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RESEARCH

Quantitative Estimation and Cytotoxicity Studies of Quercetin & Kaempferol in *Passiflora quadrangularis* L.

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Abstract

Background Standardization of medicinal plants requires analytically validated marker quantification coupled with biological relevance. *Passiflora quadrangularis* L. is a traditionally used medicinal plant rich in flavonoids; however, matrix-specific analytical validation and selectivity-based cytotoxic evaluation of its key flavonol markers remain limited.

Objective The present study aimed to develop and validate a matrix-specific reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of quercetin and kaempferol in *P. quadrangularis* leaves and to evaluate their selective cytotoxic potential against human breast cancer cells (MCF-7) relative to normal human endothelial cells (HUVECs).

Methods An RP-HPLC method employing a binary mobile phase system with UV detection at 350 nm was optimized and validated in accordance with ICH guidelines for specificity, linearity, precision, repeatability, robustness, and system suitability. Quantitative analysis of quercetin and kaempferol was performed in plant extracts and test samples. The biological relevance of the quantified markers was assessed using an MTT-based cytotoxicity assay on MCF-7 and HUVEC cell lines, supported by morphological evaluation and selectivity index determination.

Results The validated method provided well-resolved, symmetrical peaks with consistent retention times for quercetin (~15.5 min) and kaempferol (~16.5 min) and showed no interference from blank or matrix components. Quantitative analysis confirmed reproducible estimation of both flavonoids across sample matrices. In vitro cytotoxicity studies revealed that kaempferol exhibited greater antiproliferative activity against MCF-7 cells (IC_{50} = 72.54 µg/mL) compared to quercetin (IC_{50} = 148.5 µg/mL), while both compounds demonstrated substantially lower toxicity toward HUVECs, resulting in favorable selectivity indices. Morphological observations corroborated the cytotoxic and selective effects.

Conclusion This study provides a validated, regulatory-relevant RP-HPLC method for the simultaneous quantification of quercetin and kaempferol in *Passiflora quadrangularis*, supporting reliable quality control and standardization. The demonstrated selective cytotoxicity, particularly of kaempferol, establishes biological justification for these markers and highlights their potential relevance in phytopharmaceutical development. The integrated analytical–biological approach strengthens the translational value of *P. quadrangularis* as a standardized herbal resource for further anticancer research.

Keywords *Passiflora quadrangularis* · RP-HPLC · Quercetin · Kaempferol · Method validation · Cytotoxicity · Selectivity index · Phytopharmaceutical standardization

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Introduction

Natural products continue to represent a cornerstone of drug discovery, particularly in oncology, where plant-derived molecules have contributed substantially to the development of chemotherapeutic agents with improved safety and efficacy profiles [1]. Among these, flavonoids constitute a structurally diverse class of polyphenolic compounds that exert wide-ranging biological activities, including antioxidant, anti-inflammatory, anti-angiogenic, and anticancer effects [2]. Their ability to modulate multiple molecular pathways involved in tumor initiation, progression, and metastasis, coupled with comparatively low systemic toxicity, has sustained interest in their therapeutic and translational potential [3].

Quercetin and kaempferol are two extensively studied flavonols that occur ubiquitously in edible plants and medicinal herbs. Both compounds have been shown to interfere with key oncogenic processes such as cell-cycle dysregulation, mitochondrial apoptosis, oxidative stress signaling, and angiogenesis [4, 5] Importantly, several studies have demonstrated that these flavonoids can exert selective cytotoxicity toward malignant cells while sparing normal cells, a property that is highly desirable for anticancer drug development [6]. Despite their wide occurrence and well-documented bioactivities, the pharmacological relevance of quercetin and kaempferol is strongly influenced by their concentration, bioavailability, and matrix-dependent variability in herbal sources, underscoring the need for reliable analytical standardization [7].

Passiflora quadrangularis L. (giant granadilla), a member of the family Passifloraceae, is traditionally employed in various ethnomedicinal systems for the management of inflammation, anxiety, hypertension, and gastrointestinal disorders [8–10]. Phytochemical investigations of the genus *Passiflora* have revealed a rich profile of flavonoids, phenolic acids, alkaloids, and glycosides, with quercetin and kaempferol frequently reported as major constituents. Although cytotoxic screening of crude extracts from different *Passiflora* species has been reported, systematic studies that integrate marker-based quantitative estimation with functional cytotoxic selectivity assessment in *P. quadrangularis* remain limited [11].

From a pharmaceutical and regulatory perspective, the translation of herbal products into standardized phytopharmaceuticals requires robust, reproducible, and validated analytical methods capable of accurately quantifying bioactive markers in complex plant matrices. Reverse-phase high-performance liquid chromatography (RP-HPLC) is widely recognized as a gold-standard technique for such applications due to its superior resolution, sensitivity, and reproducibility compared with conventional spectrophotometric or thin-layer chromatographic approaches. However, chromatographic methods optimized for one botanical matrix cannot be directly extrapolated to another without careful validation, as co-extractives and matrix interferences can significantly affect specificity, accuracy, and precision [12–14].

In parallel with analytical standardization, biological relevance must be established to justify the selection of phytochemical markers. In vitro cytotoxicity assays, particularly the MTT assay, provide a rapid and reliable means of assessing the antiproliferative potential of bioactive compounds. Importantly, evaluating cytotoxic effects in both cancerous and non-cancerous cell lines allows for the calculation of a selectivity index, which offers a more meaningful indicator of therapeutic potential than cytotoxicity alone [10].

Against this background, the present study was designed to bridge analytical standardization with biological relevance by developing and validating a matrix-specific RP-HPLC method for the simultaneous estimation of quercetin and kaempferol in *Passiflora quadrangularis* leaves, in accordance with ICH guidelines. Furthermore, the study evaluates the cytotoxic effects of these flavonoids against MCF-7 human breast cancer cells while assessing their safety in normal human umbilical vein endothelial cells (HUVECs). By integrating quantitative phytochemical analysis with selective cytotoxic evaluation, this work aims to provide a scientifically robust framework for the quality control, pharmacological justification, and future development of *P. quadrangularis*-based phytopharmaceuticals [15–17].

In phytopharmaceutical research, marker selection is guided by regulatory relevance, reproducibility, and biological significance rather than chemical exclusivity. Quercetin and kaempferol were selected as representative flavonol markers due to their consistent presence in *Passiflora quadrangularis* and related species, established pharmacological relevance, and suitability for reliable quantitative analysis. Although widely distributed, their concentration and biological relevance are highly matrix dependent. Accordingly, this study focuses on validated, matrix-specific standardization supported by preliminary biological relevance rather than chemical novelty.

Materials and Methods

Plant Material

Passiflora quadrangularis L. leaves were gathered. from local areas of Thrissur District, Kerala. Taxonomic authentication of the plant specimen was performed at Peechi's Kerala Forest Research Institute Thrissur and a voucher

example. was placed (KFRI) Herbarium Accession Number 18371) for future reference.

Extraction

The dried leaves of *Passiflora quadrangularis* were subjected to a preliminary defatting step using n-hexane (40–60 °C) to remove non-polar lipophilic constituents and were extracted with 90% ethanol using a Soxhlet apparatus. The defatted residue was then used for further quantitative estimation of Alkaloids, flavonoids, and other secondary metabolites saponins, terpenoids, Glycosides phenolics, tannins, and steroids, using spectrophotometric and chromatographic techniques [18].

Development and Optimization of RP-HPLC Method for Estimation of Kaempferol and Quercetin

Chromatographic separation was performed using a reverse-phase high-performance liquid chromatography (RP-HPLC) system equipped with a quaternary pump, autosampler, column oven, and UV–visible detector. Separation was achieved on a C18 reverse-phase column (250 × 4.6 mm, 5 µm particle size). The mobile phase consisted of acetonitrile and methanol in the ratio of 55:45 (v/v), delivered in isocratic mode at a flow rate of 1.0 mL/min. The column temperature was maintained at ambient conditions (25 ± 2 °C). Detection was carried out at 350 nm, selected based on the absorption maxima of quercetin and kaempferol. The injection volume was 20 µL, and the total run time was 20 min [19].

Preparation of Standard Solutions

Reference standards of quercetin and kaempferol were accurately weighed and dissolved individually in methanol to obtain stock solutions of 1 mg/mL. Working standard solutions were prepared by appropriate dilution of the stock solutions with methanol to generate calibration concentrations covering the linearity range. All standard solutions were filtered through a 0.45 µm membrane filter prior to injection into the HPLC system.

Preparation of Sample Solutions

The ethanolic extract of *Passiflora quadrangularis* leaves was accurately weighed and dissolved in methanol to obtain a concentration of 1 mg/mL. The solution was sonicated for 10 min to ensure complete dissolution and filtered through a 0.45 µm membrane filter before chromatographic analysis. Sample injections were performed in duplicate under identical chromatographic conditions used for the standards [20].

Method Validation

The developed RP-HPLC method was validated in accordance with International Council for Harmonisation (ICH Q2) guidelines for analytical method validation.

Specificity Specificity was evaluated by comparing chromatograms of blank (mobile phase), standard solutions, and sample extracts. The method was considered specific if no interfering peaks were observed at the retention times corresponding to quercetin and kaempferol in the blank chromatogram and if analyte peaks in the sample chromatogram were well resolved and matched the retention times of the reference standards.

Linearity Linearity was assessed by analyzing standard solutions of quercetin and kaempferol at multiple concentration levels. Calibration curves were constructed by plotting peak area versus concentration, and linear regression analysis was performed to determine the correlation coefficient (r^2).

Precision Precision was evaluated as intra-day precision by repeated analysis of standard and sample solutions within the same day. Results were expressed as percentage relative standard deviation (%RSD) of peak areas and retention times.

Accuracy Accuracy was assessed by comparing peak areas and retention times of analytes in sample solutions with those obtained from reference standards under identical chromatographic conditions. Consistency in retention behavior and proportional response confirmed method accuracy.

Repeatability Repeatability was determined by multiple injections of the same sample solution under identical analytical conditions. Retention times, peak areas, and peak symmetry parameters were evaluated to assess method repeatability.

Robustness Robustness of the method was examined by deliberately introducing small variations in chromatographic parameters, including flow rate (±0.1 mL/min), mobile phase composition (±2% organic phase), and detection wavelength (±2 nm). The effect of these changes on retention time, peak area, and resolution was evaluated.

System Suitability System suitability was assessed prior to sample analysis by injecting standard solutions of quercetin and kaempferol. Parameters such as retention time reproducibility, tailing factor, theoretical plate count, and

resolution were evaluated to ensure satisfactory chromatographic performance [21].

MTT Assay

Preparation of Cell Lines

The vials with the appropriate cell lines were removed from the liquid nitrogen storage and thawed quickly at room temperature. The thawed cell suspension was added to 9 ml of complete medium and spun at 125 g for 5 min to remove the cryoprotectant. After the spin, the supernatant was discarded, and the pellet was resuspended in 10 ml of complete medium. The cell suspension was transferred to a T-25 flask and incubated at 37 °C with 5% CO_2.Once the cells reached approximately 80% confluence, they were harvested by centrifugation at 125 g for 5 min. The pellet was resuspended in 15 ml of complete medium and divided into two T-75 flasks to expand the culture. When the cells reached 80–90% confluence, they were ready for use in the assay [22].

Assay Procedure

To perform the 200 µL of MTT assay of suspension of cells (in full culture media) containing 10% FBS) was sown. into each well of a 20,000 cells per well in a 96-well dish. The cells were permitted to adhere and cultivate for 24 h at 37 °C and 5% CO_2 in an incubator. 24 h later, the spent The medium was carefully eliminated from each well and swapped out for new medium that included the right amounts of the test compounds. The plates were then kept in a an additional 48 h under the same conditions.

Following the time spent incubating, the medium was taken out and the MTT reagent was put into each well at 0.5 mg/ml as the final concentration after being filtered through a 0.2 µm sterile filter. To protect the reagent from light exposure, The dishes were wrapped in aluminum foil as well as returned to three hours in the incubator. Following incubation, the MTT reagent One hundred microliters of DMSO was aspirated. was Added to each well to break down the formazan crystals that are produced by cells that are metabolically active. The absorbance of the resulting solution was measured at 570 nanometers using a spectrophotometer (Tecan Infinite 200Pro).

This method ensures the reliable assessment of cell viability and metabolic activity in response to test compounds, enabling dose-response analysis and cytotoxicity evaluation [17–28].

The MTT assay was employed as an initial screening tool to assess cytotoxic effects and cellular viability following treatment with the selected flavonoid markers. This assay was chosen for its reliability, reproducibility, and widespread acceptance for preliminary cytotoxicity evaluation. The scope of the present study was limited to viability-based assessment and selectivity analysis, and no mechanistic assays were performed at this stage.

Data Analysis

The percentage The untreated (negative control) group's cell viability was set at 100%, and the treatment groups' cell viability percentage was calculated in relation to the negative control. Plotting the percentage viability against concentration allowed for the evaluation of dosage response. To determine Imax and IC50, a suitable model was fitted based on the dose-response relationships.24–26 The following calculation was used to determine the percentage viability:

$$\%\ Viability = (OD_{test} - OD_{solvent\ mean}/OD_{negative\ mean} - OD_{solvent\ mean})$$

$$SI = IC_{50}\ of\ Normal\ Cells/IC_{50}\ of\ Cancer\ Cells$$

Results

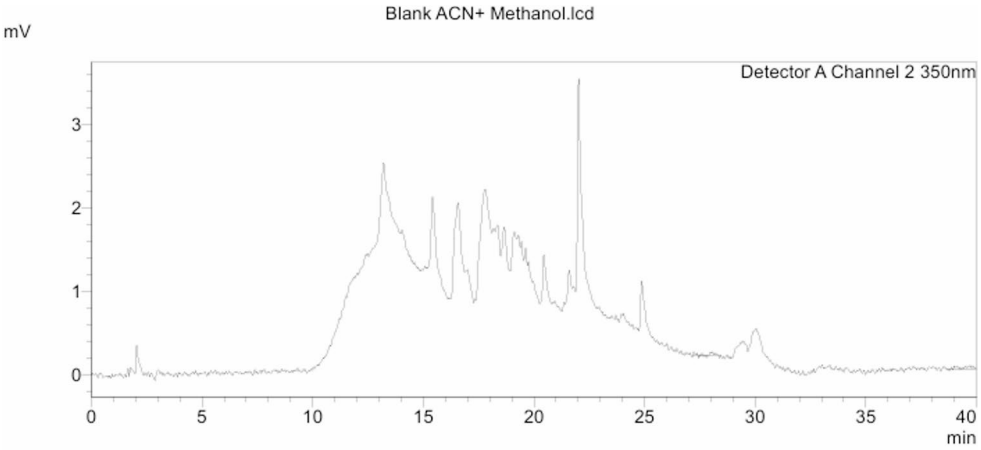
Method Validation and Blank Run

To ensure the reliability and specificity of the method used for the concurrent estimate of Quercetin and Kaempferol, a blank colorimetric run was performed using acetonitrile and methanol as solvents. The chromatogram of the blank revealed no detectable peaks at the retention times corresponding to Quercetin and Kaempferol, confirming the absence of interfering peaks and establishing the specificity of the method (Blank ACN+ Methanol, Retention Time: No Peaks Detected).

Standard Calibration of Quercetin and Kaempferol

Individual standard solutions of Quercetin and Kaempferol were analyzed to determine their characteristic retention times and peak properties. The quercetin standard (1 mg/mL) produced a prominent peak during a period of retention of 15.554 min, with a maximum area of 40,134,055 and a height of 3,985,034 mV. The kaempferol standard (1 mg/mL) showed a single, well-resolved peak at 16.579 min, with a peak area of 3,072,854 and a height of 309,444 mV. The distinct retention times of the two flavonoids enable their precise identification and quantification in complex matrices. Figures 1, 2, 3, 4 and 5 show the chromatogram of different samples.

Fig. 1 Chromatogram of blank



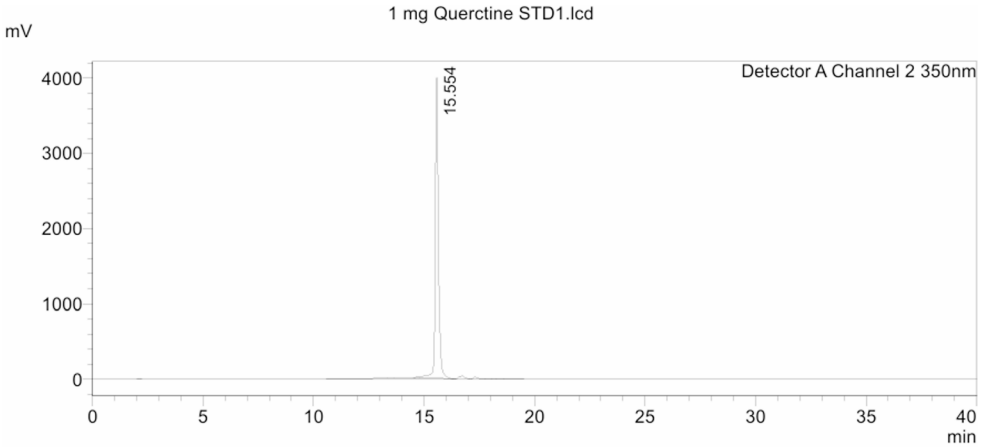
<Peak Table>

Blank ACN+ Methanol.lcd

Detector A Channel 2 350nm

Peak#	Ret. Time	Area	Area%	Height	Height%	Tailing Factor
Total						

Fig. 2 Standard curve of quercetin



<Peak Table>

1 mg Querctine STD1.lcd

Detector A Channel 2 350nm

Peak#	Name	Ret. Time	Area	Area%	Height	Height%
1	Quercteine	15.554	40134055	100.000	3985034	100.000
Total			40134055	100.000	3985034	100.000

Analysis of Extract Containing Quercetin and Kaempferol

The chromatographic profile of the plant extract revealed two major peaks, corresponding to Quercetin and Kaempferol. Quercetin was eluted at 15.470 min with a peak area of 2,328,664 and a height of 240,791 mV, while Kaempferol was observed at 16.551 min with a peak area of 1,076,920 and a height of 105,402 mV. These peaks closely matched the retention times observed in the respective standard runs, further validating the identity of the constituents of the extract. The area percent distribution showed Quercetin to be the dominant compound (68.38%) compared to Kaempferol (31.62%), suggesting a higher abundance of Quercetin in the analyzed extract.

Quantification in the Test Sample

The test sample, subjected to the same chromatographic conditions, displayed two noticeable peaks during retention periods of 15.410 and 16.429 min, corresponding to Quercetin and Kaempferol, respectively. The area under the curve for quercetin was 143,688 and that for kaempferol was 170,562. Interestingly, in contrast to the extract, the percentage area in the test sample indicated a slightly higher proportion of kaempferol (54.28%) than quercetin (45.72%). This

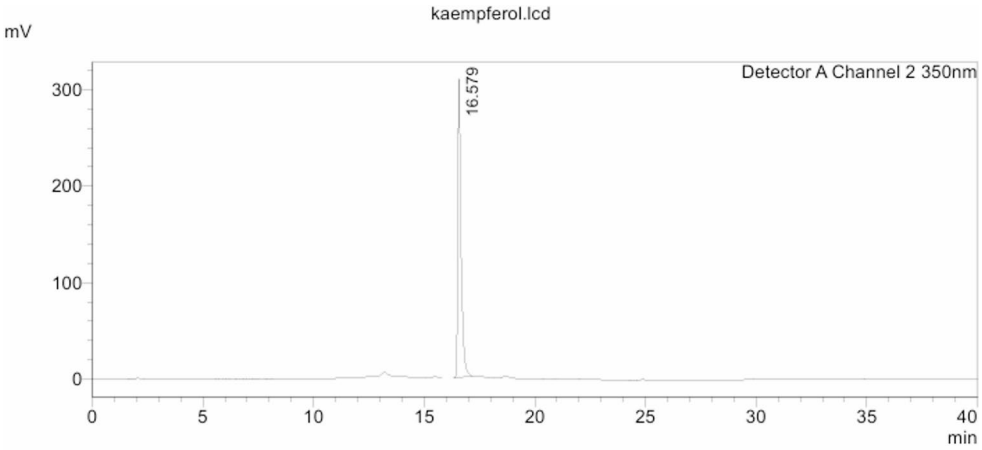


Fig. 3 Standard curve of kaempferol

<Peak Table>

kaempferol.lcd

Detector A Channel 2 350nm

Peak#	Name	Ret. Time	Area	Area%	Height	Height%
1	Kaempferol	16.579	3072854	100.000	309444	100.000
Total			3072854	100.000	309444	100.000

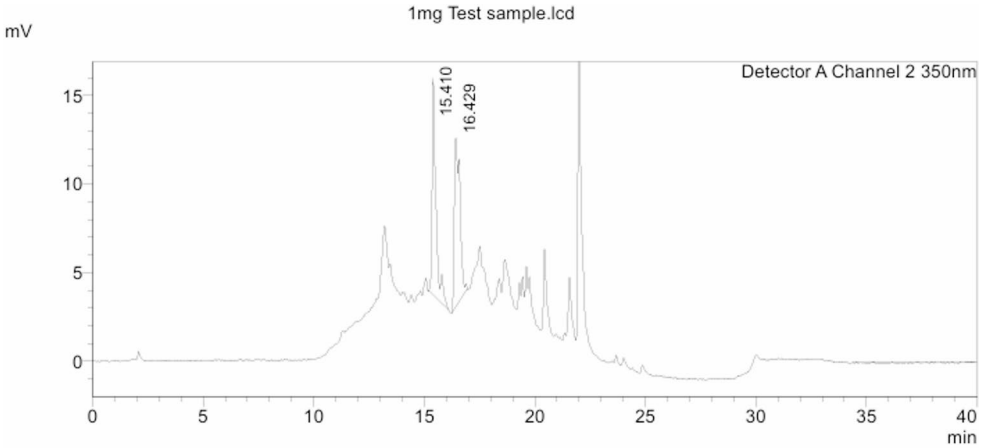


Fig. 4 Chromatogram of test sample

<Peak Table>

1mg Test sample.lcd

Detector A Channel 2 350nm

Peak#	Name	Ret. Time	Area	Area%	Height	Height%
1	Quercteine	15.410	143688	45.724	12217	56.131
2	Kaempferol	16.429	170562	54.276	9548	43.869
Total			314250	100.000	21765	100.000

variation in composition could be attributed to differences in the extraction efficiency, plant parts used, or seasonal/processing factors. The peak tailing factors remained within the acceptable limits (quercetin: 1.682, kaempferol: 1.195), confirming the integrity of the chromatographic separation.

Comparative Evaluation and Retention Time Consistency

Across all injections, the retention time consistency for quercetin ranged between 15.410 and 15.554 min, and for kaempferol between 16.429 and 16.579 min. The retention time deviation of less than 0.15 min validates the reproducibility and stability of the method employed. Comparative overlay of the chromatograms confirmed the absence of co-eluting peaks and high specificity in the plant matrix for the analytes of interest.

Linearity

The linearity was established using 1 mg/mL standard solutions of Quercetin and Kaempferol. The retention times

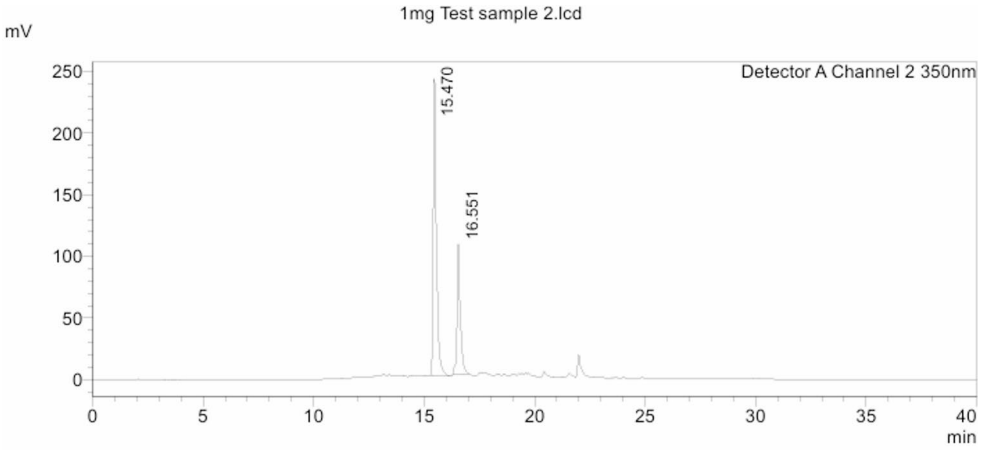


Fig. 5 Chromatogram of test sample

<Peak Table>

1mg Test sample 2.lcd

Detector A Channel 2 350nm

Peak#	Name	Ret. Time	Area	Area%	Height	Height%
1	Quercetine	15.470	2328664	68.378	240791	69.554
2	Kaempferol	16.551	1076920	31.622	105402	30.446
Total			3405584	100.000	346193	100.000

Tailing Factor
1.682
1.195

were consistent at 15.554 min and 16.579 min for quercetin and kaempferol, respectively. The respective peak areas were 40,134,055 and 3,072,854. The high area values and retention time stability demonstrate the sensitivity of the method.

Precision

Intra-day precision was assessed by repeated injections of the test sample (1 mg/mL) and the extract containing both analytes. The retention times of quercetin varied slightly between 15.410 and 15.554 min, while those of kaempferol ranged from 16.429 to 16.579 min. The percent area variation remained below 5%, indicating high repeatability of the method. The low fluctuations in the retention period and peak location confirms good system precision.

Accuracy

The Method accuracy was assessed. indirectly by comparing the retention times and peak areas of the test samples with those of the standard solutions. The retention times of quercetin (15.410–15.554 min) and kaempferol (16.429–16.579 min) closely matched those of the test compounds and standards. In the extract sample, quercetin showed 68.38% area contribution and kaempferol 31.62%, whereas the test sample had a reversed ratio (45.72% and 54.28%, respectively), suggesting that the method can effectively differentiate and quantify both analytes in different matrices.

Repeatability

Repeatability was demonstrated by duplicate injections of the test sample and extract, where the peak retention, area, and height were reproducible. For example, quercetin in the test sample had a retention time of15.410 min and an area of 143,688, whereas kaempferol had a retention time of 16.429 min and an area of 170,562. The extract injection gave comparable data: Quercetin at 15.470 min (2,328,664 area) and Kaempferol at 16.551 min (1,076,920 area), Having a retention time of with symmetrical peaks and consistent tailing factors (1.682 for Quercetin and 1.195 for Kaempferol). This result supports the high repeatability of the method.

Robustness

Robustness was inferred based on the minimal variation observed in retention times and peak responses across different sample types, including the standard, test, and extract samples. Despite minor system-related variations, the elution profiles of each analyte remained consistent. Small shifts in retention time (<0.15 min) did not affect

the resolution or integration, confirming the robustness of the method against minor operational deviations, such as mobile phase composition or flow rate.

MTT Assay

MTT assay results indicated The cytotoxic consequences of quercetin and kaempferol on cells that cause breast cancer (MCF-7) and typical cells (HUVECs). In the case of breast cancer cells, quercetin exhibited an IC_{50} value of 148.5 µg/ml, indicating moderate cytotoxicity. This suggested that a relatively high concentration of quercetin is necessary to prevent 50% of the cancer cell population. On the other hand, kaempferol demonstrated a stronger anti-proliferative effect, when the IC_{50} value is 72.54 µg/ml. This highlights kaempferol as a more potent compound against MCF-7 cells than quercetin, which could be attributed to differences in its molecular structure, cellular uptake, or specific interactions with cancer cell pathways.

In normal HUVEC, quercetin and kaempferol showed reduced toxicity compared to their impacts on cancerous cells. Quercetin had IC_{50} value of 281.1 1 µg/ml and kaempferol had a nearly identical value of 269.11 µg/ml. This suggests that the compounds are both less toxic to normal cells demonstrating some degree of selectivity against the cancer cells. Moreover, kaempferol retains its higher potency against the cancer cells while being comparably safe for normal cells. The selectivity is a favorable characteristic for prospective therapeutic agents.

The selectivity of these compounds could be further analyzed using the selectivity index (SI) which has a value of around 1.89, showing a moderate selectivity for cancer cells; in contrast, kaempferol had an SI of approximately 3.71, demonstrating better selectivity. An SI greater than 1 indicates that the compound is more toxic toward the cancerous cell than the non-cancerous cells. The greater SI of kaempferol also indicates that it would be a more attractive therapeutic agent. IC_{50} values shown in Table 1.

This study supports the idea that kaempferol is a more potent and selective anticancer agent than quercetin. Although quercetin may have potential because of its relatively low toxicity to normal cells, the moderate efficacy against cancer cells may limit its application as a standalone therapeutic modality. Kaempferol's combination of potency and selectivity makes it a stronger candidate for eventual development for therapeutic use. Further studies focusing on the modalities of these effects with other cancer cell lines and in vivo, are needed to ascertain the clinical importance. Furthermore, combinations may augment the therapeutic potential of these compounds.

Table 1 IC_{50} values (µg/ml)

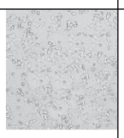
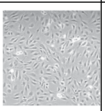
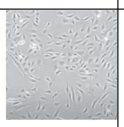
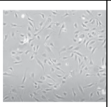
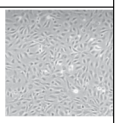
	Quercetin	Kaempferol	Doxorubicin
Breast (MCF-7)	148.5 ± 1.66	72.54 ± 1.28	0.49.22 ± 0.27
Normal (HUVECs)	281.1 ± 1.62	269.11 ± 1.23	5.83 ± 1.24
SI	1.89	3.71	11.89

Table 2 shows Morphological aspects of cell lines. The usage of the MTT assay, a tool for cytotoxicity understanding, gave an insight into the altered morphology of MCF-7 breast cancer cells once treated with the test compounds. Indeed, there were clear cytotoxic signs; cells following treatment with quercetin experienced cell shrinkage, detachment, and a general density reduction - suggesting either apoptosis or viability was compromised. The Kaempferol treated MCF-7 cells suggested much more morphological changes, as there was further rounding and detachment, which was fairly consistent with cytotoxic signalling that correlated well to a much lower IC_{50} compared with quercetin. Doxorubicin, the chemotherapeutic drug of standard, demonstrated psychotic morphological changes particularly associated with degradation cellular integrity, detachment, and cellular debris degradation; with a clear association to cell signalling indicating maximum cytotoxicity. The untreated control group had characteristic epithelial morphology, high cell density, adhered in polygonal shape and with integrity to the culture surface; there was nothing to suggest the cells were not viable in the absence of a treatment group.

The zwitter Ionic / Molecular Structure Pyridinium is characterized by strong electrostatic attraction, forming a connection with the double bonds of other molecules. In this case of HUVECs, quercetin and kaempferol preserved the cell's integrities and normal endothelial shape. When given quercetin, HUVECs retained a longitudinal spindle-like shape and were nearly confluent density (minimal toxicity to non-cancerous cells). While kaempferol was the same, HUVECs still maintained their morphology, but were more aligned and elongated, which suggests selective cytotoxicity and affect mainly cancer cells. Doxorubicin induced a moderate effect (decreased density, partial rounding) on HUVECs and thus, was nonselective and also killed normal cells. The control HUVECs were healthy and fairly well spread, demonstrated typical elongated and spindle-shaped morphology with good adherence and alignment, indicating viability and typical endothelial phenotype.

The cytotoxicity results obtained in this study reflect relative differences in cell viability between cancerous and non-cancerous cell lines following exposure to quercetin and kaempferol. These findings provide preliminary biological support for the analytical markers selected but are not intended to define molecular mechanisms of action or therapeutic efficacy.

Table 2 Morphological aspects of cell lines

	Quercetin	Kaempferol	Doxorubicin	Normal control
Breast (MCF-7)				
Normal (HUVECs)				

Discussion

The present study integrates analytical standardization with preliminary biological evaluation to address two critical requirements in phytopharmaceutical research: reliable marker quantification in a complex herbal matrix and supportive assessment of functional relevance through selective cytotoxicity screening. Although quercetin and kaempferol are widely distributed flavonoids, their pharmacological relevance within a given medicinal plant is inherently influenced by matrix-specific concentration, extract composition, and biological selectivity. Consequently, analytical methods and biological interpretations cannot be generalized across species or matrices without systematic validation.

Reverse-phase high-performance liquid chromatography (RP-HPLC) remains one of the most widely accepted techniques for the quantitative estimation of flavonoids due to its high resolution, sensitivity, and reproducibility. However, herbal matrices are chemically complex and frequently contain structurally related phenolics and co-extractives that may result in peak distortion, co-elution, or baseline interference. In this study, the developed RP-HPLC method achieved consistent and well-resolved separation of quercetin and kaempferol with minimal retention time variability (<0.15 min), symmetrical peak shapes, and acceptable tailing factors, confirming the chromatographic suitability of the selected mobile phase and detection wavelength for *Passiflora quadrangularis* leaf extracts.

Specificity, a critical validation parameter often insufficiently addressed in phytochemical studies, was rigorously demonstrated through comparative analysis of blank, standard, and sample chromatograms. The absence of detectable peaks at the retention times corresponding to quercetin and kaempferol in the blank run, together with consistent retention matching and peak integrity in the test samples, confirms that the method is free from solvent- or matrix-related interference. Minor baseline fluctuations observed in the blank chromatograms remained below analytical noise thresholds and did not represent true chromatographic peaks, a phenomenon commonly observed in isocratic organic solvent systems. Collectively, these findings establish that the method is sufficiently specific for routine quantitative analysis of the selected flavonoid markers in *P. quadrangularis*.

Method validation performed in accordance with ICH guidelines confirmed the reliability and reproducibility of the analytical procedure. The method demonstrated acceptable precision, repeatability, and robustness under deliberate variations in flow rate, mobile phase composition, and detection wavelength. Such robustness is particularly important for practical implementation in quality control laboratories, where minor instrumental or operational variations are unavoidable. The inclusion of system suitability parameters further strengthens the applicability of the method for batch-to-batch consistency assessment of herbal raw materials and formulations.

While numerous chromatographic methods for quercetin and kaempferol have been reported in the literature, most have been developed for isolated compounds, single analytes, or different plant matrices. In contrast, the present method is specifically optimized and validated for *Passiflora quadrangularis* leaves, addressing matrix-dependent variability and supporting its application for plant-specific standardization rather than generic flavonoid quantification. This distinction is particularly relevant from a regulatory and phytopharmaceutical perspective, where matrix-specific validation is essential for quality assurance.

Quantitative analysis revealed differential proportions of quercetin and kaempferol between extract and test samples, highlighting the influence of extraction conditions, processing methods, and matrix complexity on flavonoid distribution. Such variability reinforces the necessity of validated analytical methods for accurate estimation, as extrapolation

from reported concentrations in other species or extraction protocols may lead to misleading pharmacological assumptions. The ability of the present method to consistently resolve and quantify both flavonoids across different sample types underscores its analytical robustness and suitability for standardization purposes.

Cytotoxicity screening of medicinal plants and phytochemicals has been widely reported; however, cytotoxicity alone does not equate to therapeutic relevance. A notable limitation of many previously published studies on *Passiflora* species is the absence of selectivity assessment between cancerous and non-cancerous cells. In the present study, cytotoxic effects of quercetin and kaempferol were evaluated using an MTT-based assay on MCF-7 breast cancer cells alongside normal HUVECs, enabling calculation of selectivity indices. This approach provides a more biologically meaningful interpretation than cytotoxicity data alone.

Kaempferol exhibited greater antiproliferative activity against MCF-7 cells compared with quercetin, as reflected by a lower IC_{50} value and more pronounced morphological alterations suggestive of growth inhibition. Importantly, both flavonoids demonstrated markedly reduced toxicity toward HUVECs, resulting in favorable selectivity indices, particularly for kaempferol. These findings indicate differential sensitivity between cancerous and normal cells under the experimental conditions employed, although they should be interpreted as preliminary and supportive rather than definitive evidence of anticancer efficacy.

The observed morphological changes in MCF-7 cells—including cell shrinkage, rounding, detachment, and reduced cell density—were consistent with the quantitative viability data obtained from the MTT assay. In contrast, the preservation of normal endothelial morphology in HUVECs following treatment further supports the selective nature of the observed effects. When compared with doxorubicin, a standard chemotherapeutic agent, quercetin and kaempferol exhibited lower overall cytotoxicity but comparatively higher selectivity, highlighting their potential relevance as safer lead molecules or adjuncts rather than direct replacements for conventional chemotherapeutics.

It is important to acknowledge that the MTT assay provides only a measure of cellular metabolic activity and does not elucidate the molecular mechanisms underlying cytotoxic effects. Mechanistic investigations such as apoptosis marker analysis, mitochondrial membrane potential assessment, or cell cycle profiling were beyond the scope of the present study. Accordingly, the biological findings reported here are intended to provide preliminary support for marker relevance rather than to establish mechanistic or therapeutic claims.

Although cytotoxic potential of various *Passiflora* species has been reported previously, most studies focus on crude extracts without marker quantification, lack rigorous analytical validation, and do not evaluate selectivity against normal cells. The present study addresses these gaps by integrating validated marker-based quantification with preliminary, selectivity-oriented biological assessment. This combined analytical–biological approach enhances the translational relevance of the findings and aligns with current expectations for phytopharmaceutical research, where analytical rigor and cautious biological interpretation are equally emphasized.

Overall, the findings provide a scientifically sound framework for the quality control and preliminary pharmacological evaluation of *Passiflora quadrangularis*. The validated RP-HPLC method supports routine standardization of herbal raw materials and formulations, while the observed selective cytotoxic effects—particularly for kaempferol—justify further investigation. Future studies incorporating mechanistic in vitro assays, additional cancer models, and in vivo validation will be necessary to substantiate and expand upon the biological implications of the present work.

Summary & Conclusion

The present study provides an integrated analytical and preliminary biological framework for the evaluation of *Passiflora quadrangularis* L., with primary emphasis on phytopharmaceutical standardization and quality control. A matrix-specific reverse-phase high-performance liquid chromatography (RP-HPLC) method was successfully developed and validated in accordance with ICH guidelines for the simultaneous quantification of quercetin and kaempferol. The method demonstrated acceptable specificity, precision, repeatability, robustness, and system suitability, confirming its reliability for routine analytical application and regulatory-oriented evaluation of herbal raw materials and formulations.

Quantitative analysis enabled consistent and reproducible estimation of both flavonoid markers across different sample matrices, highlighting the necessity of plant- and matrix-specific method validation rather than reliance on generic analytical procedures. Such validated marker-based quantification is a fundamental requirement for ensuring batch-to-batch consistency, supporting quality assurance, and facilitating the standardization of *P. quadrangularis* in phytopharmaceutical research.

In addition to analytical validation, the study incorporated a preliminary biological assessment using an MTT-based cytotoxicity assay to provide supportive functional relevance for the selected markers. Kaempferol exhibited greater antiproliferative activity against MCF-7 breast cancer cells compared with quercetin, while both flavonoids

demonstrated relatively lower toxicity toward normal human endothelial cells, resulting in favorable selectivity indices. These observations indicate differential cellular sensitivity under the experimental conditions employed; however, they should be interpreted as preliminary and supportive rather than definitive evidence of anticancer efficacy.

Collectively, the integration of validated analytical methodology with selectivity-oriented cytotoxicity screening enhances the translational relevance of this work within its defined scope. The findings align with current regulatory and scientific expectations for phytopharmaceutical research, where analytical rigor, reproducibility, and cautious biological interpretation are essential. The validated RP-HPLC method offers a practical tool for quality assurance, while the preliminary biological findings provide a rationale for future investigations. Further mechanistic in vitro studies, expanded biological models, and in vivo validation will be required to substantiate and extend the pharmacological implications of *P. quadrangularis*–derived flavonoids.

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Declarations

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