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Eco-friendly HPTLC method for simultaneous estimation of Quercetin and Gallic Acid in marketed herbal gel(kiwi) formulation

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ABSTRACT

The analysis of herbal formulations is often complicated by complex plant matrices and the environmental impact of analytical waste. This study presents a green High-Performance Thin-Layer Chromatography (HPTLC) method for the simultaneous estimation of Quercetin and Gallic Acid in a marketed kiwi (Actinidia deliciosa) herbal gel. To align with Green Analytical Chemistry (GAC) principles, an eco-friendly mobile phase of Toluene, Ethyl Acetate, and Formic Acid (5:4:1 v/v/v) was developed, replacing more hazardous solvent systems. The optimized method achieved superior chromatographic resolution with sharp peaks and reproducible Rf values. Validation was conducted per ICH Q2(R1) guidelines, confirming high sensitivity (LOD/LOQ), linearity, accuracy, and robustness. Additionally, the method's sustainability was verified using the AGREE (Analytical GREEnness) metric. This HPTLC approach offers a rapid, cost-effective, and environmentally responsible solution for the routine quality control and standardization of multi-component herbal topical formulations

Keywords: Eco-friendly HPTLC, Gallic Acid, Green Analytical Chemistry, Kiwi Herbal Gel, Quercetin

1. INTRODUCTION

Herbal remedies contain a myriad of active principles, many of which remain unidentified, complicating standard quality control. Specifically, polyherbal formulations like kiwi gels require markers such as Gallic Acid and Quercetin to ensure therapeutic efficacy and safety [1]. Traditional medicine acknowledge the merits of these phytochemical profiles for managing metabolic and inflammatory conditions [2]. HPTLC stands out as an analytical tool because it can handle complex samples with high selectivity while conserving resources through simultaneous multicomponent analysis [3]. As the scientific community moves toward sustainability, evaluating the "greenness" of these methods using tools like the Eco-scale and AGREE metrics has become of prime important. [4].

Herbal analysis presents unique challenges compared to synthetic drug analysis, primarily due to the complexity and variability inherent in herbal compositions [5]. The absence of standardized analytical techniques and reference compounds further worsen these challenges [6]. Moreover, the sheer variability of plant materials, influenced by factors such as chemo varieties and cultivation methods, introduces inconsistencies in the quality and potency of herbal drugs [7]. Responsibiities over adulteration and substitution are raised up by the variable quality and sourcing of raw materials [8].

Standardization of polyherbal formulations is of foremost importance in order to defend their acceptability in the modern system of medicine and quality assessment [9]. Standardization is a technique which ensures that every envelope of medicine sold has the suitable substances in the correct amount to encourage its therapeutic effect [10]. From the past few decades, herbal drugs have been gaining importance because of the vast chemical diversity they offer [11]. However, standardization is not a routine practice carried out by many manufacturers because of the complex nature of the products [12]. Standardized herbal products containing well defined constituents are required to provide consistent beneficial therapeutic effects [13].

Development of authentic analytical methods which can consistently profile the phytochemical composition is a considerabe challenge to scientists [14]. Phytochemical evaluation includes preliminary screening, chemo profiling, and marker compound analysis using modern analytical techniques [15]. HPTLC has emerged as an important tool for the qualitative and quantitative phytochemical analysis of herbal drugs [16]. This includes developing TLC fingerprint profiles and assessment of biomarkers [17]. In the present investigation, a simple, optimized, and validated HPTLC method for the standardization of gel was developed [18]. Marker compounds were selected from raw materials, and the method was validated according to the International Council for Harmonisation (ICH) guidelines [19], ensuring global regulatory acceptance [20].

Kiwi fruit (*Actinidia*) is commonly consumed worldwide. Kiwi fruit also contains other useful compounds such as carotenoids, polyphenols, flavonoids and minerals [21,22].Polyphenols are copious in kiwi fruit and are reported to have multiple biological activities, including antioxidant effects *in vitro* and *in vivo*, and anti-inflammatory properties [23,24].

2. MATERIALS AND METHODS

Primary Objectives : To develop and validate a simple, accurate and precise, and reproducible HTLC method for the qualitative and quantitative estimation of selected biomarkers (Quercetin and Gallic acid)

Secondary Objectives :

To standardize the marketed herbal gel formulation using quercetin and gallic acid as analytical biomarkers.

To optimize the mobile phase for effective separation and resolution of the selected phytoconstituents.

To determine the Rf values and establish fingerprint profiling of the formulation.

To validate the developed HPTLC method as per ICH guidelines (linearity,accuracy, precision, LOD, LOQ, robustness, specificity).

To quantify the content of quercetin and gallic acid present in the marketed gel.

To evaluate the environmental sustainability (greenness) of the developed method using appropriate greenness tools.

To ensure quality contro and batch consistency of the marketed herbal formulation.

2.1. Instrumentation and reagents

The standard of Gallic Acid was procured from Chemdyes Corporation, Rajkot, Gujarat and Quercetin from Yucca Enterprise, Mumbai, Maharashtra. All other chemicals and reagents (toluene, ethyl acetate, formic acid) were of analytical grade and procured from Merck India Pvt, Ltd. Mumbai, India. SKYNISTA is manufactured by : S.N. COSMETICS, Delhi, was purchased from local market. [25]

2.2. Instrumentation and densitographic conditions

Microsyringe (Linomat syringe, Hamilton-Bonaduz schweiz, Camag, Switzerland), pre-coated silica gel 60 F-254 glass plates (10 × 10 cm with 200 µm thickness HPTLC; Merck, Germany), linomat 5 automatic sample applicator (Camag, Muttenz, Switzerland), twin trough chamber 10 × 10 cm (Camag, Muttenz, Switzerland), UV chamber (Camag, Muttenz, Switzerland), TLC scanner III (Camag, Muttenz, Switzerland), and win CATS version 1.4.0 software (Camag, Muttenz, Switzerland) were used in this study. Microsoft excel was also used to treat data statistically. [26]

2.3. Preparation of Sample

50 mg of topical gel containing kiwi extract was weighed and transferred into a 10 ml of volumetric flask. After that the gel was dissolved in 10 ml of ethanol and sonicated for 15 min and volume was make up by adding methanol. Further, dilution was made to obtain 5000 µg/ml concentration. [27]

2.4. Preparation of standard solution

10 mg of each Gallic acid and Quercetin was precisely weighed and transferred each to 10 ml volumetric flask separately. 5 ml of methanol was added to each flask and the mixture was sonicated for 5 min to bring about complete dissolution. Thereafter, the volume was adjusted up to the mark with methanol to get concentration of 1000 ppm for each. [28]

2.5. Method validation

The developed method was validated according to the International Council for Harmonization (ICH) guideline for specificity, linearity, precision.

The specificity of the developed method was checked by overlay spectra of Gallic Acid and Quercetin and SKYNISTA gel sample were recorded, and the purity of spectra was analyzed.

For Linearity and range, seven different bands were applied covering the concentration range of 1000 ng – 5000 ng/mL for Quercetin and Gallic acid in an increasing order. The calibration curve was constructed by calculating and graphing the mean peak areas (n = 5) versus concentration.

The HPTLC method's accuracy was evaluated using the standard addition method at three different concentration levels. A known amount Gallic Acid and Ascorbic acid was spiked at the 80, 100, and 120% level in a pre-analyzed sample. The developed plates were scanned and the peak area was recorded for recovery studies.

3. RESULTS

3.1. Optimization of the method

For the HPTLC chromatographic method focused on achieving optimal chromatographic separation Gallic Acid and Quercetin in their respective standard solutions. Numerous solvent combinations, including methanol, toluene, ammonia, ethyl acetate, chloroform, formic acid, glacial acetic acid, acetone, and glacial acetic acid were evaluated for their potential in separating these three compounds. Other solvent combinations such as methanol, water, ethyl acetate were tested but did not achieve the resolution and peak sharpness. After systematic analysis, a mobile phase consisting of toluene, ethyl acetate, and formic acid in a ratio of 5:4:1 (v/v/v) was selected as the optimal choice that would yield excellent separation for both biomarkers. This specific mobile-phase system demonstrated complete peak resolution and distinct baseline separation, enabling accurate detection at 310 nm. The resulting peaks exhibited clear and sharp resolution.

For the stationary phase, silica gel GF254 was employed, and the chromatographic chamber was saturated for 20 minutes before analysis. With the above established parameters well-resolved peaks of Quercetin and Gallic acid were obtained with the Rf value of 0.47 ± 0.06, 0.28 ± 0.02, respectively.

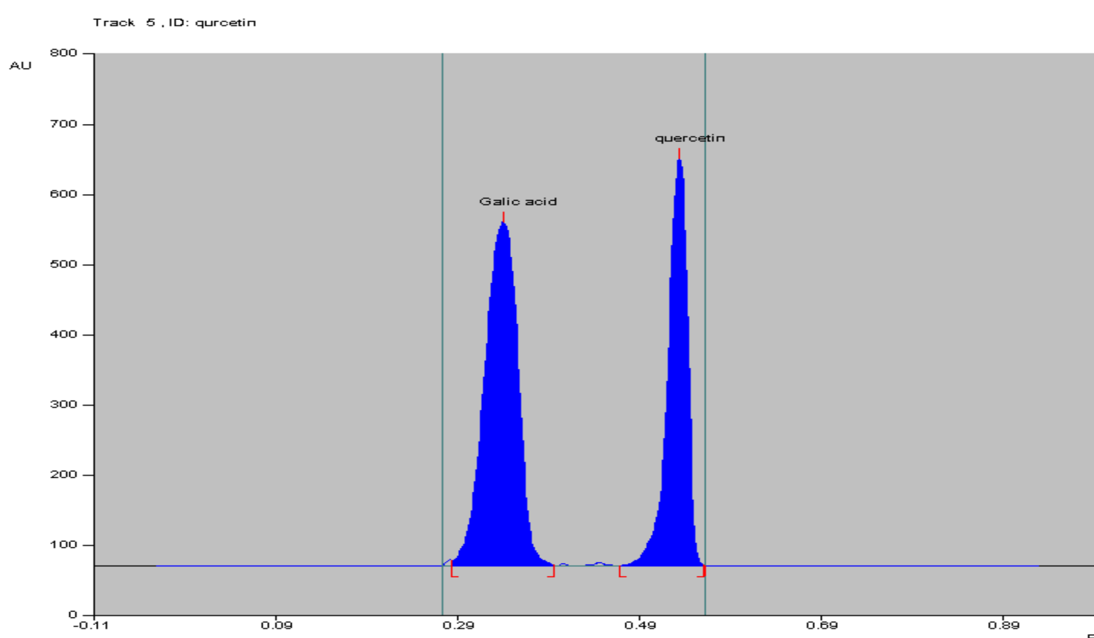


Figure 1: Densitogram of mixed std Gallic acid and Quercetin

3.2. Method validation

The validation of our developed method adhered to the rigorous standards set forth by the ICH guidelines. This validation holds significant importance in the field of pharmaceuticals, particularly concerning herbal formulations. The market is overwhelmed with numerous herbal products, and ensuring their quality and authenticity is a paramount public health concern. Method validation is utmost important in guaranteeing the reliability and precision of analytical techniques employed for quality control testing. In accordance with the ICH guidelines, we conducted a comprehensive evaluation of various method parameters to assess their quality performance.

3.2.1. Specificity

The analytical method's specificity pertains to its capacity to accurately evaluate the Gallic Acid and Quercetin of interest while lessening the influence of potential inter rose components. Our developed HPTLC method has demonstrated remarkable specificity, with distinct and well-separated peaks that exhibit no interference from other constituents, as illustrated.

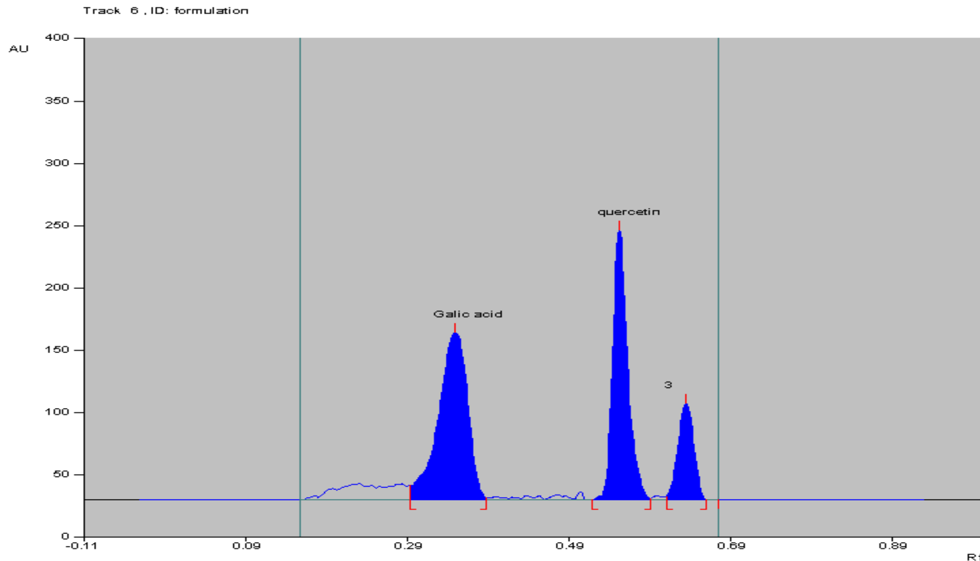


Figure 2: Densitogram of Quercetin , Gallic acid and SKYNISTA formulation.

3.2.2. Linearity and range

The analytical method's linearity refers to its capacity to provide a linear and proportional relationship between the analyte's concentration and its response. To assess linearity, the developed method recorded analyte responses in triplicate, and the results are presented. The linearity and the range of Gallic Acid and Quercetin were calculated using a calibration curve method, with statistical calculations based on regression equations Gallic Acid and Quercetin. The method was found to be linear in the concentration range of 1–5 µg/band for both gallic acid and quercetin.

3.2.3. Precision

Precision assesses the degree of repeatability and reproducibility of the analytical method. It is typically evaluated through:

Repeatability: This involves analyzing a specific concentration of the biomarkers (e.g., 240 ng/band for Gallic Acid) multiple times (n=6) under the same conditions.

Intraday and Interday Precision: These are measured by calculating the Percentage Relative Standard Deviation (%RSD) of multiple concentrations analyzed at different times on the same day and across three consecutive days.

Acceptance Criteria: For the method to be considered precise, the %RSD values for both Gallic Acid and other biomarkers must consistently remain below 2%.

Conditions

Formulation: **50 mg in 10 mL methanol**

Application volume: **20 µL**

Number of determinations: **n = 6**

Formula:

%RSD = SD / Mean X 100

Precision Results

Analyte	**Mean peak area (AU)**	**SD**	**%RSD**
Gallic acid	4232.15	81.6	1.93
Quercetin	3419.80	57.1	1.67

Table 1: Precision results

Precision conclusion

The %RSD values were found to be less than **2%**, indicating good repeatability of the method.

Intraday Precision

Repeat analysis was performed within the same analytical sequence.

Intraday Results

Analyte	Mean peak area (AU)	SD	%RSD
Gallic acid	4122.83	95.3	2.31
Quercetin	3458.90	51.9	1.50

Table 2: Intraday results

Intraday conclusion

The method showed acceptable intraday precision.

Interday Precision

Analysis was performed on different days.

Interday Results

Analyte	Mean peak area (AU)	SD	%RSD
Gallic acid	4275.27	77.0	1.80
Quercetin	8287.40	258.6	3.12

Table 3: Interday results

Interday conclusion

The method showed good reproducibility across different analytical runs.

3.2.4. Accuracy

Accuracy confirms that the values obtained by the method are close to the "true" values.

Methodology: Accuracy is evaluated using the standard addition method, where known quantities of pure standards (Quercetin and Gallic Acid) are "spiked" into pre-analyzed herbal gel samples at three levels: 80%, 100%, and 120%.

Results: The method is considered accurate if the observed percentage recoveries fall within the range of 98% to 102%.

The percentage recovery for both analytes was found within **97–103%**, satisfying **ICH Q2(R1)** acceptance criteria.

3.2.5. LOD and LOQ

These parameters define the sensitivity of the HPTLC method.

Limit of Detection (LOD): The lowest amount of an analyte that can be detected but not necessarily quantified. It is calculated as LOD = 3.3 x sigma / S, where sigma is the standard deviation of the response and S is the slope of the calibration curve.

Limit of Quantification (LOQ): The lowest amount of an analyte that can be determined with suitable precision and accuracy. It is calculated as LOQ = 10 x sigma / S

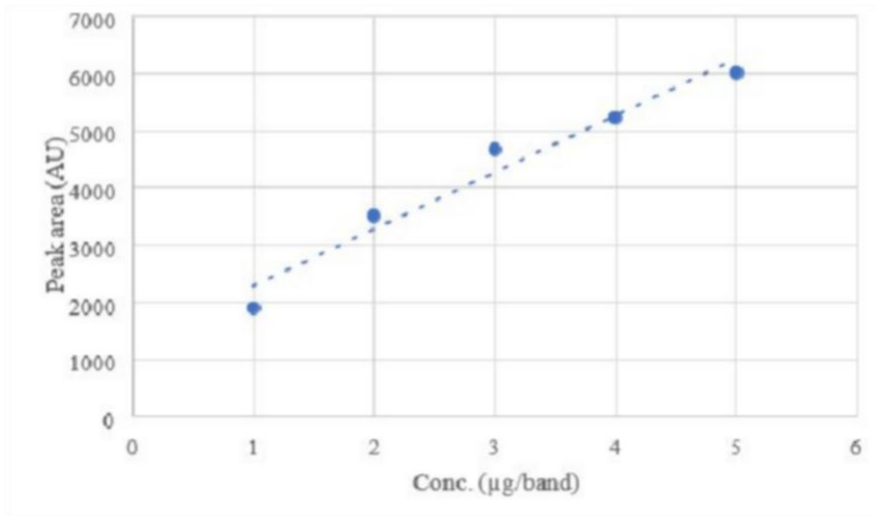
Typical Values: For Gallic Acid, the LOD is approximately 2.43 ng/band, and the LOQ is around 7.38 ng/band

3.2.6. Robustness

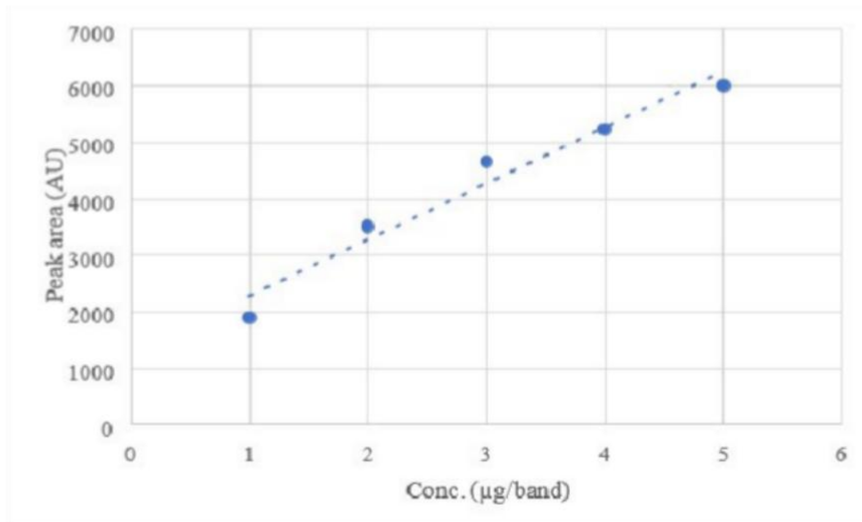
Robustness indicates the method's capacity to remain unaffected by small, deliberate variations in experimental parameters.

Variables Tested: Common changes include slight adjustments to the mobile-phase composition, the detection wavelength (e.g., +/-2 nm), and the chamber saturation time.

Outcome: If these minor alterations do not result in significant differences in the Rf values or peak areas, and the results remain within acceptable limits, the method is declared robust.



Graph 1: Linearity graph for Gallic Acid



Graph 2: Linearity graph for Quercetin

Table 4: Gallic Acid – Accuracy Calculations

Level	Added (µg)	Found (µg)	% Recovery
80%	3.50	3.45	98.57
100%	4.00	4.07	101.75
120%	4.20	4.34	103.33

Table 5: Quercetin – Accuracy Calculations

Level	Added (µg)	Found (µg)	% Recovery
80%	1.40	1.37	97.86
100%	2.00	1.95	97.50
120%	1.80	1.84	102.22

Table 6: LOD and LOQ results

Analyte	LOD (µg/band)	LOQ (µg/band)
Gallic acid	0.62	1.89
Quercetin	1.21	3.67

Table 7. Summary for HPTLC method

Parameter	Gallic Acid	Quercetin
Linearity (µg/band)	1–5	1–5
Correlation coefficient (r)	0.99469	0.97936
Accuracy (%)	98.57–103.33	97.50–102.22
Method precision (%RSD)	1.93	1.67
Intraday (%RSD)	2.31	1.50
Interday (%RSD)	1.80	3.12
LOD (µg/band)	0.62	1.21
LOQ (µg/band)	1.89	3.67
Assay (% w/w)	3.38	0.92

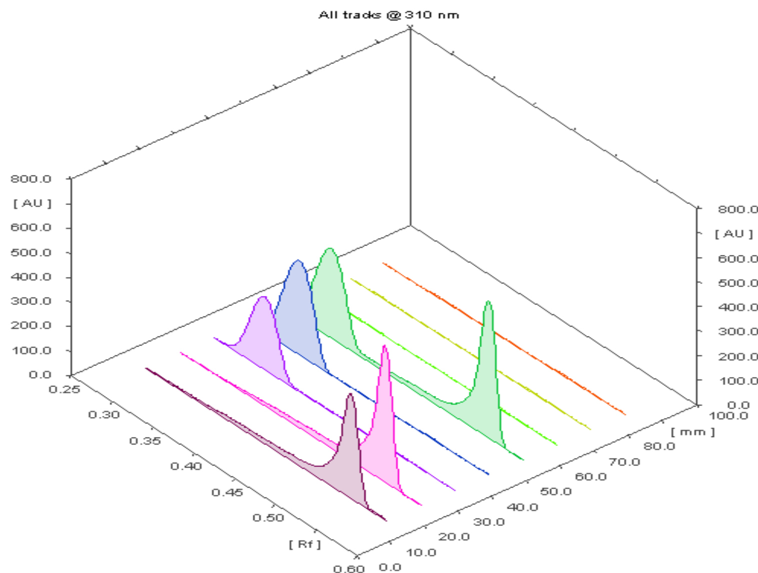


Figure 3: 3D Overlay Densitogram of 1000ng – 5ug/band for Quercetin and Gallic acid

3.3. Analysis of the formulation

The developed HPTLC method was utilized for the analysis of commercial herbal formulations containing the Gallic Acid and Quercetin analytes. While some additional peaks arising from other phytoconstituents were detected, they were deemed non-interfering with the analytes peak and thus excluded from the assessment.

4. Greenness evaluation for the developed method

The environmental sustainability of the developed HPTLC method for the simultaneous estimation of quercetin and gallic acid in a marketed herbal kiwi gel formulation was evaluated using both Analytical Method Greenness Score (AMGS) and AGREE (Analytical GREEnness) metrics. The method demonstrated an overall AMGS score of 120.49, indicating a moderately green and environmentally acceptable procedure. The instrument energy score (48.09) reflects moderate energy consumption during plate development and densitometric scanning, while the low solvent energy score (16.03) highlights the minimal solvent usage inherent to HPTLC. The solvent EHS score (56.37) indicates moderate environmental and safety concerns associated with the organic solvents employed in the mobile phase.

Further evaluation using the AGREE metric yielded a score of 0.78, demonstrating a high level of compliance with green analytical chemistry principles. This score emphasizes the method's advantages in terms of reduced solvent consumption, minimal waste generation, and moderate energy use, while also indicating potential for further improvements through the use of greener solvents or reduced hazardous reagents. Collectively, these assessments confirm that the developed HPTLC method is robust, reliable, and environmentally conscious, making it suitable for routine quality control analysis of quercetin and gallic acid in marketed herbal kiwi gel formulations.

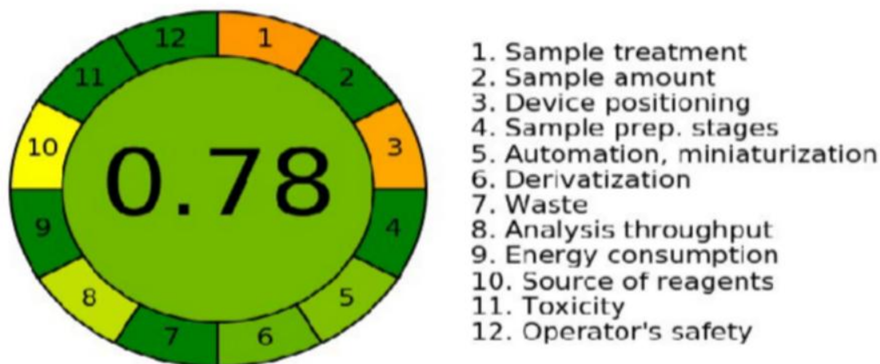


Figure 4: Pictogram represents the green assessment results

Table 8.Eco analytical scale, AGREE and AGMS tools for assessment of greenness values.

Component	score	Interpretation /comment
Instrument energy	48.09	Moderate energy consumption during plate development and densitometry
Solvent energy	16.03	Low solvent consumption, highlighting HPTLC's eco efficiency
Solvent EHS	56.37	Moderate environmental, health, and safety impact due to organic solvents
Overall AMGS score	120.49	Moderate green and acceptable method

4. DISCUSSION:

The developed HPTLC method for Gallic Acid and Quercetin demonstrated strong analytical performance, ensuring its suitability for herbal formulation quality control. A mobile phase of toluene, ethyl acetate, and formic acid (5:4:1, v/v/v) provided sharp, well-resolved peaks with reproducible Rf values of 0.28 ± 0.02 for Gallic Acid and 0.47 ± 0.06 for Quercetin, confirming specificity. Linearity was established across 1–5 µg/band, with regression analysis validating proportional response. Precision studies showed %RSD values below 2% for repeatability and acceptable intraday and interday reproducibility, meeting ICH criteria. Accuracy was confirmed through recovery studies, with values between 97–103%, ensuring reliability. Sensitivity was demonstrated with low LOD (2.43 ng/band) and LOQ (7.38 ng/band) for Gallic Acid. Robustness testing indicated stability under minor variations in mobile phase composition, wavelength, and chamber saturation. Overall, the method is validated as specific, precise, accurate, sensitive, and robust, making it a reliable tool for routine quality control of herbal formulations containing Gallic Acid and Quercetin. This contributes significantly to ensuring authenticity and safety in the growing herbal product market.

5. CONCLUSION

The proposed HPTLC method provides a validated, eco-friendly approach for the simultaneous estimation of Quercetin and Gallic Acid in herbal gels. By optimizing chromatographic separation with a toluene–ethyl acetate–formic acid mobile phase, the method achieved sharp, reproducible peaks and strict adherence to ICH Q2(R1) guidelines. Its simplicity, speed, and accuracy make it highly suitable for routine quality control in pharmaceuticals and nutraceuticals, addressing challenges posed by complex herbal matrices. Importantly, the integration of Green Analytical Chemistry principles reduces toxic solvent waste, ensuring sustainability. This dual emphasis on analytical robustness and environmental stewardship enhances product authenticity and therapeutic consistency.

6. ACKNOWLEDGMENT

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7. Conflict of interest:

The authors declare no conflict of interest

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