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A Process of Sonication-Assisted Extraction and Quantification of Baicalein in *Clerodendrum serratum* Linn. Leaf Extract through QbD Supported Green Photometric Method

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KEYWORDS:

Analytical Quality by Design, Baicalein, Green Analytical Chemistry, Photometric Method, Sonication Extraction.

ABSTRACT:

Introduction: Herbal medication standardization necessitates simple, dependable, and ecologically friendly analytical procedures. Clerodendrum serratum Linn. is a medicinal plant high in flavonoids, with baicalein acting as a key bioactive indicator for quality assessment.

Objectives: The current study intended to develop and validate a green, QbD-supported UV photometric method for measuring baicalein in Clerodendrum serratum Linn. leaf extract after sonication-assisted extraction.

Methods: A UV-based analytical technique was devised that uses methanol and water (25:75 v/v) as solvent and detects at 275 nm. Analytical Quality by Design (AQbD) concepts were followed, including risk assessment and optimization via Central Composite Design. Sonication-assisted extraction was used to increase the recovery of baicalein from plant material. The approach was validated using ICH criteria. Forced degradation tests were carried out under various stress situations to confirm the stability-indicating capacity. The greenness of the procedure was evaluated using the AGREE and GAPI tools.

Results: The technique demonstrated excellent linearity in the range of 2-10 µg/mL (r^2=0.9997), with low LOD (0.24 µg/mL) and LOQ (0.75 µg/mL), indicating great sensitivity. The precision and accuracy levels were acceptable (%RSD < 2; recovery 98-102%). Degradation investigations have proven that the approach indicates stability. The extract contained 98.60 ± 0.55% baicalein, corresponding to 1.57 ± 0.0058 mg/g of dried plant material. The AGREE score of 0.70 suggested favorable environmental compatibility.

Conclusion: The proposed method is simple, precise, eco-friendly, and suitable for routine quality control and standardization of baicalein in Clerodendrum serratum Linn. Leaf extract.

Introduction

Modern drug from natural sources still constitute a major segment of drug development and medicinal plants are the major actors of traditional healthcare systems all over the world. In fact, a significant portion of the world's population at present time is dependent on herbal medicines for their primary healthcare needs, which calls for assurance of the safety, efficacy, and quality of the materials used [1]. Herbal drugs may be prepared from unprocessed plant materials, extracts, or finished herbal products [2]. Standardization is a must for therapeutic consistency. It should comprise of a thorough check of physicochemical and analytical characteristics to reduce differences between batches [3]. Besides that, it is a must in polyherbal formulations in which several components work synergistically to

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give therapeutic effects. Good standardization not only leads to better consistency and safety, it can also result in higher acceptance by the regulators [4,5].

Clerodendrum serratum Linn. is a medicinal plant that has been used extensively in traditional practices like Ayurveda, Siddha, and Unani for treating inflammation, respiratory problems, and infections. The plant has different phytoconstituents such as flavonoids phenolics sterols, etc. These constituents have been reported as a source for antioxidant antimicrobial anti-inflammatory, and other related activities [6]. Baicalein (5,6,7-trihydroxyflavone) among other compounds is considered one of the important bioactive markers that possess documented pharmacological effects of being an antioxidant, anti-inflammatory, antimicrobial, and anticancer agent [7]. Its clinical usefulness might be limited due to poor bioavailability; however, baicalein still can be a good marker compound for analytical standardization because of its biological significance [8]. Its occurrence in Clerodendrum serratum also justifies its use in quality assessment, more so in view of the pharmacological relevance of flavonoids [9].

Chromatographic techniques like High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS) have been extensively employed to determine the levels of flavonoids. Nevertheless, these techniques usually entail sophisticated equipment, thorough sample processing, and large volumes of solvents, thus rendering them unsuitable for regular use. On the other hand, UV spectrophotometry provides a simple, fast, and inexpensive option for the regular analysis of herbal extracts. In line with enhancing method dependability and uniformity, the application of Quality by Design (QbD) concept was implemented which highlights the planning of development, gaining knowledge about process, and risk assessment in line with ICH guidelines [10]. Analytical Quality by Design (AQbD) brings these principles into analytical method development by determining and regulating critical variables. In addition, the use of Design of Experiments (DoE) provides a way to the effective improvement of analytical conditions [11,12].

The Green Analytical Chemistry (GAC) movement promotes the use of methods and materials with the least environmental impact while maintaining analysis performance, e.g. using fewer and less harmful solvents, reducing energy consumption and waste generation [13]. Although there have been progresses in analytical techniques, only a few studies have managed to combine AQbD and green analytical principles for the analysis of herbal drugs. Hence, this study has been conducted towards the development and validation of a green UV spectrophotometric method for the quantification of baicalein in Clerodendrum serratum Linn. by including concepts of AQbD and GAC to establish a dependable, sustainable and simple analytical method.

1. Materials and Methods

Instruments and apparatus

A Shimadzu UV-1800 spectrophotometer was used to measure absorbance. Bath sonicator was used for the solubility of marker in given solution. A Sartorius electronic balance was used to accurately calculate sample weights [14].

Chemicals and reagents

Analytical-grade methanol was used as the solvent, and the diluent was prepared using deionized water generated by a Milli-Q® Direct system, Baicalein was provided as gift sample from the BLD Pharmatech (India) Pvt Ltd., Hyderabad, India [15].

Selection of Solvent

The methanol was used as the main solvent based on its excellent dissolving power for Baicalein and its compatibility with UV analyses. Subsequent dilutions were made using a blend of methanol and deionized water (25:75 v/v). The decision to go with this combination was made following the initial tests, which showed that it gave good quality spectra, hardly any interferences, and stable baseline features [16,17].

Selection of Wavelength for Analysis

The wavelength of maximum absorbance (max) was found by scanning a standard Baicalein solution (10 g/mL) from 200 to 800 nm. A strong sharp peak was seen at 275 nm and this wavelength was chosen for the quantitative analysis to get the best sensitivity and accuracy [18].

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Preparation of Standard Solutions

Ten milligrams of Baicalein were dissolved in methanol to prepare a standard stock solution (1000 g/mL). The stock solution was then diluted appropriately to get a working concentration of 100 g/mL. Further serial dilutions were made with the solvent system chosen for calibration and validation. Solutions were made fresh and kept away from light to prevent deterioration [19].

Method Development

A UV spectrophotometric technique for Baicalein estimation was developed emphasizing simple, sensitive, and reproducible features. Main factors like solvent system, wavelength, and calibration were fixed by preliminary experiments. The method followed AQbD concepts to make it more robust, reduce variations and deliver consistent, dependable, and economical performances for regularly used analytical purposes [20,21].

AQbD-Based Method Optimization

Following the AQbD approach the analytical method was methodically optimized. Initially, an Analytical Target Profile (ATP) was set to determine desired performance standards such as accuracy and precision. Some of Impact Analytical Attributes and Method Parameters which include composition of the solvent and conditions of the scanning were identified. By applying an Ishikawa diagram for risk assessment, a number of possible sources of variability were identified which facilitated the prioritization of key factors and was used to direct the efficient experimental optimization [21].

Design of Experiments (DoE)

In order to analyze the influence of selected variables and identify the best analytical conditions, the authors decided to use a Design of Experiments (DoE) method. A Central Composite Design (CCD) was picked because it can assess interactions and quadratic effects in a very efficient way. Two factors, solvent ratio (A) and scanning speed (B) were independently changed at three levels. The design created nine experimental runs, which included factorial and center points. Absorbance at 275 nm was the chosen response variable, as it is the one that directly depicts the method performance. Experimental data were analyzed with Design Expert software to get a second-order polynomial model that relates variables to response. Different statistical methods, i.e. ANOVA, regression and testing of model significance were employed to validate the model. Moreover, response surface and contour plots were drawn to depict the interactions of variables and to lay out the optimal design space [21].

Forced Degradation Studies

To evaluate whether the method is good enough for stability study, forced degradation experiments were performed. Baicalein was exposed to different stress factors such as UV light heat oxidation, acidic hydrolysis, and alkaline hydrolysis. In case of UV degradation, the samples were kept under UV light for a certain duration. Thermal degradation was carried out by heating drug samples at a high temperature, whereas oxidative degradation was done by treating the drug with hydrogen peroxide solution. Acid and base hydrolysis were performed using the respective solutions of hydrochloric acid and sodium hydroxide. Following these treatments, the samples were diluted suitably and the developed UV method was used to analyze them. Spectral variations were traced to understand drug decomposition and to verify that the method can specifically distinguish analyte from the other degradation products [22].

Method Validation

The developed UV spectrophotometric method was validated as per ICH Q2(R1) guidelines [14-23]. Specificity was checked by matching the spectra of the blank solvent with that of the Baicalein standard. No interfering peaks appeared at 275 nm. This indicates that the procedure exclusively determines the analyte without any impact from excipients, impurities, or degradation products. Linearity was confirmed at five concentration levels in the range of 210 g/mL. A correlation plot between concentration and absorbance indicated a directly proportional relationship, thereby giving evidence to a uniform analytical response over the selected concentration range. The method's limit of detection (LOD) and limit of quantification (LOQ) were obtained from the standard deviation of the response and the slope of the calibration curve. These data showed the ability of the analytical method to recognize and assign Baicalein even at low concentration levels. Precision was measured by intra-day and inter-day

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studies, and the results were reported as %RSD. Low values of %RSD showed the good repeatability of the method and intermediate precision. Accuracy was checked through recovery experiments carried out at three different concentration levels: 80%, 100% and 120%. The findings of robustness and ruggedness tests showed that small changes in analytical conditions and changing the analyst or instrument did not substantially alter the results, hence it confirms the reliability of the method.

Estimation of Baicalein in Plant Extract through Sonication-Assisted Extraction

Fresh leaves of *Clerodendrum serratum* Linn. were collected, washed to remove impurities, and dried under shade to protect the thermolabile constituents. The dried material was ground to a fine powder and passed through a sieve to ensure uniformity. The extracts were prepared, one using 200 mL ethanol. For the extraction, 10 g powder, in a conical flask, was subjected to ultrasonic treatment for 30 to 45 min, which increased solvent penetration and improved extraction efficiency. The extracts were filtered and concentrated under reduced pressure using a rotary evaporator to yield semi-solid residues, which were further dried and stored. Different dilutions of the extract were prepared in solvent system as used for standard preparation. The prepared samples were analyzed at 275 nm using the developed UV method. Baicalein content was calculated from the linear regression equation of the calibration curve [24,25].

Greenness Evaluation

The green credentials of the developed analytical method were evaluated through both the AGREE (Analytical GREEnness metric) and GAPI (Green Analytical Procedure Index) tools. Such tools judge the method based on criteria like solvent toxicity, energy usage, waste production, and overall environmental impact. AGREE metric quantifies the greenness of the method in the form of a numerical score whereas GAPI shows the environmental profile via a visual pictogram. This assessment was done to guarantee that the developed method is in line with the Green Analytical Chemistry principles and is ready to be used in sustainable analytical practices [26, 27].

2. Results and Discussion

We have systematically evaluated the UV spectrophotometric method developed for the estimation of Baicalein in leaves of selected plant, using AQbD principles, and the results obtained proved the method's capability for routine analytical applications.

Spectral Analysis and Method Development

Baicalein displayed a sharp absorption maximum at 275 nm in methanol: water (25:75 v/v), which supported the choice of this compound for the analysis. The spectral line was quite symmetric and with a constant baseline, showing that there was almost no disturbance. Such circumstances allowed to achieve a very sensitive and highly reproducible method. Parameters adjusted, initially derived from screening experiments and subsequently AQbD-based finalization, have been listed in Table 1.

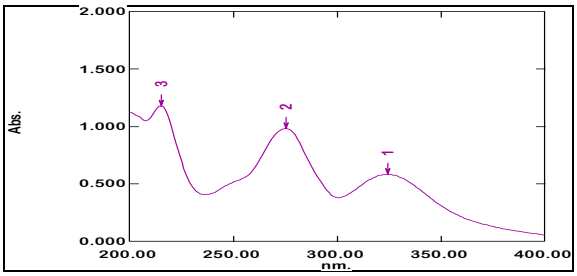

Figure 1. UV-Spectrum of Baicalein (10µg/ml)

Table 1. Developed Photometric Method Specifications

SN.	Parameters/ATP	Description
1	Analytical Technique	UV Spectroscopy
2	Instrument	UV-Spectrophotometer
3	Instrument Model	Shimadzu
4	Make	UV-1800
5	Software	UV- probe
6	Target Compound	Baicalein
7	Solvent System	Methanol: Deionized water
8	Wavelength	275nm

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AQbD-Based Optimization

Analytical Target Profile (ATP) was determined so as to assure the precision, speed, and dependability in the quantification of Baicalein by UV spectrophotometric method. Table 2 (ATP for Baicalein), lists the ATP parameters such as target analyte, analytical technique, and validation requirements.

Table 2. ATP for Photometric Analysis of Baicalein

SN	ATP Parameters	Target
1	Target Analyte	Baicalein
2	Target Sample	Clerodendrum serratum extract
3	Method category	UV-spectrophotometric method
4	Instrument	UV-spectrophotometer
5	Nature of analyte	Solid (Solution)
6	Standard stock solution preparation	Dilution of the main drug in a linear manner
7	Application of Method	Estimation of Baicalein
8	Validation parameters	Accuracy, selectivity, ruggedness, robustness, precision, linearity, specificity and sensitivity.

Risk Assessment

The risk assessment was conducted with an Ishikawa fishbone diagram (Figure 2: Fishbone diagram for method variables), through which major factors like solvent composition and scanning speed turned out to be critical contributors to analytical performance. Such a structured review made it possible to single out the Critical Method Parameters (CMPs) for targeted optimization.

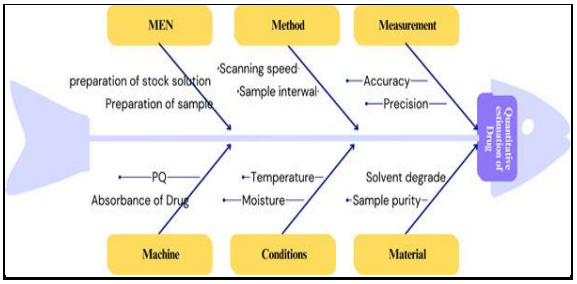

Figure 2. Ishikawa Fishbone Diagram

DoE-Based Method Optimization

Central Composite Design (CCD) was a method applied to understand the effect of various independent variables on the effectiveness of the method systematically. The factor levels chosen are shown in Table 3, and the various experiment settings are given in Table 4. The analysis using a quadratic polynomial equation of the solvent ratio (A), scanning speed (B), and absorbance at 275 nm suggests that both factors have a significant influence on the response, including the non-linear contributions of both variables. Model validity was tested using ANOVA, with a p-value less than 0.05 indicating a statistically significant result, and the F-value showing the model's ability to predict well. The coefficient of determination (R), being nearly one, indicates a very strong relationship between the observed and predicted results. The use of diagnostic tools such as normal probability and predicted versus actual plots confirmed that the model was a good fit to the data. Variable interactions were visualized using response surface and contour plots, which indicated that the best solvent composition and scanning conditions lead to the highest absorbance. The interaction between solvent ratio and scanning speed was depicted in the response surface and contour plots (Figure 3 and 4). It was shown that the highest absorbance was obtained at moderate solvent ratios and optimized scanning speeds. The overlay plot (Figure 5: Design space plot) provided a visual representation of the area within which the method achieves the best results.

Table 3. Selection of levels for CCD of Baicalein by UV method

Independent Variable	Levels		
	+1	0	-1
Scanning Speed (A)	**+1 (High)**	**0 (Medium)**	**-1 (Low)**
Solvent Ratio (B)	**100 %**	**75%**	**50%**

Table 4. Experimental design matrix by CCD for Baicalein UV method

Run	Factor 1 (A: Solvent Ratio)	Factor 2 (B: Scanning Speed)	Response 1 (Absorbance)
1	100	-1	1.01

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2	50	-1	1.05
3	100	1	1
4	75	1	0.972
5	100	0	1.01
6	75	0	0.946
7	50	1	1.01
8	75	1	0.964
9	50	1	1.05

Table 5. ANOVA Results for UV Method

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0081	5	0.0016	11	0.0381	**Significant**
A-solvent ratio	0.0014	1	0.0014	9.13	0.0567	
B-scanning speed	0.0004	1	0.0004	2.77	0.1946	
AB	0.0002	1	0.0002	1.52	0.3051	
A^2	0	1	0	0.1861	0.6953	
B^2	0.0039	1	0.0039	26.63	0.0141	
Residual	0.0004	3	0.0001			
Cor Total	0.0086	8				

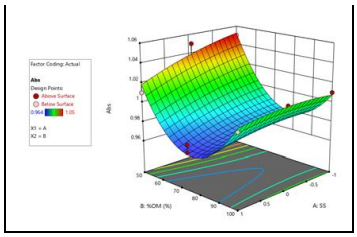

Figure 3. 3-D Response Surface Graph

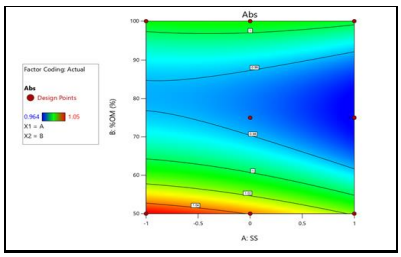

Figure 4. 2-D Contour Graph

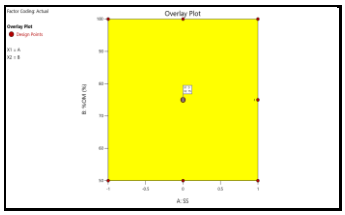

Figure 5. Overlay Graph Showing the Design Space

Forced Degradation Studies

To verify that the developed method is stability-indicating, forced degradation studies were carried out under various conditions of (Table 6). Irradiation with UV light over 48 hours turned out to be a cause of a big drop in absorbance which was the evidence of photodegradation and light sensitivity to a certain extent. Heating at 70 ^{0}C, hardly changed absorbance and the compound was considered to be reasonably heat stable. However, Baicalein exposed to hydrogen peroxide affected by oxidation. In an acidic medium (0.1 N HCl), a decline in absorbance was observed, which was only partial degradation, indicated. The decrease in alkaline condition (0.1 N NaOH) was considerably more, which means that Baicalein is more unstable in the basic media as compared to the acidic ones. From the obtained results, one can conclude that Baicalein is sensitive to oxidative conditions, while the UV and alkalinity exert only a moderate effect, and the heat affects at lower rate. Totally, no spectral interference was observed at 275 nm, which proves the specificity and the stability-indicating capability of the method.

Table 6. Forced Degradation Results of Baicalein

Stress Condition	Treatment Conditions	Absorbance	% Degradation
UV Degradation	UV exposure for 48 h	0.945	10.00%
Thermal Degradation	70°C for 48 h	0.982	6.48%
Oxidative Degradation	30% H_2O_2, 2 h	0.870	17.14%
Acidic Hydrolysis	0.1 N HCl, reflux 2 h	0.928	11.62%
Alkaline Hydrolysis	0.1 N NaOH, reflux 2 h	0.910	13.33%

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Method Validation

Specificity and Selectivity

The UV spectrum of methanol: water solvent as blank and Baicalein as showed in Figure 6 and Figure 7 showed no interference at 275 nm, which confirms the specificity and selectivity of the method.

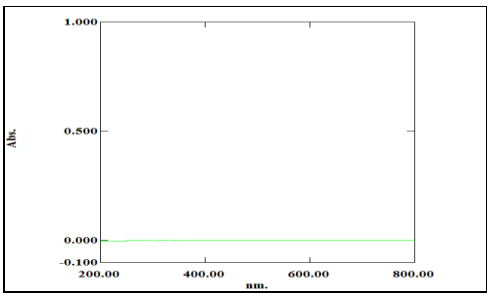

Figure 6. UV Spectrum of Solvent

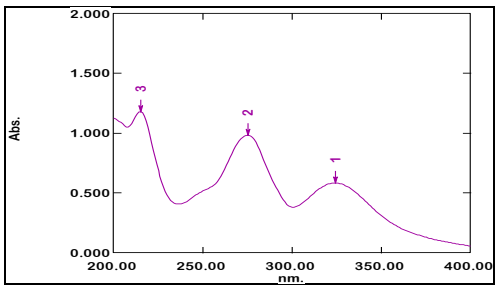

Figure 7. UV-Spectrum of Baicalein

Linearity and Range

The calibration graph made using concentrations of 2 to 10 g/mL showed very good linearity, with a correlation coefficient (r) of 0.9997. Table 7 (Linearity data of Baicalein) lists the linearity data, whereas Figure 8 (Calibration curve) depicts the calibration curve. In addition, they verified uniform absorbance at all concentrations through their overlay spectra (Figure 9: Linearity overlay spectrum).

Table 7. Linearity data of Baicalein by UV spectroscopic method

S N.	Concentration	Absorbance 1	Absorbance 2	Absorbance 3	Absorbance
1	2ug/ml	0.237	0.236	0.240	0.237
2	4ug/ml	0.443	0.434	0.435	0.437
3	6ug/ml	0.643	0.641	0.645	0.642
4	8ug/ml	0.833	0.831	0.838	0.833
5	10ug/ml	1.050	1.058	1.055	1.054

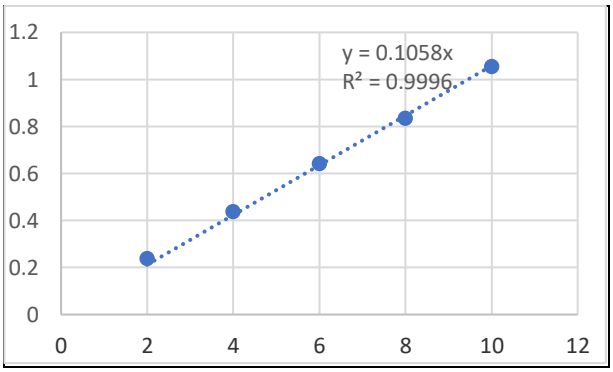

Figure 8. Calibration Curve of Baicalein

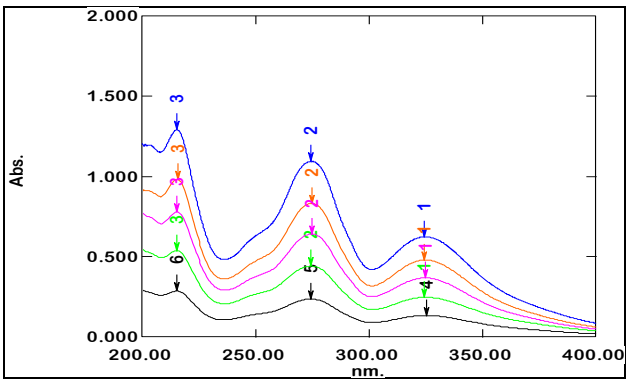

Figure 9. Overlay Spectrum of Baicalein

LOD and LOQ

The calculated values of LOD and LOQ were found to be 0.24 g/mL and 0.75 g/mL, respectively which shows the method is adequately sensitive to the detection of Baicalein.

Precision

In precision analysis studies, %RSD values were less than 2% for both intra-day and inter-day measurements (Table 8: Precision data), which indicates the method is not only highly repeatable but also possesses good intermediate precision.

Ruggedness and Robustness

The ruggedness results (Table 9: Ruggedness data) showed little change in results when the analysis was done by different analysts and using different instruments. In the same way, the robustness experiments (Table 10: Robustness data) showed that variations in wavelength and other experimental conditions hardly changed the results, which points to the method's dependability.

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Accuracy

The accuracy analysis was studied at 80%, 100%, and 120% levels which showed the % recovery within acceptable values (Table 11: Recovery data), showing the accuracy of the proposed method.

Table 8. Precision Data of Baicalein by UV Spectroscopic Method

Precision	Concentration (µg/ml)	Mean Absorbance	Standard Deviation	%RSD
Intraday	2 µg/ml	0.240	0.001	0.71
	6 µg/ml	0.635	0.001	0.18
	10 µg/ml	1.046	0.005	0.48
Interday-1	2 µg/ml	0.233	0.001	0.66
	6 µg/ml	0.638	0.004	0.72
	10 µg/ml	1.035	0.004	0.39

Table 9. Ruggedness Data of Baicalein by UV Spectroscopic Method

Precision Type	Concentration (µg/ml)	Mean Absorbance	SD	%RSD
Change in Analyst	2 µg/ml	0.244	0.000	0.24
	6 µg/ml	0.643	0.001	0.18
	10 µg/ml	1.056	0.004	0.39
Change in Instrument	2 µg/ml	0.240	0.001	0.72
	6 µg/ml	0.617	0.004	0.65
	10 µg/ml	1.024	0.001	0.17

Table 10. Robustness Data of Baicalein by UV Spectroscopic Method

Concentration (µg/ml)	Wavelength			Mean	SD	%RSD
	273	275	277			
2 µg/ml	0.245	0.245	0.242	0.24	0.14	0.71
6 µg/ml	0.637	0.635	0.635	0.64	0.12	0.18
10 µg/ml	1.055	1.054	1.046	1.05	0.23	0.47

Table 11. Recovery Data of Baicalein by UV Spectroscopic Method

Levels %	Total quantity (µg/ml)	Mean Quantity Recovered (µg/ml) ± SD	Mean % Recovery (µg/ ml) ± SD
80	8 µg/ml	7.90 ±0.03	98.75 ± 0.25
100	14 µg/ml	13.96±0.05	99.71 ± 0.36
120	20 µg/ml	19.88 ±0.04	99.40 ± 0.20

Estimation of Baicalein in Plant Extract

Using sonication-assisted extraction we were able to effectively extract Baicalein from *Clerodendrum serratum* Linn. Leaves. Our UV spectrophotometric method was well-executed, producing very practical results with high accuracy and precision. There is a substantial amount of Baicalein, 1.57 ± 0.0058 mg/g of dried plant material present in the plant material. The wavelength 275 nm was free from spectral disturbances, indicating the selectivity of the method.

Greenness Evaluation

To check how ecofriendly the new method is, we have used AGREE and GAPI tools. A rating of 0.70 on AGREE showed the greener analysis practices. Less solvent needed, uses of low power, leaves smaller footprint overall. Most areas in the GAPI profile turned out green, meaning little harm to ecosystems (Figure 10 and Figure 11).

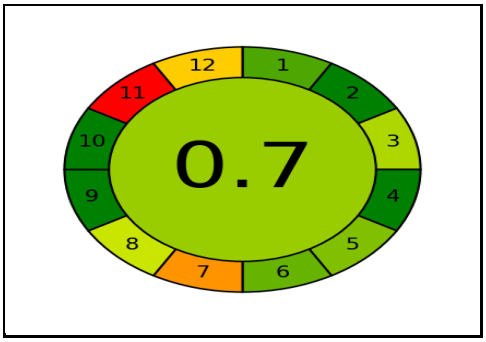

Figure 10. AGREE Greenness Score

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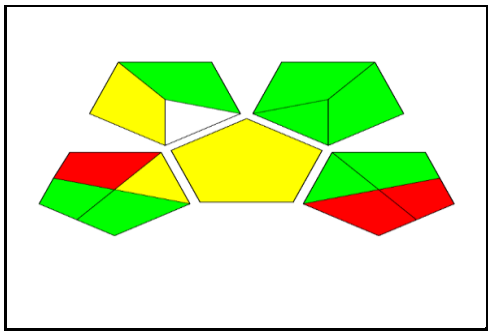

Figure 11. GAPI Pictogram

3. Conclusion

The proposed research study reported a simple and accurate photometric tool for the quantification of Baicalein in *Clerodendrum serratum* Linn. Leaves extract. The developed method was optimized using the practices and guidelines of AQbD to ensure better performance. The method validation analysis showed the simplicity, specificity, selectivity, acceptable accuracy, precision, linearity, and sensitivity, supporting its suitability for routine use. Sonication-assisted extraction improved the effectiveness of Baicalein recovery with dropping processing time. Stability evaluation confirmed the method's ability to selectively measure the analyte in stressed conditions. The method involves the use of green analytical practices, minimizing environmental impact. Overall, the method provides a practical, economical, and dependable tool for herbal standardization and quality control.

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