Ruggedness of analytical method is the degree of reproducibility of test results obtained by analysis of the same samples under a variety of conditions, such as different laboratories, different analyst, different instruments, different lots of reagent, different temperatures etc.. In order to determine the ruggedness of proposed spectrofluorimetric method, baicalein solutions were prepared and analyzed by different analysts. Sample analysis and data processing resulted into % RSD values between 0.119 and 0.146. Results of ruggedness studies revealed that proposed spectrofluorimetric method was rugged as it showed % RSD values less than 2 (table 6).

**Table 6: Ruggedness data of Spectrofluorimetric method for Baicalein**

S. No.	Concentration (ng/ml)	Analyst	Amount	% RSD
1	350	I	402.874	0.146
2	350	II	403.273	0.129
3	350	III	403.270	0.119

### Limit of quantitation (LOQ) and limit of detection (LOD)

Limit of quantification (LOQ) represents the lowermost concentration that can be analyzed with acceptable accuracy and precision. Limit of detection (LOD) of an individual analytical procedure is the lowest amount of an analyte in a sample which can be detected but not necessarily quantitated as an exact value. From the standard deviation of lower most concentration and the slope of the calibration curve, LOD and LOQ of proposed spectrofluorimetric method was found to be 2.14ng/ml and 6.48ng/ml respectively (table 7). Lower LOQ value indicated that proposed method is sensitive enough to quantify the baicalein content of samples at its lower level.

**Table 7: LOD & LOQ data for Spectrofluorimetric method for Baicalein**

1	LOD	2.14ng/ml
2	LOQ	6.48ng/ml

### Estimation of Baicalein in Oroxyllum indicum leaves powdered extract

The developed spectrofluorimetry method was successfully applied for estimation of Baicalein content in Oroxyllum indicum leaves extracts. By proposed spectrofluorimetric method, baicalein content in Soxhlet Assisted Extraction (SAE) extracts of Oroxyllum indicum was found to be  $1.45 \pm 0.0036$  and for baicalein content in Ultrasound Assisted Extraction (UAE) of Oroxyllum indicum was found to be  $1.75 \pm 0.0052$  respectively.

### CONCLUSION

The simple, precise, accurate, and sensitive Spectrofluorimetric method by the estimation for Baicalein in Oroxyllum indicum leaves powdered extract was developed and validated. Proposed method was found to be robust and rugged in nature and was successfully used for the estimation of Baicalein in Oroxyllum indicum leaves powdered.

### ACKNOWLEDGEMENT

The extra-mural grant support of DST-DPRP, Govt. of India (Ref:-VI-D&P/626/2018-19/TDT) sanctioned to P.I. Dr. Sachin S. Bhusari for the proposed research work is highly acknowledged.

### REFERENCES:

- Lawania R, Mishra A, Gupta R. Oroxyllum indicum: A Review. Pharmacognosy Journal. 2010; 2(9):304-310.
- Preety A, Sharma S. A review on Oroxyllum indicum (L.) Vent: an important medicinal tree. International Journal of Research in Biological Sciences. 2016; 6(1):7-12.
- Satya Eswari J, Dhagat S, Naik S, Dibya S. Oroxyllum indicum leaf extracts for screening of antimicrobial properties and phytochemicals. Pharm. Bioprocess. 2018; 6(1):7-14. biological Sciences. 2016; 6(1):7-12.
- Zaveri M, Gohil P, Jain P. Immunostimulant Activity of n-Butanol Fraction of Root Bark of Oroxyllum indicum, vent. Journal of Immuno toxicology. 2006; 3:83-99.
- Upaganlawar A, Tenpe CR, Yeole PG. Antiinflammatory activity of aqueous extract of Oroxyllum indicum vent. Leaves extract- preliminary study. Pharmacologyonline. 2009; 1:22-26.
- Zaveria M, Khandhar A, Jain S. Quantification of Baicalein, Chrysin, Biochanin-A and Ellagic Acid in Root Bark of Oroxyllum indicum by RP-HPLC with UV Detection. Eurasian Journal of Analytical Chemistry. 2008;
- Maddi Sesharao, Vallabaneni Madhavarao. A new validated simultaneous reversed-phase high-performance liquid chromatography assay method for estimation of two flavones (baicalein and Schrysin) in API drugs. Asian Journal of Pharmaceutical and Clinical Research. 2018; 11(1):351-356. 3(2):25-257.
- Bhusari SS, Rindhe M, Chaudhary A. Development and validation of UV-Spectrophotometric method for estimation of Picroside-ii in Picrorhiza Kurroa rhizome extracts. International Journal of Pharmacy and Biological Sciences. 2018; 8(4):177-183.
- Note for guidance on validation of analytical procedures: text and methodology. European Medicines Agency, 1995, 1-15.
- ICH Guidance on Analytical Method Validation, In: Proceedings of the international Conference of Harmonization, Geneva, 1996