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# A Concurrent Estimation of Phytoconstituents Piperine, Quercetin and Kaempferol Present in Siddha Polyherbal Formulation Kabasara Kudineer by RP-HPLC

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***Abstract***

*A straightforward, accurate, and precise technique was developed and validated for the simultaneous determination of Quercetin, Kaempferol, and Piperine in the polyherbal formulation Kabasura Kudineer. The sample was chromatographed using a C18 column (150x4.6 mm, 5µ) with a mobile phase composed of Acetonitrile and Ortho-phosphoric acid in a 45:55 ratio, flowing through the column at a rate of 1ml/min. The detection wavelength for Quercetin, Kaempferol, and Piperine was set at 254 nm. The retention times for Quercetin, Kaempferol, and Piperine were found to be 3.2 mins, 4.4 mins, and 13.4 mins, respectively. The precision, as indicated by the % RSD for Quercetin, Kaempferol, and Piperine, was found to be within acceptable limits. The LOD and LOQ values for Quercetin, Kaempferol, and Piperine were also within acceptable limits. The correlation coefficient for the three phytoconstituents was calculated to be 0.999.*

**Keywords:** Quercetin, Kaempferol , Piperine, RP-HPLC, Kabasura Kudineer.

## INTRODUCTION

Alternative medicine systems, including homeopathy, naturopathy, yoga, unani, siddha, and ayurveda, have authentically coexisted with allopathy. The assessment and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic study data are significantly influenced by the use of bioanalytical method validation (BMV), which is used for the quantitative determination of pharmaceuticals and their metabolites in biological fluids. In general, regulatory filings are supported by these studies. The quality of the underlying bioanalytical data directly affects the importance of these investigations.

Chaudhari *et al.,* developed a sensitive, selective, accurate, and precise reverse-phase HPLC method for simultaneous analysis of quercetin and piperine in Nanostructured Lipid Carriers (NLCs) [1]. The chromatographic separation was achieved using a Hypersil gold C-18 column and a mobile phase consisting of acetonitrile and HPLC-grade water (pH 2.6 with 2%v/v glacial acetic acid) in isocratic elution mode. The method exhibited specificity for simultaneous analysis in the presence of NLCs matrix, high accuracy (>90%), and precision (%RSD < 2%). The validated method was applied to determine drug entrapment efficiency and drug loading in NLCs formulations, demonstrating reliability for in vitro cumulative drug release studies [2].

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Jain *et, al.,* developed a simple, accurate, and robust RP-HPLC method for simultaneous estimation of ferulic acid, quercetin, piperine, and thymol in a marketed Ayurvedic formulation.[3] Utilizing a shim pack GIST C-18 column and a mobile phase of acetonitrile: 0.04 M potassium dihydrogen ortho-phosphate buffer (pH 3.0), the

method demonstrated good linearity, precision, accuracy, and robustness. The method, validated according to ICH Q2 (R1) guidelines, was successfully applied to quantify these markers in a marketed Ayurvedic formulation, making it suitable for quality and safety assessments [4-7].

Quercetin, a polyphenolic flavonoid, exhibits potential chemopreventive properties. Although the precise mechanism of action remains incompletely understood, various in vitro effects have been observed, including reduced expression of mutant p53 protein and p21-ras oncogene, initiation of cell cycle arrest at the G1 phase, and inhibition of heat shock protein synthesis. Additionally, quercetin demonstrates synergy and reversal of multidrug resistance when combined with chemotherapeutic drugs in vitro. Quercetin, as a flavonoid abundantly found in numerous foods and herbs, is a common component of a regular diet. It serves various roles, including antibacterial agent, antioxidant, protein kinase inhibitor, antineoplastic agent, inhibitor of EC 1.10.99.2 (ribosyldihydronicotinamide dehydrogenase - quinone), plant metabolite, phytoestrogen, radical scavenger, chelator, Aurora kinase inhibitor, and geroprotector. Characterized as a pentahydroxyflavone and a 7-hydroxyflavonol, quercetin exists as the conjugate acid of quercetin-7-olate.

Kaempferol, classified as a tetrahydroxyflavone due to the arrangement of its four hydroxy groups at positions 3, 5, 7, and 4', serves as an antioxidant, actively mitigating oxidative stress. Belonging to the flavonols group, it is characterized as a 7-hydroxyflavonol and a tetrahydroxyflavone. As a conjugate acid, it forms a kaempferol oxoanion. Kaempferol, a naturally occurring flavonoid, has been successfully isolated from various plant sources such as Delphinium, Witch-hazel, grapefruit, and others. Presenting as a yellow crystalline solid with a melting point ranging from 276 to 278 degrees Celsius, it exhibits slight solubility in water while being readily soluble in hot ethanol and diethyl ether. Widely distributed in nature, kaempferol is a natural product identified in organisms like Lotus ucrainicus, Visnea mocanera, and other sources for which relevant data is available.

Piperine is an N-acylpiperidine, featuring piperidine substitution by a (1E,3E)-1-(1,3-benzodioxol-5-yl)-5-oxopenta-1,3-dien-5-yl group at the nitrogen atom. This alkaloid is derived from the plant Piper nigrum and is recognized for its role as an NF-kappaB inhibitor, serving as a plant metabolite, a component in food, and a metabolite found in human blood serum. Classified as a member of benzodioxoles, N-acylpiperidine, piperidine alkaloid, and tertiary carboxamide, piperine is functionally related to an (E,E)-piperic acid. Piperine occurs naturally in Macropiper, Piper boehmeriifolium, and various other organisms for which relevant data is available. Bioperine, a formulation containing piperine, has been studied in clinical trials exploring its potential in the treatment of Multiple Myeloma and Deglutition Disorders.

To develop the RP-HPLC method for the simultaneous determination of Quercetin, Kempferol and Piperine present in Nilaveembu Kudineer. The objective of study was not limited to determine the solubility and compatibility studies, development of suitable analytical methods, validate the developed analytical methods as per ICH guidelines of selected phytochemicals but also focused the application of developed HPLC methods for analysis of polyherbal formulation.

## MATERIALS AND METHODS

### Chemical and Reagents

All the Phytochemical Markers (Standards) were procured from Yucca Enterprises Mumbai, India. Polyherbal Formulation was purchased from Siddha Pharmacy, Tirupati. Silica Gel GF 254 TLC Plates were procured from local suppliers in Tirupati. All other reagents and chemicals had been purchased from Merck (Darmstadt, Germany) and were of analytical or HPLC grade. Aqua Max - Basic and Aqua Max - Ultra ultrapure water filtration systems (Young Lin, Korea) were used to purify the distilled water. When necessary, distilled water was used to clean all glassware, which was subsequently cleaned with acetic anhydride, acetone, and hot air oven drying. Prior to use, solvents were filtered and degassed.

### Instruments and Equipments

High Performance Liquid Chromatography (HPLC-UV) Detector (254 nm), Shimadzu system equipped with a quaternary low-pressure gradient solvent delivery HPLC pump equipped with vacuum degasser, SDV 50 A valve module, CTS 30 column oven, YL9150 alias auto-sampler with a variable injection and SP 930 D solvent delivery pump. The output signal was monitored and processed using Lab solution software and isocratic elution with flow rate of 1.0 mL min−1 was performed on $C_{18}$ Chromatopak (150mm×4.6×5micron) with injection volume of 10 µL. The run time was set to 20 min and column temperature was 40 °C. The column was equilibrated with mobile phase for 30–40 min prior to sample injection. The UV spectra were measured using a Lambda 25 PerkinElmer spectrophotometer at 254 nm.

**Extraction of Quercetin**

The plant materials underwent air-drying at room temperature (26°C) for a period of two weeks, followed by grinding to achieve a uniform powder [8, 9]. The extraction of leaf samples was carried out sequentially using a systematic approach. One hundred grams of the dried powder were subjected to extraction in 900 ml of various solvents (hexane, ethyl acetate, and ethanol) for 48 hours [10]. Subsequent to each extraction, the plant extract underwent filtration through Whatman filter paper. The resulting filtrate was concentrated under reduced pressure in a vacuum at 40°C for 25 minutes using a rotary evaporator [11].

**Extraction of Kaempferol**

This extraction process was iterated four times on the same leaf sample, utilizing a total of 8 L of absolute ethanol. The resulting extract was then filtered and concentrated under vacuum at 60°C until dryness using a rotary evaporator. Subsequently, the concentrated extract was distributed into 1 L of hot water, followed by the addition of chloroform. For each 100 mL of the distributed water, shaking with 30 mL of chloroform was repeated until the chloroform layer showed no color. The resulting acidic aqueous solution was shaken with ethyl acetate (EtoAc), and each round of shaking involved 30 mL of EtoAc with the collection of the acidic water layer. This process was reiterated until the yellow color in the EtoAc layer disappeared. The acidic aqueous layer underwent hydrolysis through a reflux method with 20% HCl at a pH of 1.3-1.4 for four hours, followed by a one-hour rest period. This layer was concentrated under vacuum at 40°C until dryness using an evaporator.

**Extraction of Piperine**

Cold maceration extraction was performed on 25 g of Kabasara kudineer using 300 mL of glacial acetic acid. The resulting extract was diluted with an equivalent volume of water and subjected to partitioning with chloroform in a separating funnel. Following these steps, the extract was concentrated using a Rota evaporator and then dried using anhydrous sodium sulfate. To further purify the extract, recrystallization was carried out using diethyl ether [12-14].

**Preparation of Mobile Phase Solution**

The mobile phase consists of isocratic elution with a low-pressure gradient using double-distilled HPLC grade ACN: OPA (A: B): (45:55)**.** All solutions were degassed and filtered through 0.2 µm pore size filter. Accurately weigh 1000 ml of methanol and HPLC water and they are degassed in an ultrasonic water bath and sonicated for 15 min to remove any entrapped air bubbles and mix thoroughly [15].

**Preparation of Sample Solution**

For preparation of sample solution, the crude plant material from the KABASARA KUDINEER CHOORNAM is taken and required plant material is isolated by the above manner.

**Preparation of Standard Solution**

Accurately weigh 1mg of standard andrographolide and genistein and dissolve in a 10 ml volumetric flask with a suitable diluent. Methanol showed good solubility with both drugs [16].

## RESULTS AND DISCUSSION

### Wavelength Selection

Selection of an appropriate detection wavelength is necessary in order to detect all substances simultaneously and obtaining low LODs. Thus, three separate solutions of quercetin, kaempferol, piperine was prepared and absorbance was measured over the wavelength range of 200-400 using UV-spectrophotometer. An overlaid spectrum shown most of the components demonstrating lambda max at 254 nm. UV Spectrum of 100 µgm/ml of Andrographolide and 100 µgm /ml of Genistein is recorded by scanning in the range of 200 to 800 nm From the UV Spectrum Wavelength selected is 220 nm.

### Method Validation

After method development, its validation was performed as per ICH and USP guidelines in terms of range, precision, accuracy, specificity, LOD, LOQ, robustness, ruggedness and system suitability. In this study, the system suitability parameters for the analysis of phytochemical compounds, including quercetin, kaempferol, and piperine, were evaluated (Table 1).

**Table 1.** System suitability parameters for phytochemical compounds.

| Parameters | Quercetin | Kaempferol | Piperine |
|---|---|---|---|
| Rt | 3.133 | 4.258 | 13.350 |
| Theoretical plates | 1401 | 3293 | 7459 |
| Resolution | - | 1.912 | 11.243 |
| Tailing factor | 1.257 | 1.211 | 1.152 |

The interval between the peak and lower concentration of analyte in the sample is known as the range of an analytical method. Five combinations of the working standards encompassing the range of 70%–130% of the target concentration were used to assess the method range. After filtering the solutions using 0.45 µm membrane filters, each dilution was injected into the chromatographic apparatus in triplicate, and the response area was noted. Peak area was plotted against concentration to create calibration curves, and regression equations were then calculated.

The degree of agreement between individual results obtained by repeatedly applying the process to multiple samplings of a homogenous sample is known as precision. Six replicate injections of a prepared sample with concentrations of 25, 50, 75, 100, 150, 175, 200 µg $mL^{-1}$ for K, Q and P respectively, were used to determine the inter-day precision of the procedure and were analyzed on the same day(Fig.1). The precision study indicated consistent retention times with low standard deviations, ensuring the reliability and repeatability of the analytical method (Table 2).

**Table 2.** Precision study for Phytocompounds.

| Precision | Quercetin | Kaempferol | Piperine |
|---|---|---|---|
| | ***Rt*** | ***Rt*** | ***Rt*** |
| 1 | 3.105 | 4.324 | 6.18 |
| 2 | 3.143 | 4.417 | 6.33 |
| 3 | 3.275 | 4.320 | 6.34 |
| 4 | 3.195 | 4.355 | 6.34 |
| 5 | 3.106 | 4.367 | 6.34 |
| 6 | 3.296 | 4.372 | 6.34 |
| Mean | 3.186667 | 4.3405 | 13.3435 |
| SD | 0.076277 | 0.011369 | 0.01163 |
| % RSD | 0.023936 | 0.261924 | 0.000872 |

The percentage relative standard deviation (%RSD) for the selected analytes was discovered to be 0.02, 0.26, and 0.0008%, in that order.

Linearity data demonstrated a high correlation ($R^2 > 0.998$) between concentration and peak area for each compound, supported by corresponding linearity graphs (Figures 2) (Table .3).

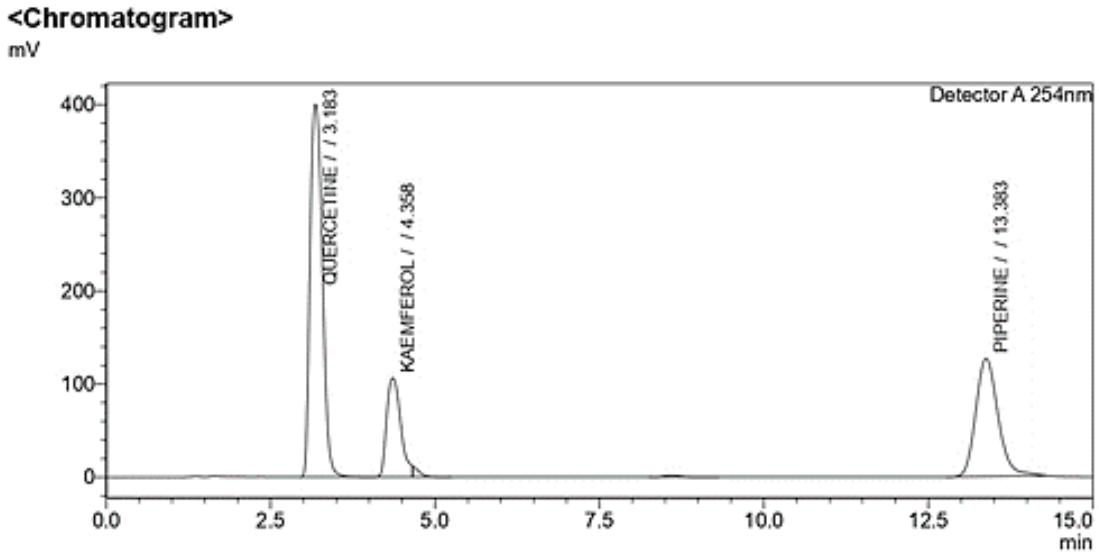
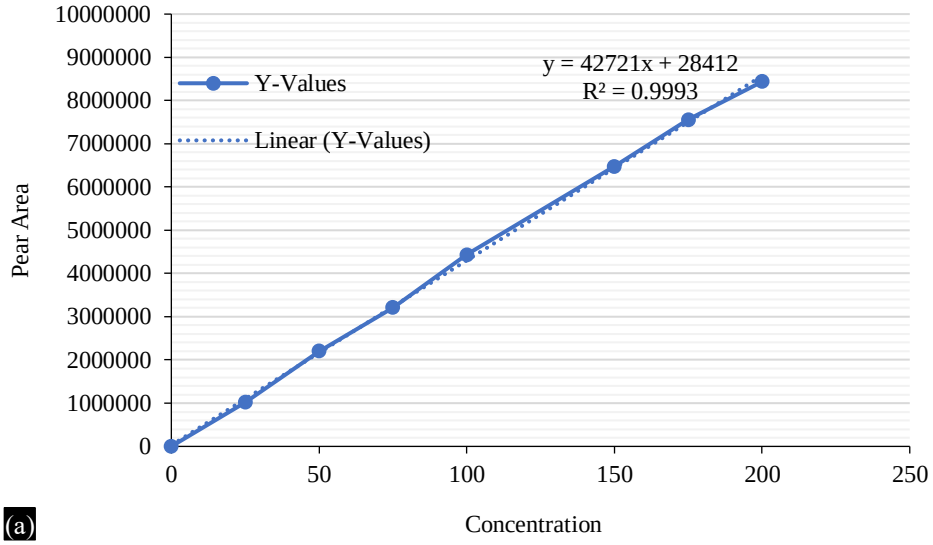



**Figure 1.** Precision chromatogram.

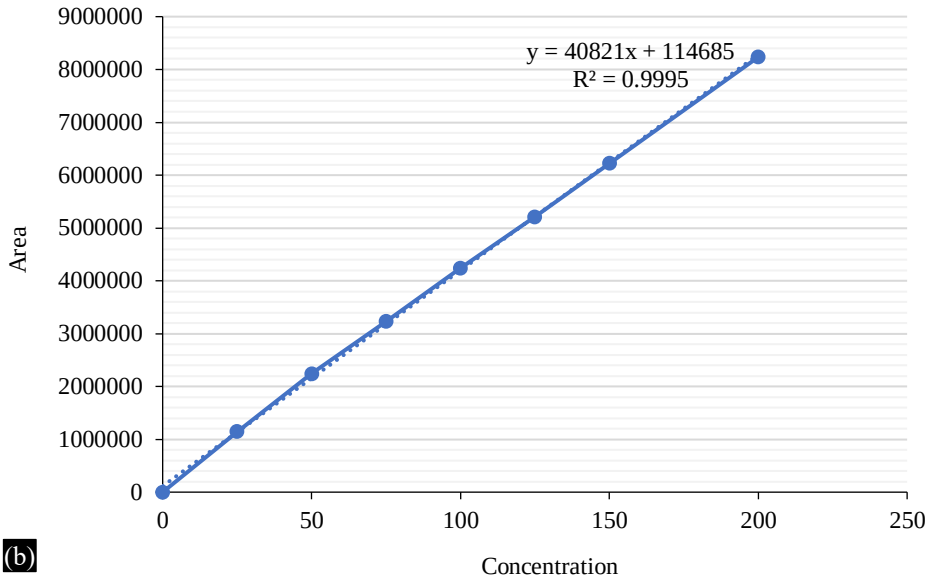
**Table 3.** Linearity Data for Phytochemicals.

| Concentration (µg/ml) | Peak Area *Quercetin* | Peak Area *Kaempferol* | Peak Area *Piperine* |
|---|---|---|---|
| 25 | 1015901 | 1142125 | 1005552 |
| 50 | 2204121 | 2239074 | 1936556 |
| 75 | 3209088 | 3228163 | 2820895 |
| 100 | 4433253 | 4237252 | 3700844 |
| 150 | 6479256 | 5212340 | 5197548 |
| 175 | 7558418 | 6219401 | 6008779 |
| 200 | 8436074 | 8234561 | 6920891 |

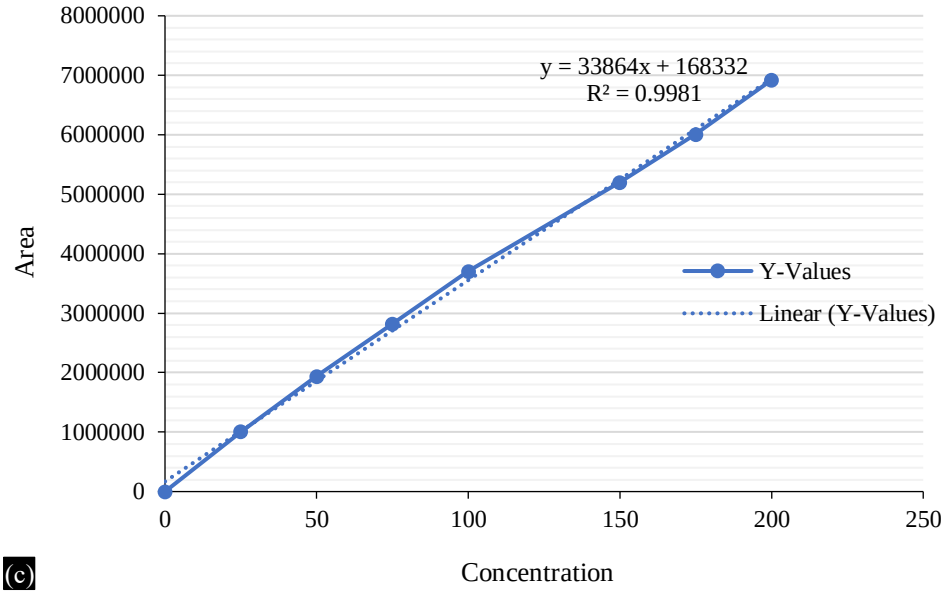



(a)




(b)




(c)

**Figure 2.** Linearity of (a) Piperine, (b) Kaempferol, (c) Quercetin.

The robustness is the ability to remain unaffected by small but deliberate variations in method. The robustness of the proposed method was validated by changing flow rate and column oven temperature parameters in the range of 70–130% of label claimed.

Robustness testing revealed that deliberate changes in column temperature and flow rate had minimal impact on the chromatographic parameters, confirming the method's robustness (Table 4) (Fig.3).

The method's accuracy was assessed through a recovery study conducted by ICH guidelines. The sample solution was mixed with a known concentration of standard solution which was equal to 50, 100, and 150% of the label claimed. Three injections of the sample solution at these concentrations were used to determine the accuracy, and the findings were presented as in Table 5.

## DISCUSSION

HPLC chromatograms from the standard solutions exhibited effective peak separation with resolution values exceeding 2. The within-day and between-day percentage relative standard deviations (% RSD)

for five repeated injections were consistently below 2%. The calibration curve, derived from plotting peak areas at various concentrations, demonstrated excellent linearity (correlation coefficient $r^2 > 0.99$). Accuracy, assessed through percentage recovery of spiked standards, consistently fell within the range of 100% to 150%. Other validation parameters, including the limit of quantitation (LOQ) and limit of detection (LOD), as well as robustness, were also determined and presented.

**Table 4.** Results of robustness study.

| Parameters | Deliberate Changes | Quercetin | Kaempferol | Zingerone |
|---|---|---|---|---|
| Column Temperature (-) | 39°C | 3.1 | 4.217 | 12.992 |
| Column Temperature (+) | 41°C | 3.125 | 4.242 | 12.925 |
| AVG | | 3.1125 | 4.2295 | 12.9585 |
| SD | | 0.0125 | 0.0125 | 0.0335 |
| RSD | | 0.401606 | 0.295543 | 0.258518 |
| Flow Rate (-) | 0.9 ml | 3.492 | 4.398 | 12.998 |
| Flow Rate (+) | 1.1 ml | 3.459 | 4.348 | 12.98 |
| AVG | | 3.4755 | 4.373 | 12.989 |
| SD | | 0.0165 | 0.025 | 0.009 |
| RSD | | 0.474752 | 0.57169 | 0.069289 |

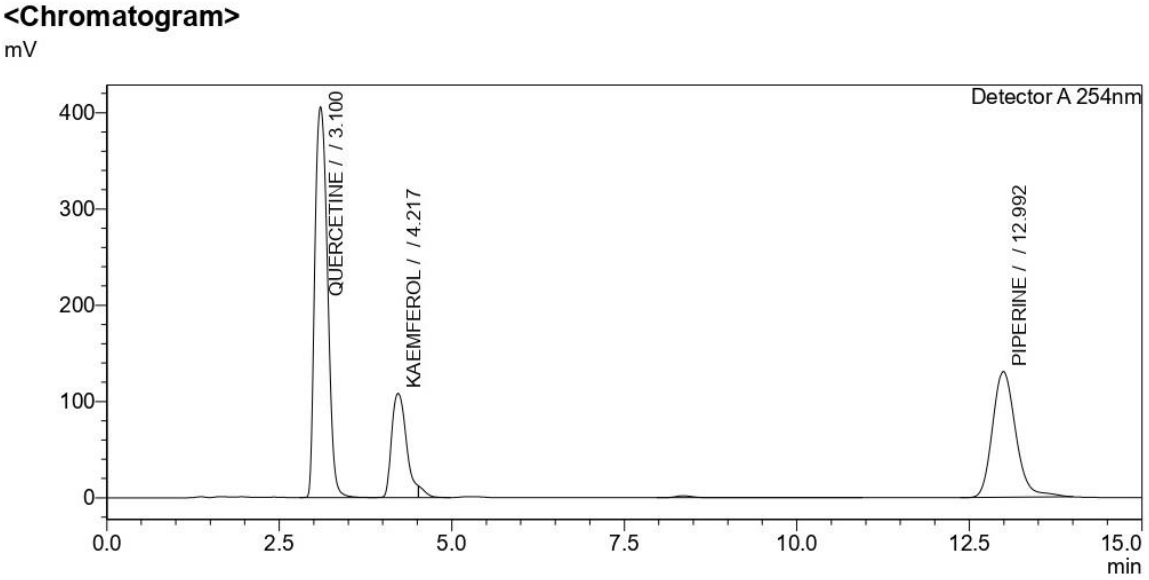



**Figure 3.** Robustness Chromatogram.

**Table 5.** Accuracy data of phytocompounds.

| Parameters (ppm) | % Recovery | | |
|---|---|---|---|
| | *Quercetin* | *Kaempferol* | *Piperine* |
| 50 | 100.96 | 99.17 | 99.69 |
| 100 | 100.83 | 100.67 | 100.76 |
| 150 | 102.39 | 102.28 | 101.33 |
| Average | 101.3933 | 100.7067 | 100.5933 |
| %RSD | 0.86 | 1.55 | 0.83 |

The retention time (Rt), theoretical plates, resolution, and tailing factor were determined for each compound, providing essential information about the chromatographic separation. The chromatograms of individual compounds and their mixtures illustrate the separation efficiency and peak characteristics. Additionally, accuracy data showed satisfactory percent recoveries, indicating the reliability of the

method for the quantification of quercetin, kaempferol, and piperine in samples. Overall, these comprehensive results validate the analytical method's suitability for the precise and accurate determination of these phytochemical compounds in various samples.

The comprehensive validation results indicated the reliability of this HPLC method, making it suitable for the analysis of the five compounds. This developed method was subsequently employed to quantify piperine, quercetin, and kaempferol in ethanol extracts of Kabasura Kudineer (KK). These chemical markers play a crucial role in quality control, aiding in the standardization of the herbal extract and formulation. The study particularly emphasized the neuroprotective effects associated with piperine, quercetin, and kaempferol.

Piperine, a prominent constituent from Piper nigrum, has demonstrated cognitive improvement and protective effects on dopaminergic neurons, indicating potential benefits in Parkinson's disease models. Additionally, quercetin and kaempferol from Nelumbo nucifera exhibit antidepressant and cardioprotective effects. Quercetin, in particular, has shown efficacy in alleviating oxidative stress, tauopathy, and astrogliosis.

The HPLC method developed in this study allows simultaneous analysis of these three key compounds in KK, offering an advantage over previous methods. This enhanced method contributes to the consistent and reliable quantification of active compounds, ensuring the quality and potential therapeutic activity of KK across different production batches.

## CONCLUSION

An HPLC method, demonstrating reliability and accuracy, has been formulated and validated for the quantification of three key compounds—piperine, kaempferol, and quercetin—in the KK formula. This method is deemed appropriate for routine quality control assessments of the KK formula. The analytical conditions employed ensure effective resolution of the three active compounds. Following validation in line with ICH guidelines, the method successfully met all crucial parameters, including robustness. These findings are anticipated to serve as valuable quality control framework for standardizing the KK formula.

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