Development And Validation of a Rapid RP-HPLC Method For Simultaneous Quantification of Five Phytoestrogens in Pueraria Tuberosa Root Extract

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Abstract
A rapid and validated RP-HPLC method was developed for the simultaneous quantification of five key phytoestrogens puerarin, genistein, daidzein, biochanin-A, and quercetin in Pueraria tuberosa root extract. Chromatographic separation was achieved within 12 minutes using a C18 column with gradient elution of 0.1% orthophosphoric acid in water and acetonitrile at a flow rate of 2.0 mL/min. Detection was performed at 205 nm with an injection volume of 10 µL. The method was validated according to regulatory guidelines, evaluating system suitability, precision, accuracy, specificity, linearity, limit of detection (LOD), and limit of quantification (LOQ). Excellent linearity ($r^2 > 0.999$) and high precision (RSD < 2%) were observed for all analytes. The novelty of this study lies in the development of a rapid and validated method for simultaneous estimation of five phytoestrogens within a short analytical run time. The method is simple, sensitive, and reliable, making it suitable for quality control, standardization, and routine analysis of P. tuberosa and its herbal formulations.

Keywords: Pueraria tuberosa, RP-HPLC, Method validation, Quality control, Standardization, phytoestrogens

1. Introduction

Seed purity, defined as the proportion of seeds that accu-rately represent the declared cultivar,
For ages, traditional medical systems have been essential to healthcare, especially in less fortunate countries like India where plant-based treatments are widely used to prevent and treat a wide range of illnesses. The tremendous structural variety and biological activity of natural compounds obtained from medicinal plants make them a valuable source of therapeutic medicines [1]. The World Health Organization (WHO) reports that over 80% of the world's population receives basic healthcare from traditional medicine, which mostly uses preparations derived from plants. Numerous bioactive substances found in medicinal plants contribute to their therapeutic qualities, and their potential to treat infectious and chronic illnesses including cancer, diabetes, and cardiovascular conditions has been extensively studied [2]. Interest in natural products as substitute sources of bioactive chemicals with better safety profiles has increased due to rising concerns about the side effects of synthetic medications and the rise of antimicrobial resistance.
Numerous secondary metabolites, including as alkaloids, flavonoids, phenolics, tannins, glycosides, steroids, and volatile oils, are known to be abundant in plants and to be responsible for a variety of pharmacological actions. Numerous biological characteristics, including antibacterial, antioxidant, anti-inflammatory, anticancer, and antidiabetic activities, have been shown by these phytochemicals. Thus, the investigation of bioactive chemicals produced from plants has emerged as a crucial field of study for both the identification of new therapeutic agents and the verification of the conventional claims made about herbal remedies [3]. Understanding the chemical makeup and pharmacological potential of medicinal plants depends heavily on scientific studies including the extraction, isolation, and characterisation of phytoconstituents. Phytochemicals found in plant-based medications are often identified and characterised using sophisticated analytical techniques including mass spectrometry and chromatography [4][5].
P. tuberosa (Willd.) DC., sometimes referred to as Vidarikand or Indian Kudzu, is a perennial woody climber that is found across India and is a member of the Fabaceae family. The plant is considered as a significant medicinal species in the Ayurvedic Pharmacopoeia of India and is acknowledged in ancient medical systems like Ayurveda and Siddha. Traditionally, P. tuberosa's tuberous roots have been used as a tonic, demulcent, refrigerant, aphrodisiac, and galactagogue to treat a variety of conditions, such as rheumatism, bronchitis, fever, inflammation, and urinary diseases [6]. The tubers are also used in traditional medicine to boost overall vigour, cleanse blood, and increase fertility. Numerous biological activities of P. tuberosa, such as antihyperglycemic, antihyperlipidemic, hepatoprotective, and anti-implantation properties, have been described by pharmacological research. The presence of bioactive phytoconstituents such isoflavonoids and phytoestrogens, such as puerarin, daidzein, genistein, puetuberosanol, and tuberosin, is primarily responsible for this plant's medicinal potential [7].
Because of their many pharmacological characteristics and capacity to function as phytoestrogens, which mimic the biological action of oestrogen, isoflavonoids found in Pueraria species have garnered significant scientific attention. Puerarin is regarded as one of these compounds' main active ingredients and has been shown to have a variety of therapeutic benefits, including neuroprotective, antidiabetic, cardioprotective, and antioxidant properties. The prospective uses of these phytoestrogenic substances in the treatment of menopausal symptoms, cardiovascular illnesses, and metabolic disorders are also being thoroughly studied. As a result, Pueraria species have drawn more interest in the creation of functional foods, herbal remedies, and nutraceuticals that include bioactive isoflavonoids [8].

Despite the enormous therapeutic value of medicinal plants, it is still very difficult to guarantee the quality, safety, and effectiveness of herbal materials because of differences in harvesting techniques, environmental factors, geographic origin, and processing techniques. Therefore, in order to ensure the identification, purity, and consistency of medicinal plants in herbal compositions, standardisation is crucial. Standardisation entails the discovery and measurement of distinctive bioactive marker molecules, which function as trustworthy markers for herbal material quality monitoring. Phytoconstituents found in medicinal plants are often characterised and quantitatively determined using contemporary analytical techniques like Fourier transform infrared spectroscopy (FTIR), liquid chromatography-mass spectrometry (LCMS), and high-performance liquid chromatography (HPLC) [9] [10].

2. Material and methods

2.1 Plant Material and extraction

The tuberous roots of P. tuberosa (Willd.) DC. were collected from Ghotavade village, Pune, India. The plant material was authenticated at the Agharkar Research Institute (ARI), Pune, and a voucher specimen was deposited under the authentication number AUTH 24-21. The collected tubers were cleaned to remove adhering soil and other impurities, shade-dried at room temperature, and pulverized into a fine powder for further experimental analysis.

Finely powdered root material (50 g) was extracted with 400 mL ethanol under magnetic stirring (200 rpm) for 20 h at room temperature. The extract was filtered through Whatman No. 40 filter paper and concentrated using a rotary evaporator at 40 °C under reduced pressure to obtain a crude extract, which was stored at low temperature until quantitative analysis.

2.2 Chemicals and Reagents

All solvents and reagents used in the study were of HPLC grade. O-phosphoric acid (OPA) and acetonitrile (ACN) were procured from Merck Specialities Pvt. Ltd., Mumbai, India. Ultrapure water was obtained from a Milli-Q water purification system (Milli-Q EQ 7000, Q-POD with Millipak 0.22 µm filter, Merck Millipore, Germany) and used throughout the study for preparation of mobile phases and solutions.

2.3 Reference Standards

The reference standards of puerarin, daidzein, genistein, and biochanin-A were purchased from TCI Chemicals (Tokyo, Japan). Quercetin standard was procured from Sigma-Aldrich (St. Louis, MO, USA). All reference standards were of analytical grade and were used for preparation of calibration solutions and method validation studies.

2.4 Instrumentation and chromatographic condition:

Chromatographic analysis was carried out using a Shimadzu LC-2050 High Performance Liquid Chromatography (HPLC) system equipped with a photodiode array (PDA) detector. The chromatographic separation was performed using different reversed-phase columns during method development, including GL Sciences Inertsil C18 (250 × 4.6 mm, 5 µm), YMC J-Sphere (150 × 4.6 mm, 4 µm), and YMC-Pack ODS-AM (150 × 6 mm, 5 µm). Data acquisition and processing were performed using the LabSolutions software provided with the Shimadzu HPLC system.

Chromatographic separation of the selected phytoestrogens was achieved on a YMC-Pack ODS-AM C18 column (150 × 6 mm, 5 µm) using a binary gradient elution system. The mobile phase consisted of 0.1% O-phosphoric acid in water (Solvent A) and acetonitrile (Solvent B). The flow rate was maintained at 2.0 mL min^{-1}, and detection was carried out at a wavelength of 205 nm using a PDA detector. The total run time was 12 minutes, which allowed efficient separation of the target phytoconstituents including puerarin, daidzein, genistein, quercetin, and biochanin-A.

2.5 Preparation of Standard Stock Solutions

Standard stock solutions of the reference compounds puerarin, daidzein, quercetin, genistein, and biochanin-A were prepared individually in ethanol. The standards were prepared at concentrations of 155 µg mL^{-1} for puerarin, 65 µg mL^{-1} for daidzein, 50 µg mL^{-1} for quercetin, 55 µg mL^{-1} for genistein, and 60 µg mL^{-1} for biochanin-A. All solutions were mixed thoroughly to ensure complete dissolution and were used for further HPLC analysis.

2.6 Preparation of Sample Solution

The fine powder of P. tuberosa root was accurately weighed and dissolved in ethanol to prepare a 40000-ppm sample solution. The mixture was stirred properly to ensure complete dissolution.

The volumetric flask containing the solution was then placed in an ultrasonic bath for 60 min to facilitate efficient extraction. An ultrasonicator equipped with a chiller system was used to maintain the temperature at ambient conditions during the sonication process. After sonication, the extract was subjected to centrifugation using an Eppendorf centrifuge 5910 Ri at 5000 rpm for 15 min. The clear supernatant obtained was carefully collected and filtered through a 0.22 µm nylon syringe filter (Ecotest) before being used for HPLC analysis.

2.7 Method validation parameters

The developed analytical method was validated according to established analytical validation guidelines to ensure its suitability for the quantitative determination of the selected phytochemicals. The validation parameters evaluated included system suitability, specificity, precision, limit of detection (LOD), limit of quantification (LOQ), linearity, and accuracy.

2.7.1. System Suitability: System suitability tests were performed to verify the performance and reliability of the chromatographic system prior to sample analysis. The evaluation was carried out by injecting the standard solution six

times under the optimized chromatographic conditions. System performance was assessed by examining parameters such as percentage relative standard deviation (%RSD) of peak areas, peak asymmetry (tailing factor), and chromatographic resolution between adjacent peaks. The acceptance criteria were defined as %RSD of replicate injections not exceeding 2.0%, peak asymmetry not greater than 2.0, and chromatographic resolution not less than 2.0.

2.7.2. Specificity: The specificity of the method was evaluated to confirm that the analytes could be accurately detected and quantified in the presence of other components in the sample matrix. Blank solutions, individual standard solutions, and spiked sample solutions were analyzed under the optimized chromatographic conditions. The obtained chromatograms were examined to verify the absence of interfering peaks at the retention times corresponding to the analytes. Peak purity analysis was also performed to confirm the homogeneity of the analyte peaks and to ensure that no co-eluting components were present.

2.7.3. Precision: The precision of the analytical method was evaluated at three different levels: system precision, method precision, and intermediate precision.

System Precision: System precision was determined by performing replicate injections of the standard solution under the same chromatographic conditions. The consistency of the chromatographic system was evaluated based on the variation in the detector response obtained from these injections.

Method Precision: Method precision (repeatability) was assessed by preparing multiple independent sample preparations according to the developed analytical procedure and analyzing them under identical experimental conditions. The variability among the obtained results was used to evaluate the repeatability of the analytical method.

Intermediate Precision: Intermediate precision was evaluated to determine the reproducibility of the method under normal laboratory conditions. The analysis was performed by different analysts and/or on different days using the same chromatographic conditions, and the variability in the analytical results was examined.

2.7.4. Determination of Limit of Detection (LOD) and Limit of Quantification (LOQ): The limit of detection (LOD) was determined using the visual detection method, which involves analyzing progressively diluted solutions of the analytes to identify the lowest concentration that can be reliably detected by the chromatographic system. The limit of quantification (LOQ) was established as a concentration corresponding to three times the LOD value, ensuring reliable quantification with acceptable precision.

2.7.5. LOD and LOQ Precision: The precision at the LOD and LOQ levels was evaluated by performing replicate injections of solutions prepared at these concentrations. The repeatability of the detector response at these low concentration levels was assessed to confirm the reliability of the analytical method near the detection and quantification limits.

2.7.6. Linearity: Linearity of the method was evaluated by preparing a series of standard solutions covering a concentration range corresponding to 25% to 200% of the standard concentration. Each concentration level was analyzed under the optimized chromatographic conditions. Calibration curves were constructed by plotting the peak area against the corresponding analyte concentration, and linear regression analysis was used to assess the relationship between detector response and analyte concentration.

2.7.7. Accuracy: The accuracy of the developed analytical method was evaluated through recovery studies performed at different concentration levels ranging from the LOQ level to 150% of the standard concentration. Known amounts of the standard compounds were spiked into the sample matrix and analyzed using the developed chromatographic procedure. The percentage recovery of the analytes was calculated to assess the accuracy of the method.

2.7.8. Precision at Different Concentration Levels: The precision of the method across the working concentration range was further evaluated by analyzing samples prepared at 50%, 100%, and 150% of the standard concentration. Replicate analyses were performed at each concentration level under identical experimental conditions to assess the repeatability of the method at different concentration levels.

Table 1. Analytical parameters of phytoestrogens

Phyto-estrogen	Retention time (min)	Regression equation (n=8)	Regression coefficient (R^2)	Calibration range, µg/mL	RSD, %	LOD, µg/mL	Peak purity
Puerarin	2.766	y = 14750x - 24201	0.9996	38.63-309.04	1.41	0.44	1.0
Diadezein	6.072	y = 30596x - 16532	0.9999	16.32-130.56	1.08	0.13	1.0
Quercetin	6.439	y = 36518x - 7914.6	0.9999	12.72-101.76	1.10	0.10	1.0
Genistein	7.321	y = 31823x - 11640	0.9999	14.08-112.64	1.13	0.11	1.0
Biochanin-A	7.985	y = 28776x - 3394.2	0.9999	15.28-122.24	0.60	0.12	0.999

Table 2. Recovery study of phytoestrogens

Recovery level	Phyto-estrogen	Concentration (ppm)	Recovery, %
LOQ	Puerarin	1.35	94.66
	Diadezein	0.44	99.81
	Quercetin	0.35	97.03
	Genistein	0.37	100.14
	Biochanin-A	0.40	93.23
50%	Puerarin	77.26	100.91
	Diadezein	32.64	100.96
	Quercetin	25.44	101.14
	Genistein	28.16	100.83

	Biochanin-A	30.56	100.92
100%	Puerarin	154.52	98.96
	Diadezein	65.28	100.42
	Quercetin	50.88	100.30
	Genistein	56.32	100.33
	Biochanin-A	61.12	100.57
150%	Puerarin	231.78	98.38
	Diadezein	97.92	100.97
	Quercetin	76.32	100.52
	Genistein	84.48	100.46
	Biochanin-A	91.68	100.63

3. Results and discussion

3.1Optimization of RP-HPLC Method

To obtain effective separation and satisfactory peak symmetry for five phytoestrogens puerarin, daidzein, quercetin, genistein, and biochanin-A chromatographic settings were methodically optimised. A number of experiments were carried out by altering the gradient program, column chemistry, and mobile phase composition.

First, a gradient system of 0.1% orthophosphoric acid (OPA) and acetonitrile was used to try separation on a C18 column. In these circumstances, the remaining analytes displayed moderate separation, although Puerarin eluted extremely early with poor retention and an undesirable peak shape.

Puerarin's retention and peak symmetry were enhanced by substituting 0.1% formic acid for the aqueous phase in order to improve chromatographic behaviour and retention. Peak broadening and some baseline disruptions were still noted, though. Retention was somewhat enhanced by further altering the acidic environment using a mixture of OPA, acetonitrile, and glacial acetic acid; nevertheless, peak tailing and baseline instability remained.

A YMC J-Sphere column was then used to assess column chemistry, which resulted in better retention for puerarin; nevertheless, the peaks of quercetin and daidzein were tightly eluted with inadequate resolution.

A YMC-Pack ODS-AM column (150 × 6 mm, 5 µm) with a gradient mobile phase made of 0.1% OPA and acetonitrile was ultimately used to obtain the best chromatographic separation. With crisp, symmetrical peaks and a steady baseline, all five analytes were successfully resolved in a brief run time under these circumstances. In instance, close-eluting chemicals like quercetin and daidzein showed adequate resolution.

The UV spectra of the selected phytoestrogens were recorded using a PDA detector to determine the optimal detection wavelength. All analytes exhibited significant absorbance at 205 nm, which was selected as the detection wavelength to ensure adequate sensitivity and simultaneous detection of all compounds. The spectral analysis confirmed the suitability of the selected wavelength for accurate quantification. The spectra of individual component is showed in Figure 1.

As a result, the optimised chromatographic conditions were deemed appropriate for the quantitative measurement of phytoestrogens in P. tuberosa root extract and subsequent technique validation.

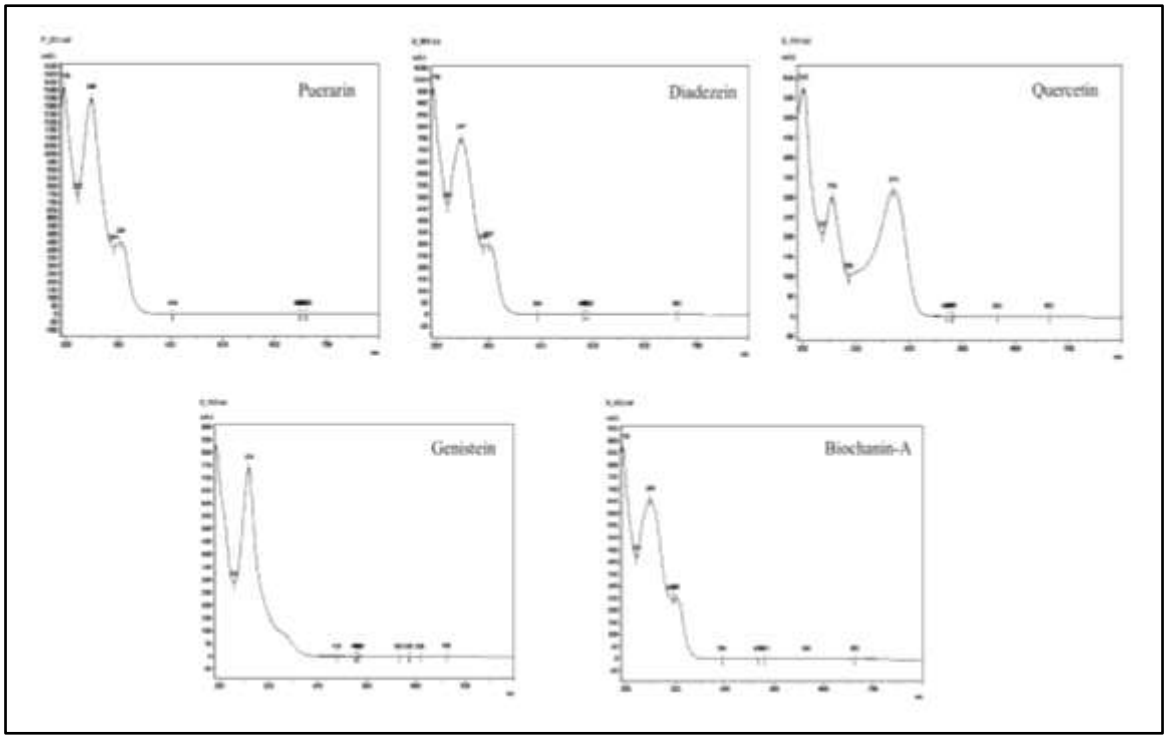


Figure 1. UV spectra of five phytoestrogens showing absorbance at 205 nm.

3.2 Validation of RP-HPLC method

The established RP-HPLC methodology for the concurrent quantification of phytoestrogenic compounds underwent

rigorous validation in accordance with the analytical validation principles articulated by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals guidelines. The validation investigation encompassed the assessment of system suitability, specificity, precision, linearity, limits of detection and quantification, and accuracy, all aimed at confirming the dependability and appropriateness of the proposed analytical technique [11].

Before the commencement of sample analysis, system suitability assessments were conducted to ascertain the optimal operation of the chromatographic system and the adequacy of the analytical conditions. Six replicate injections of the composite standard solution were subjected to analysis under optimized chromatographic conditions. The relative standard deviation (%RSD) of the peak areas for all analytes was determined to be below 2.0%, demonstrating exceptional repeatability of the chromatographic system. Moreover, the asymmetry coefficients associated with all peaks remained within the prescribed parameters (≤ 2.0), thereby validating the symmetrical morphology of the peaks and the efficacy of the chromatographic column. Satisfactory chromatographic resolution between contiguous peaks was attained, evidenced by resolution values exceeding 2.0, which illustrates that the established chromatographic parameters facilitated adequate separation of all intended analytes [12].

The specificity of the method was evaluated to ensure precise identification and quantification of target analytes amidst matrix components. Chromatographic profiles of blank, standard, and spiked solutions were analysed. The findings showed no interfering peaks at the analytes' retention times, signifying the developed RP-HPLC method's high selectivity for the chosen phytoestrogens. Additionally, peak purity analysis verified the uniformity of the chromatographic peaks, indicating that the signals detected are solely from the target analytes, free from co-eluting impurities.

The precision of the method was assessed through system, method, and intermediate precision evaluations to ensure analytical reproducibility. System precision was evaluated by six injections of the standard solution under consistent chromatographic conditions. The %RSD values of peak areas were acceptable (<2%), indicating system consistency and stability. Method precision (intra-day) was assessed by analyzing several independently prepared samples under identical conditions. The %RSD values obtained indicated strong repeatability of the analytical procedure. Intermediate precision was evaluated over different days, confirming method robustness and reproducibility amid laboratory variations. In all instances, %RSD values remained within acceptable limits, demonstrating the method's reliability and consistency in analytical results [13].

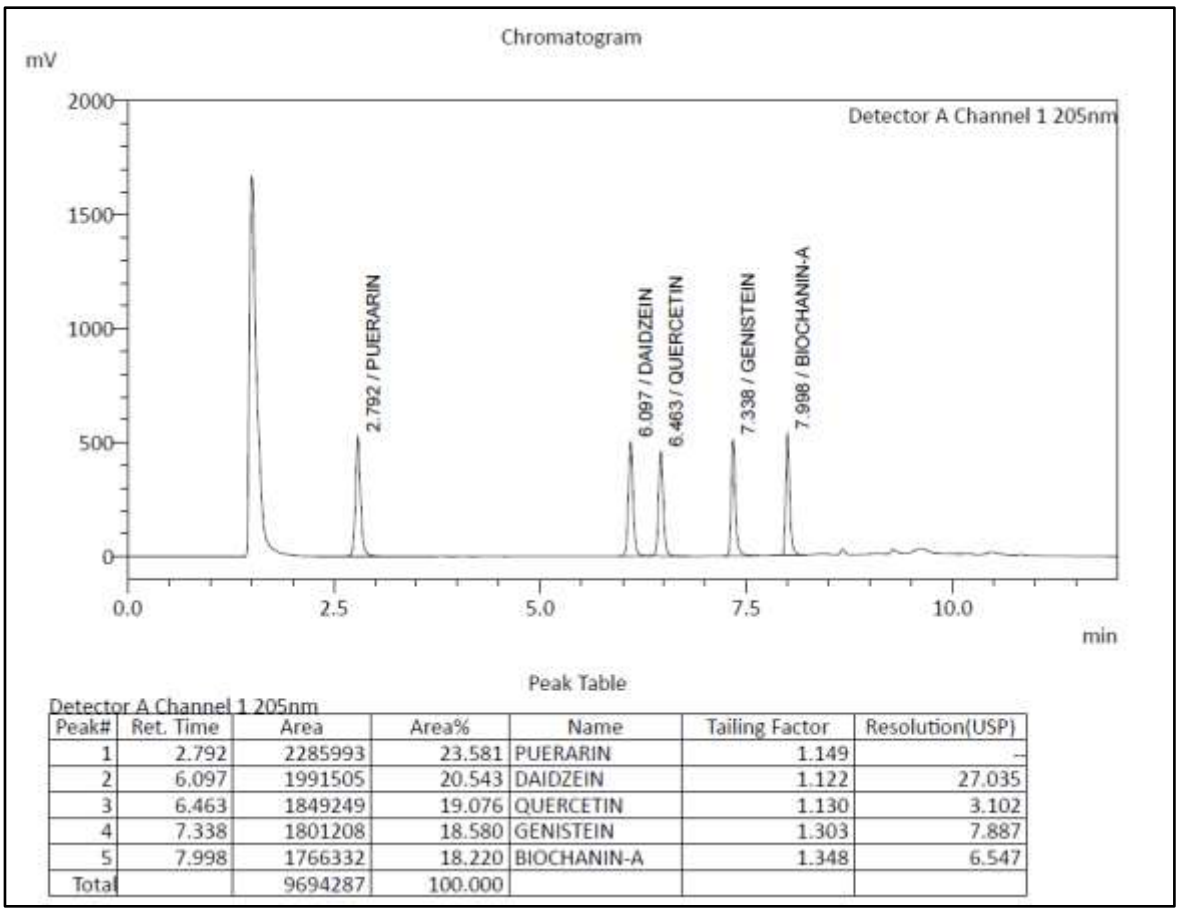
By calculating the limits of detection (LOD) and quantification (LOQ), the analytical method's sensitivity was assessed. By gradually diluting the standard solutions until the lowest concentration generating a discernible chromatographic signal above the baseline noise was noted, the LOD was determined using a visual detection technique. The lowest concentration at which the analyte could be measured with reasonable accuracy was known as the LOQ, and it was defined as three times the LOD concentration. Replicate injections of the appropriate solutions were used to assess the precision at both LOD and LOQ levels. Even at very low concentration levels, the obtained %RSD values were below 5%, showing good repeatability and proving the new method's sufficient sensitivity.

For each analyte, the method's linearity was assessed across a concentration range that corresponded to 25%-200% of the standard concentration. Plotting peak area against the relevant analyte concentration allowed for the construction of calibration curves. With correlation values (R^2) more than 0.999 for every chemical, linear regression analysis showed a strong linear connection between detector response and analyte concentration. These findings verify that the established RP-HPLC technology offers accurate quantitative response over a wide range of concentrations [14].

By introducing known amounts of standards into the sample matrix at various concentration levels, ranging from the LOQ level to 150% of the standard concentration, recovery tests were used to assess the analytical procedure's accuracy. The method's ability to precisely quantify the target analytes in the sample matrix was shown by the obtained recovery values. While recoveries at 50%, 100%, and 150% concentration levels fell between 98 and 102%, recoveries at the LOQ level varied between 93 and 102%. These findings show that the approach has outstanding accuracy over the whole operating range.

Precision tests were also conducted at 50%, 100%, and 150% of the standard concentration to further validate the method's dependability across various concentration levels. The analytical approach maintains constant accuracy throughout low, medium, and high concentration levels, as seen by the %RSD values produced at various concentration levels falling within acceptable bounds.

Overall, the validation findings show that the proposed RP-HPLC technique has excellent linearity across the concentration range under investigation and is highly specific, sensitive, accurate, and exact. These results demonstrate the validity and suitability of the suggested analytical method for the simultaneous measurement of phytoestrogenic chemicals in P. tuberosa root extract.



Peak Table

Detector A Channel 1 205nm

Peak#	Ret. Time	Area	Area%	Name	Tailing Factor	Resolution(USP)
1	2.792	2285993	23.581	PUERARIN	1.149	
2	6.097	1991505	20.543	DAIDZEIN	1.122	27.035
3	6.463	1849249	19.076	QUERCETIN	1.130	3.102
4	7.338	1801208	18.580	GENISTEIN	1.303	7.887
5	7.998	1766332	18.220	BIOCHANIN-A	1.348	6.547
Total		9694287	100.000			

Figure 2. Chromatogram showing results of System suitability test

3.3Quantification of phytochemicals in P. tuberosa root extract

The optimized and rigorously validated RP-HPLC methodology was effectively employed for the quantitative assessment of specific phytoestrogens present in the ethanolic extract derived from the tuberous roots of P. tuberosa. A sample solution of the extract was meticulously prepared at a concentration of 40,000 ppm and subsequently injected into the meticulously developed HPLC system under precisely optimized chromatographic parameters.

The analytical results elucidated the presence of five predominant bioactive phytoestrogens, specifically puerarin, daidzein, quercetin, genistein, and biochanin-A, within the extract. Among the quantified phytoestrogens, puerarin was identified as the most prevalent constituent, exhibiting a content of 2.18% in the extract. The remaining compounds were detected at comparatively lower concentrations, including genistein (0.17%), quercetin (0.15%), biochanin-A (0.08%), and daidzein (0.07%).

The quantitative analysis indicated that puerarin is the predominant phytoestrogen present in the P. tuberosa tuberous root extract. Puerarin is a well-documented isoflavonoid known for its antidiabetic, antioxidant, and cardioprotective properties, which may significantly contribute to the therapeutic potential of the plant. The presence of other phytoestrogens such as genistein, daidzein, quercetin, and biochanin-A, although in comparatively lower concentrations, may collectively enhance the pharmacological activity through synergistic effects. Therefore, the quantitative profiling of these bioactive constituents provides an important basis for the standardization and quality control of P. tuberosa and its herbal formulations, particularly for its potential use in the management of metabolic disorders [15].

4. Conclusion

In this study, a validated RP-HPLC method was developed for the simultaneous quantification of five phytoestrogens in P. tuberosa extracts. The method achieved efficient separation of all compounds in 12 minutes with sufficient column re-equilibration for further analyses. High sensitivity was demonstrated, allowing accurate quantification of the target phytoestrogens.

Quantitative analysis identified puerarin as the predominant compound (2.18%), followed by genistein, quercetin, biochanin-A, and daidzein. The novelty of this study lies in the development of a rapid, validated, and simultaneous RP-HPLC method for the estimation of five phytoestrogens in P. tuberosa, which has not been extensively reported with such a short analytical run time and comprehensive validation.

The proposed method provides a robust and efficient analytical tool for the quality control and standardization of P. tuberosa and its herbal formulations. Furthermore, this method can be extended for routine analysis, pharmacokinetic studies, and bioavailability assessment of phytoestrogens, as well as for the standardization of other phytoestrogen-rich medicinal plants. Future studies may focus on the application of this method in complex polyherbal formulations and its integration with advanced detection techniques such as LC–MS for enhanced sensitivity and structural confirmation.

Highlights:

1. Rapid RP-HPLC method developed for 5 phytoestrogens
2. Complete separation achieved within 12 minutes
3. Method validated as per ICH guidelines
4. High sensitivity and reproducibility ($R^2 > 0.999$)
5. Suitable for herbal standardization and QC

Abbreviations:

HPLC	:	High Performance Liquid Chromatography
RP-HPLC	:	Reverse Phase High Performance Liquid Chromatography
PDA	:	Photodiode Array
OPA	:	Orthophosphoric Acid
ACN	:	Acetonitrile
LOD	:	Limit of Detection
LOQ	:	Limit of Quantification
RSD	:	Relative Standard Deviation
FTIR	:	Fourier Transform Infrared Spectroscopy
LC-MS	:	Liquid Chromatography-Mass Spectrometry
WHO	:	World Health Organization
ICH	:	International Council for Harmonisation
ARI	:	Agharkar Research Institute
ppm	:	Parts Per Million
µg/mL	:	Microgram per milliliter
mL/min	:	Milliliter per minute
°C	:	Degree Celsius
rpm	:	Revolutions per minute

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Conflicts of interest: The authors of this work declare that they have no conflicts of interest.

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