

Original Article



Investigating a Simple and Sensitive High-performance Liquid Chromatography Method for Simultaneous Determination of Apigenin, Catalpol, and Gallic Acid Contents in *Plantago Lanceolata* L. and *Plantago Major* L.

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ABSTRACT

Background and Objectives: *Plantago* is a diverse genus of the Plantaginaceae family. *Plantago lanceolata* L. (*P. lanceolata*) and *Plantago major* L. (*P. major*) are used commercially worldwide as a traditional treatment for many diseases. A sensitive, simple, and reliable high-performance liquid chromatography (HPLC) method was developed to simultaneously quantify the three active ingredients: apigenin, catalpol, and gallic acid in *P. lanceolata* and *P. major*.

Methods: HPLC analysis was carried out using C₈ and C₁₈ columns. The mobile phase comprised acetonitrile, orthophosphoric acid, or formic acid (different ratio V/V) with flow rates of 0.4, 0.8, and 1 mL/min. The eluted peaks were detected at 204, 210, 256, and 330 nm. The crude extracts were separated using the liquid-liquid extraction method.

Results: HPLC analysis was performed using the C₈ column with the mobile phase consisting of acetonitrile–orthophosphoric acid (1:1%) at a 1 mL/min flow rate. The detection of the eluted peaks was observed at 204 nm. Using this protocol, the detection and quantification limits for apigenin, catalpol, and gallic acid were 0.007 and 0.022 µg/mL, 0.04 and 0.14 µg/mL, 0.02 and 0.073 µg/mL, respectively. The calibration curve's correlation coefficient indicated good linearity (r>0.9996, 0.9991, and 0.9978), with average recoveries for the three compounds between 100.02, 95.98, and 108.30%, respectively. Meanwhile, the intra-day and inter-day accuracy averages ranged from 100.07 to 99.95%, respectively. The results showed that using dichloromethane extracts of *Plantago* species leaves produced the highest yield of apigenin (1.08 and 0.58 µg/mg). At the same time, gallic acid was more abundant in methanolic, butanol, and aqueous extracts of *P. lanceolata* (3.33 µg/mg) and *P. major* (3.95, 4.34, and 4.72 µg/mg). The aqueous extract of *P. lanceolata* leaf and *P. major* root also showed more catalpol content (9.339 and 2.451 µg/mg).

Conclusion: The developed method indicated reliable results with reproducibility, high accuracy in an analytical run, repeatability, acceptable intermediate precision, reproducibility, and stability of these working solutions. To the best of our knowledge, this study is the first report for the simple, simultaneous quantification of three compounds in *Plantago* spp. using HPLC.

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Introduction

Recently, medicinal plants have attracted significant attention due to their therapeutic and biological properties. Numerous studies have reported the potential of medicinal plants in various traditional, alternative, and complementary systems

[1]. Plantago genus belonging to the Plantaginaceae family includes about 275 species [2]. *Plantago* species are used commercially worldwide as food and in the traditional treatment of many diseases [2].

Ribwort plantain (*Plantago lanceolata* L. and great plantain (*Plantago major* L. are the most well-known *Plantago* species, which exhibited beneficial pharmaceutical properties. *P. lanceolata* has been used for medicinal purposes to treat various diseases, such as wound healing [3], respiratory system disorders, reproductive system, digestive organs, blood circulation, anti-inflammation [4], and anti-cancer, in addition to its anti-bacterial properties [5-7]. *P. major* is effective as an anti-oxidant [8], wound healing [9], anti-diabetic [10], anti-inflammatory [11], anti-bacterial, and anti-cancer [12-14]. *Plantago* species have many medicinally active ingredients, such as iridoids, flavonoids, phenolic acids, triterpenes, and phenylpropanoid glycosides [2].

There is currently considerable interest in studying the biological activity of plant extracts. Plants containing iridoids have attracted more attention [15, 16]. Iridoids are a large group of monoterpenoids [17] that induce a defensive role in plants [18]. Iridoids are widespread in Lamiales, such as Cornales, Gentianales, and Plantago [19]. The iridoid glycosides aucubin and catalpol are the biologically active secondary metabolites utilized as analytical and chemotaxonomic markers to determine the quality of plant-derived extracts [20]. In herbal samples, the iridoid glycosides with aucubin and catalpol are approximately 2% to 3%. The stage of plant maturity determines the content of iridoids. The iridoid content in old leaves is extremely low, while young leaves show a maximum of 9% iridoid. Aucubin is a major compound in older leaves, while catalpol is the dominant constituent in young leaves [21]. Iridoids have various biological activities, such as anti-tumor, anti-inflammatory, hepato-protective, and neuro-protective activities [22]. Catalpol has various therapeutic functions, such as protecting against liver damage and lowering high blood sugar [23]. Flavonoids, phenolic, and tannins compounds comprise most medicinal plants' secondary metabolites [24]. Apigenin is a subclass of flavonoids and a non-mutagenic flavone with less toxicity [25]. Apigenin

has notable properties, including anti-oxidant, anti-proliferative, anti-tumor, apoptosis, anti-inflammatory [26], and anti-bacterial activity [27]. Gallic acid is one of the essential bioactive agents that showed numerous medicinal properties, including anti-microbial, anti-cancer [28], anti-viral [29], anti-inflammatory, anti-oxidative, and anti-neoplastic properties [30].

Many studies have shown that apigenin, catalpol, and gallic acid present in *P. lanceolata* and *P. major* have therapeutic potential; however, the methods used to determine their amount in previous studies were not optimized and crude extracts of plants were often used, highlighting the need for further research and more detailed information. This study introduced a simple, selective, and stable method for detecting these compounds in different fractions of *P. lanceolata* and *P. major* leaves and roots using high-performance liquid chromatography (HPLC). The goal of choosing a solvent system is to find the best type of extract for detecting these compounds. To the best of the authors' knowledge, this is the first report on the simultaneous quantification of apigenin, catalpol, and gallic acid in these species.

Materials and Methods

Instrument and high-performance liquid chromatography apparatuses

The system was equipped with a pump (Waters Corp., Milford, MA, USA) and a UV detector (WATER, model Breeze, USA) used at wavelengths 204, 210, 256, and 330 nm, with the outputs recorded using the Breeze software. The apigenin, catalpol, and gallic acid were conducted through two types of columns, the Phenomenex-C₈ column (150 mm×4.6 mm, the particle size of 5 μm, USA) and Thermo Hypersil-C₁₈ column (150 mm×4.6 mm, the particle size of 5 μm, Germany) equipped with the same packed guard column. The mobile phase includes an organic solvent, acetonitrile, and eluents orthophosphoric acid or formic acid (different ratio V/V) with flow rates of 0.4, 0.8, and 1 mL/min. An aliquot of 20 μL was injected into the HPLC system using a loop injector (Rheodyne 7725i, Cotati, CA, USA). The column temperature was kept at 25°C.

Chemicals

Apigenin (98% purity), catalpol (96%), and gallic acid (96%) were provided by the Sigma-Aldrich (Aldrich Division; Steinheim, Germany) company. Acetonitrile, water, and methanol were HPLC, gradient grade.

Preparation of stock solutions and working solutions

Apigenin stock solutions were made in dimethyl sulfoxide (DMSO) and HPLC grade methanol (5:95%, V/V). Each standard was separately dissolved in sterile purified water to prepare catalpol and gallic acid and then was stored at 4°C for gallic acid and 20°C for apigenin and catalpol. Working standard solutions used to optimize and validate the method were prepared in solvents using the dilution method at concentrations of 1.5625 to 100 µg/mL for catalpol and 0.15625 to 10 µg/mL for apigenin and gallic acid.

Plant sample

P. lanceolata and *P. major* were collected as wild plants in September 2018 from the University of Zanjan, Iran, research field. The voucher specimens were prepared in triplicates for each analysis and authenticated at the Department of Pharmacognosy, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan. The plant samples were washed to remove dust particles. The leaf and root parts of the two species of *Plantago* were isolated, air-dried, and then powdered by an electric mixer-grinder.

Sample preparation by the fraction method (liquid-liquid extraction)

An amount of 250 g of dried and powdered leaf and root parts of *Plantago* spp were weighed, and extraction was carried out for 16 h via reflex method using petroleum ether as a solvent (to remove nonpolar compounds) followed by methanol with the same period. The methanol extract was separated using The methanol extract was separated using dichloromethane, ethyl acetate, n-butanol, and aqueous phase via the liquid-liquid extraction method in a separatory funnel [31]. Using filter paper, the aqueous fraction was filtered for plant fiber removal.

Extracts were then concentrated to dryness under a vacuum using a rotary evaporator and dried at room temperature for seven days. Dried extracts were re-dissolved in HPLC-grade methanol for HPLC analysis to obtain stock solutions, and then all of the solutions were filtered through a 0.22-µm-pore-size filter.

Standard curves

The proper volume of one of the above-mentioned working solutions for the preparation of the standard curve points equivalent to 1.5625, 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL catalpol and 0.15625, 0.3125, 0.625,

1.25, 2.5, 5 and 10 µg/mL apigenin and gallic acid, respectively, was produced. The calibration curves were formed by plotting the mentioned concentrations against the corresponding peak regions.

Quality control samples preparation

Different samples were spiked with an appropriate volume of the corresponding standard solution to prepare the quality control (QC) samples daily. A final concentration equivalent to a low level (0.15625, 1.5625, 0.15625 µg/mL), middle level (1.25, 12.5, 1.25 µg/mL), and high level (10, 100, 10 µg/mL) of apigenin, catalpol, and gallic acid, were prepared, respectively. The following methods are similar to those described above.

Method validation

The well-organized methods were validated based on the USP and ICH instructions for analytical methods validation [32]. For this purpose, the procedure was confirmed for the analysis's linearity, limit of detection (LOD) and limit of quantification (LOQ), precision, accuracy, stability, and recovery according to the Food and Drug Administration industry guideline principles.

Linearity

The linearity of the method was investigated using the regression coefficient (R^2) of the calibration curves generated by seven concentrations of standards. The analysis of the blank samples was performed to ensure that no interferences existed but was not used to construct the calibration function. The ratio of signal-to-noise was used to estimate the LOD. This parameter was considered the lowest concentration level caused by a peak region of three times the baseline noise.

Precision and accuracy

Intra-run (within-run) variations and inter-run (between-run) variations were employed. The developed HPLC method was used to prepare and analyze samples in triplicate with concentrations of QCs (from low, middle, and high regions of the standard curve) in one run and on three different runs, respectively. The coefficient of variations (CV%) of the associated concentrations was then calculated in each case.

Repeatability test

Six independent samples with apigenin, catalpol, and gallic acid concentrations of 1.25, 12.5, and 1.25 µg/mL were prepared to examine the procedure repeatability

and intermediate precision. The prepared samples were injected into the HPLC system, and the percentage of relative standard deviation (%RSD) between retention time (Rt) and peak areas was determined as the method's repeatability.

Intermediate precision

Similar to a repeatability study, six samples of fresh reagents were prepared and analyzed with a different HPLC column. The %RSD was determined between six measurements, and the %RSD between 12 measurements of the repeatability and intermediate precision tests.

Reproducibility

The mean results for the analysis of the same sample between our laboratory and two various test facilities were acquired. Then, the Equation 1 was used to calculate the percentage difference between content measurements:

$$1. \text{Reproducibility} = \frac{(\text{Highest value} - \text{Lowest value})}{(\text{Mean value})} \times 100$$

Stability freeze and thaw stability

The three concentration levels of 1.5625, 12.5, and 100 µg/mL of QC catalpol were kept at the storage temperature (-20°C) for one day and thawed unassisted at room temperature. After being completely thawed, The samples were refrozen for one day under the same conditions. They were assayed after three freezes (-20°C)-thaw cycles (room temperature).

Short-term temperature stability

Three concentration levels of QC catalpol (1.5625, 12.5, and 100 µg/mL) were placed at room temperature for a time interval of 4 h that went beyond the catalpol routine preparation time.

Long-term stability

Three concentration levels of QC catalpol (1.5625, 12.5, and 100 µg/mL) stored at low temperatures (-20°C) were investigated within a time interval of two weeks.

Robustness

The method's robustness was tested to evaluate the variation of the chromatographic conditions on catalpol determination. The mobile phase flow rate was changed to 0.4, 0.8, and 1 mL/min to determine robustness only for catalpol.

Statistical analysis

The results were reported as Mean±SD. The means were compared using the Duncan test (P<0.05) by the SPSS software, version 21.

Results

Optimization conditions

The experiment's plan was to optimize conditions for determining the content of catalpol; however, the optimal method will also be effective in determining the content of apigenin and gallic acid. After optimization of the method, three compounds were identified in the leaf and root parts of two *Plantago* species by comparison with the chromatogram of the standard constituents obtained under similar conditions. Peak areas were utilized to quantify the apigenin, catalpol, and gallic acid contents in herbal samples.

Method validation

Different chromatographic variables, including kinds of columns, UV wavelengths, and different flow rates with various mobile phase compositions, were optimized for catalpol assay by HPLC-UV. Finally, optimum separation, good peak separation, and a shorter analysis time were obtained on the C₈ column and 204 nm wavelength with a flow rate of 1.0 mL/min (Table 1).

Various mobile phase compositions with different concentrations of acetonitrile, methanol, and acetonitrile-methanol were tested as organic solvents, and formic acid or orthophosphoric acid as eluents in water were compared to achieve the best separation and resolution. Acetonitrile-orthophosphoric acid (1:1%, V/V) system was the optimum choice, giving distinct resolution and sharp peaks. In this study, the methanol peak interfered with the standard peak when methanol was used as a mobile phase. In contrast, by applying acetonitrile as a mobile phase, no peak was observed except the internal standard peak (Figure 1).

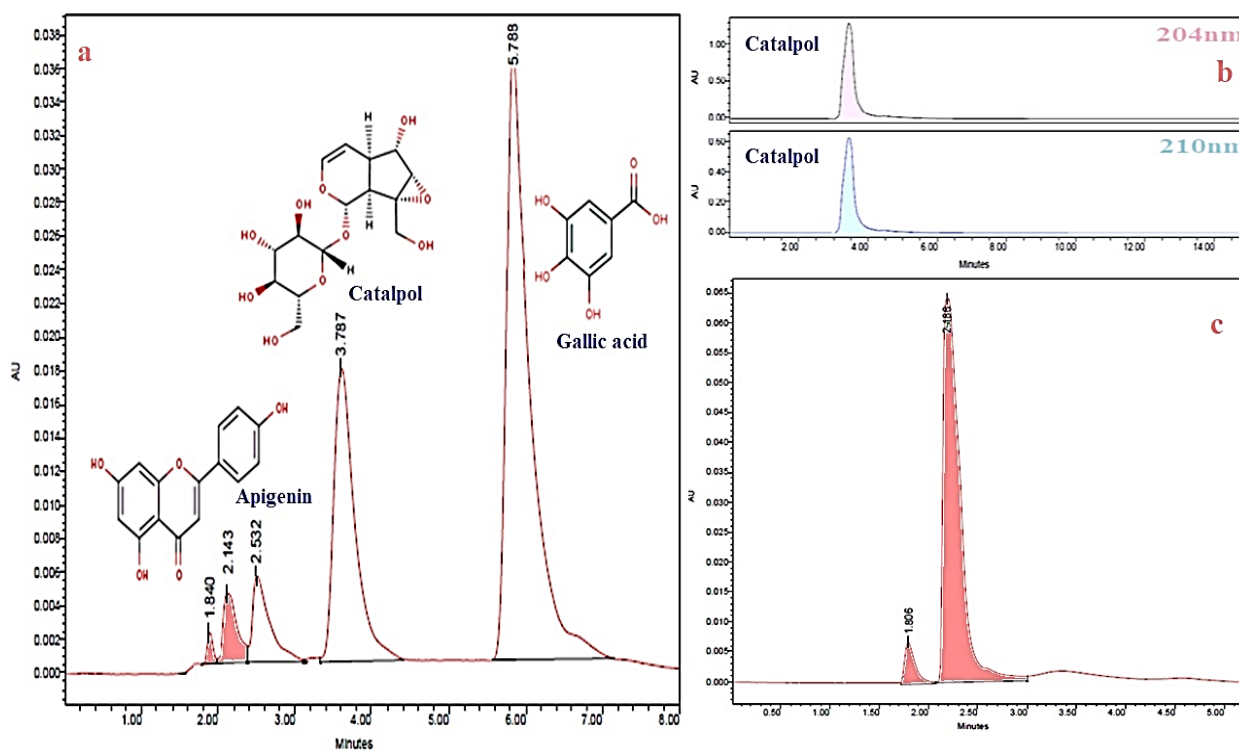
The elution times of the analytes vary with the addition of different concentrations of acetonitrile-orthophosphoric acid in the mobile phase system. As the acetonitrile concentration increased, the Rt decreased, potentially interfering with the solvent peak (Table 2). To prevent this, we used 1% acetonitrile as the optimized ratio in the mobile phase system, which shortened the detection run time without causing interference.

Table 1. Optimization of high-performance liquid chromatography conditions

Chromatographic Factor	Compared Parameter	Optimized Conditions	Main Advantages
Column	C ₈ analytical column, Phenomenex;	C ₈	No difference in C ₈ -C ₁₈ (C ₈ was selected)
	150 mm×4.6mm, particle size 5μm;		
	C ₁₈ analytical column, thermo hypersil;		
	150 mm×4.6mm, particle size 5μm		
λ UV	204, 210, 256, 330 nm	204 nm	No difference in 204-210 nm (the peaks attributed to 204 nm displayed sharper peaks)
Mobile phase	ACN-H ₂ O, ACN-MeOH-H ₂ O	ACN-H ₂ O	Better peak shape and separation, stable baseline
	Adding formic acid or orthophosphoric acid in H ₂ O	Orthophosphoric acid-H ₂ O	Better peak shape and separation
ACN: Orthophosphoric acid (%)	1:0, 1:1, 5:95, 10:90, 50:0.5, 70:1	1:1	Shorter elution time was in 1% and 5%; however, catalpol overlapped with a solvent peak when ACN was >50% and to 5 and 10%, the solvent interfered with the peak of apigenin and catalpol

ACN: Acetonitrile; MeOH: Methanol.

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Figure 1. Typical high-performance liquid chromatography chromatograms developed for apigenin, catalpol, and gallic acid assay

a) Chromatogram of standards with concentrations of 1.25, 50, and 10 μg/mL of three compounds, methanol and acetonitrile, (no perceptible interferences were observed between the matrix factors and the analyte); b) Two UV wavelengths (204 nm and 210 nm) were utilized to determine the content of catalpol; c) The two peaks are related to acetonitrile and methanol

Table 2. Elution time of apigenin, catalpol, and gallic acid analytes

Acetonitrile %	Orthophosphoric Acid%	Rt (Min) of Apigenin	Rt (Min) of Catalpol	Rt (Min) of Gallic Acid
1	0	-	3.9-4	5
1	1	2.5	3.6- 3.7	5.7
5	1	2.6	2.6	3.5
10	1	-	2.2	3.2-3.3
50	0.5	-	3.4	-
70	1	-	3.6	-
1:1methanol (V/V)	1	3.6	3.8	5.3

Rt: Retention time; Min: Minimum.

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Under established conditions, the longest and shortest analysis Rt is related to gallic acid and apigenin, respectively (Table 2).

Assay specificity

Typical chromatograms are generated by the developed method, and no perceptible interferences can be observed between the matrix factors and the analyte (Figure 1). Thus, the method developed to determine the concentrations of apigenin, catalpol, and gallic acid shows reliable results.

Linearity

Linear responses, LOD, and LOQ, were produced for apigenin, catalpol, and gallic acid in the range concentration shown in Table 3, which is appropriate for the predesignated objectives.

Intra-day and inter-day, precision, and accuracy

The intra-day precision and accuracy of the developed HPLC method were investigated through the analysis

of three sets of QC samples at low, middle, and high concentrations (Table 4). Inter-assay precision and accuracy were analyzed over three days, while the QC samples were prepared daily (Table 5). The RSD values of intra-assay and inter-assay precision were between 0.10 and 4.88%, and the accuracy was between 99.52% and 100.15%. These results indicate the developed method's reproducibility and high accuracy in an analytical run.

Repeatability test

The repeatability test of the Rt and peak area is shown in Table 6. The RSD% was between 0.76 and 9.89%, respectively. This method displayed repeatability for the apigenin, catalpol, and gallic acid assay.

Intermediate precision

Table 7 shows the results of the intermediate precision test of peak area and Rt. Accordingly, an acceptable intermediate precision is shown for apigenin, catalpol, and gallic acid assay.

Table 3. Calibration curves, regression coefficient, linearity ranges, LODs, and LOQs of external standards

Standard Chemical	Standard Curve	Regression Coefficient (R ²)	Concentration (µg/mL)	LOD	LOQ	Test Range (µg/mL)
Apigenin	y=296784x±13686	R ² =0.9996	0.15625	0.00729	0.02211	(0.15625-10)
Catalpol	y=11865x±4919.9	R ² =0.9991	1.5625	0.04949	0.14997	(1.5625-100)
Gallic acid	y=355517x±24027	R ² =0.9978	0.15625	0.024357	0.07381	(0.15625-10)

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Notes: In the typical linear regression equation for apigenin, catalpol, and gallic acid, x, and y, respectively, show the compound concentration (µg/mL) and the peak area (in arbitrary units).

LOD: Limit of detection; LOQ: Limit of quantification.

Table 4. Intra-day precision and accuracy of the HPLC method for the quantitation of the compounds (n=3)

Sample	Concentration (µg/mL)	Mean±SD	(%)	Mean±SD
		Measured Concentration	RSD	Accuracy
Catalpol	1.5625	1.54±0.35	2.30	100.03±2.33
	12.5	12.6±0.10	0.79	100.004±0.79
	100	100.26±0.64	0.64	100.002±0.64
Gallic acid	0.15625	0.15±0.08	4.88	100.15±4.81
	1.25	1.24±0.02	1.67	100.01±1.69
	10	10.15±0.34	3.38	100.07±3.32
Apigenin	0.15625	0.156±0.001	0.10	100.00±0.10
	1.25	1.23±0.03	2.11	100.02±2.13
	10	10.03±0.06	0.59	100.002±0.59

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Abbreviations: HPLC: High-performance liquid chromatography; RSD: Relative standard deviation; SD: Standard deviation.

Table 5. Inter-day precision and accuracy of the HPLC method for the quantitation of the compounds

Sample	Concentration (µg/mL)	Mean±SD	(%)	Mean±SD
		Measured Concentration	RSD	Accuracy
Catalpol	1.5625	1.56±0.025	0.16	99.98±0.16
	12.5	12.44 ±0.21	1.70	99.52 ±0.02
	100	100.08 ±0.19	0.19	100.08 ±0.001
Gallic acid	0.15625	0.15±0.0007	0.49	100.00±0.49
	1.25	1.25±0.003	0.28	100.00±0.28
	10	9.99±0.03	0.36	100.00±0.36
Apigenin	0.15625	1.56±0.003	0.24	100.00±0.24
	1.25	1.25±0.001	0.12	100.00±1.69
	10	10.09±0.14	1.37	100.01±1.36

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Abbreviations: HPLC: High-performance liquid chromatography; RSD: Relative standard deviation; SD: Standard deviation.

Table 6. Repeatability of the test results for spiked standards containing 1.25, 12.5, and 1.25 µg/mL apigenin, catalpol, and gallic acid, respectively

Sample	Mean±SD	(%)	Mean±SD	(%)
	Peak Area	RSD	Retention Time	RSD
Apigenin	438711.5±8032.05	1.83	2.54±0.05	1.88
Catalpol	129843.83±12846.95	9.89	3.66±0.03	0.88
Gallic acid	471287.95±14970.41	3.18	5.56±0.04	0.76

RSD: Relative standard deviation; SD: Standard deviation.

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Table 7. Intermediate precision of the test results for spiked standards containing 1.25, 12.5, and 1.25 mg/mL apigenin, catalpol, and gallic acid

Sample	Mean±SD	(%)	Mean±SD	(%)
	Peak	RSD	Retention Time	RSD
Apigenin	466200.33±7642.12	1.64	2.54±0.05	1.87
Catalpol	137372.16±3682.28	2.68	3.68±0.02	0.65
Gallic acid	490801.83±9274.51	1.89	5.56±0.09	1.56

RSD: Relative standard deviation; SD: Standard deviation.

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Reproducibility

The highest and the lowest test result of the spiked catalpol with 12.5 µg/mL catalpol was 142 646 and 132 842, respectively; the mean value was 137 372.16. The percentage difference was thus 7.13%, indicating a high reproducibility for the method.

Stability

Table 8 shows the freeze-thaw and short and long-term stability results for catalpol. The working solution's stability was examined at the room's temperature for 4 h. The results from this method showed the stability of these working solutions at this time.

Robustness

The robustness test was evaluated by changing the flow rate from 0.4, 0.8, and 1 mL/min. A significant change was observed in the Rt of catalpol by changing the flow rate of the mobile phase; however, there was no significant change in the integration area of catalpol. The lowest values of the RSD, mean values of Rt, and peak area are shown in Table 9.

High-performance liquid chromatography analysis of fractions

The results indicated that at 204 nm wavelength, the analyte showed no interferences per sample, and the extracts containing apigenin, catalpol, and gallic acid were represented (Figure 2).

Table 8. The stability of catalpol at different quality control levels (n=5)

Stability	1.5625 (µg/mL)	12.5 (µg/mL)	100 (µg/mL)
Short-term stability	86.68	87.46	86.39
Freeze and thaw stability	95.34	95.22	96.68
Long-term stability	88.78	86.18	87.65

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Table 9. Results from testing the robustness of the method by changing the mobile phase flow rate

Flow Rate (mL/min)	Mean±SD			RSD (%)
	Area n=3	RSD	Rt n=3	
0.4	131284±4304.98	3.47	8.63±0.02	0.23
0.8	139728±3465.35	2.48	5.54±0.06	1.09
1	137372±3682.29	2.68	3.68±0.02	0.65

Notes: The concentration of the solution of Catalpol analyzed was 12.5 µg/mL.

Abbreviations: SD, standard deviation; RSD, relative standard deviation; Rt, retention time.

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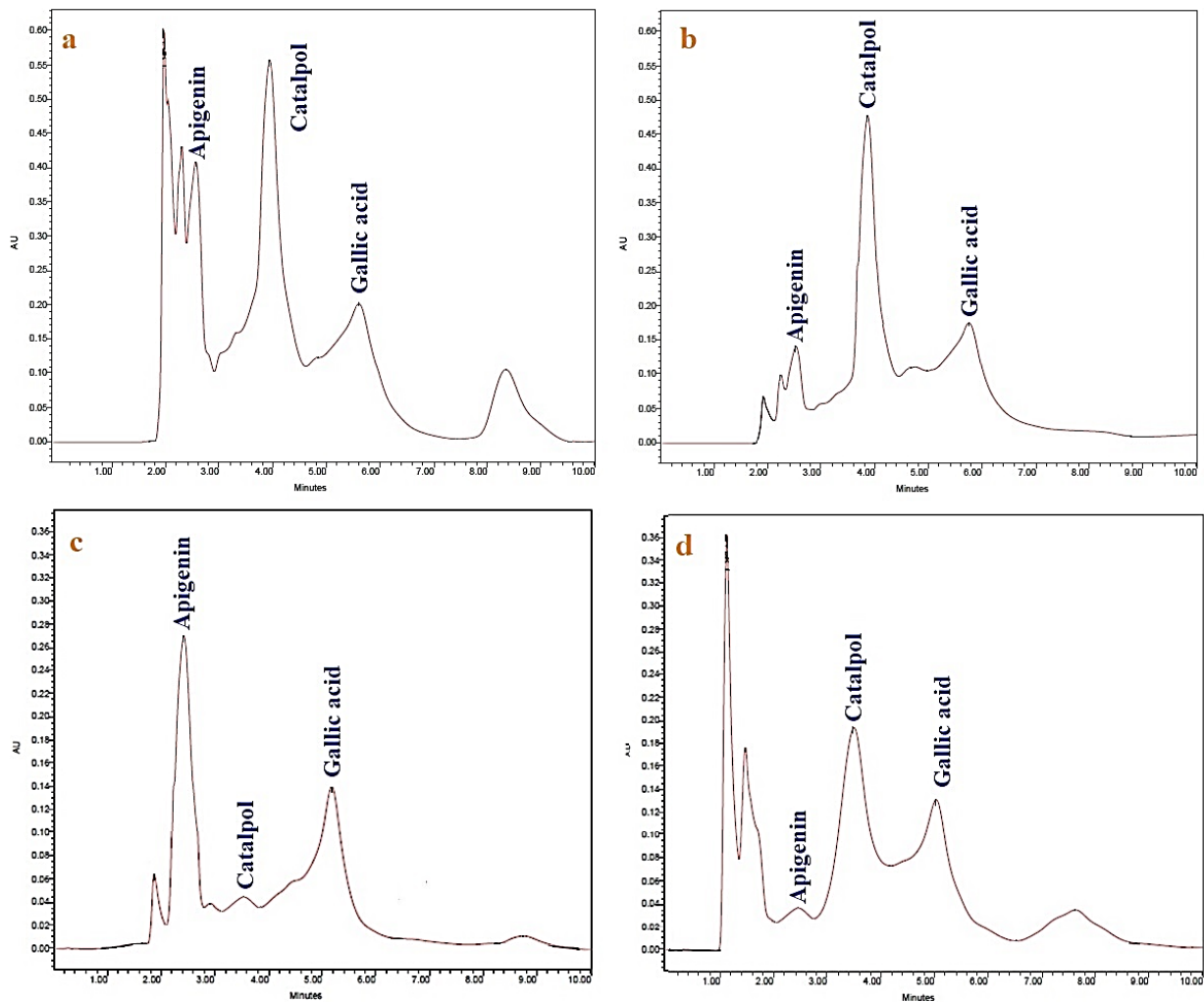


Figure 2. Typical high-performance liquid chromatography chromatograms of the developed for apigenin, catalpol, and gallic acid assay in different parts of *P. lanceolata* and *P. major*

a) and b) Methanolic extract from leaf and root part of *P. lanceolata*; c) and d) Methanolic extract from leaf and root part of *P. major*.

Optimization of solvent systems for the extraction of apigenin, catalpol, and gallic acid

Liquid extraction was performed using various solvents to find an effective solvent for extracting apigenin, catalpol, and gallic acid. After separating the extracts, the fractions containing apigenin, catalpol, and gallic acid were analyzed using HPLC. The dichloromethane extract of the leaf part of *P. lanceolata* and *P. major* produced the highest yield of apigenin (1.08 and 0.58 $\mu\text{g}/\text{mg}$ DW) compared with the root part, respectively (Table 10 and Table 11). In contrast, the aqueous extract of *P. lanceolata* leaf and *P. major* root showed more catalpol content (9.339 and 2.451 $\mu\text{g}/\text{mg}$). The content of gallic acid was higher in methanolic, butanol, and aqueous extracts of *P. lanceolata* (3.33 $\mu\text{g}/\text{mg}$) and *P. major* (3.95, 4.34, and 4.72 $\mu\text{g}/\text{mg}$) (Table 10 and Table 11).

Discussion

A reliable HPLC technique was used to evaluate the levels of three pharmaceutical compounds, including the iridoid glycoside catalpol, the flavonoid apigenin, and the phenol gallic acid, in various fractions of leaf and root parts of *Plantago* species.

Some studies have used a methanol-water mobile phase to determine the aucubin iridoid glycoside in *Plantago* species using HPLC [33, 34]. Methanol can interfere with compound detection. As a result, some studies have used acetonitrile instead of methanol [35]. In this study, when methanol was used as the mobile phase, its peak interfered with the analytes' peak. Only the internal standard peak was observed using acetonitrile as the mobile phase.

Table 10. The extraction recovery determined for apigenin, catalpol, and gallic acid from *Plantago* spp different fractions

Leaf Part	<i>P. lanceolata</i>			<i>P. major</i>		
	Apigenin (µg/mg)	Catalpol (µg/mg)	Gallic Acid (µg/mg)	Apigenin (µg/mg)	Catalpol (µg/mg)	Gallic Acid (µg/mg)
Methanol	0.6047 ^d	7.35089 ^e	3.3319 ^a	0.3159 ^d	1.35933 ^b	3.9574 ^c
RSD (%)	0.7408	0.2047	0.1014	0.3347	0.9274	0.0987
Dichloromethane	1.0829 ^a	0.21134 ^f	0.1452 ^d	0.5823 ^a	0.03591 ^f	1.4849 ^e
RSD (%)	0.5097	0.1465	0.2799	0.3211	0.2571	0.9024
Ethyl acetate	0.6465 ^c	6.64746 ^d	1.1935 ^b	0.5632 ^b	0.52489 ^d	2.5422 ^d
RSD (%)	0.1316	0.5214	0.2422	0.0500	0.0777	0.0639
Butanol	0.8343 ^b	8.59827 ^b	3.3338 ^a	0.2672 ^e	1.14364 ^c	4.3455 ^b
RSD (%)	0.0995	0.9119	0.0407	0.0093	0.4462	0.0131
Aqueous	0.3776 ^f	9.33978 ^a	3.3338 ^a	0.1024 ^f	1.47177 ^a	4.7291 ^a
RSD (%)	0.1036	1.0959	0.0811	0.0081	0.0015	0.1945

Notes: Mean of values (n=3).

The Duncan test was used for analysis ($P < 0.05$). Values followed by the same letter in a column are not significantly different.**PBR****Table 11.** The extraction recovery determined for apigenin, catalpol, and gallic acid from different root fractions of *Plantago* spp

Root Part	<i>P. lanceolata</i>			<i>P. major</i>		
	Apigenin (µg/mg)	Catalpol (µg/mg)	Gallic Acid (µg/mg)	Apigenin (µg/mg)	Catalpol (µg/mg)	Gallic Acid (µg/mg)
Methanol	0.2836 ^f	6.87115 ^b	2.0809 ^b	0.0407 ^e	0.61365 ^d	0.4692 ^c
RSD (%)	0.1057	0.0001	0.0236	0.4888	0.2119	0.1267
Dichloromethane	0.6860 ^a	1.00373 ^d	0.2584 ^e	0.0621 ^a	0.04029 ^f	0.3060 ^e
RSD (%)	0.5856	0.1101	0.0290	0.6958	1.0011	0.4599
Ethyl acetate	0.3761 ^c	2.97054 ^c	0.6130 ^d	0.0434 ^d	0.69105 ^c	0.3585 ^d
RSD (%)	0.8679	0.0839	0.7399	0.4031	0.3560	0.1561
Butanol	0.3587 ^d	2.91261 ^c	1.3803 ^c	0.0501 ^c	1.61171 ^b	1.1472 ^b
RSD (%)	0.2766	0.1079	0.1749	0.8894	0.0200	0.1155
Aqueous	0.4885 ^b	8.95773 ^a	3.4528 ^a	0.0263 ^f	2.45199 ^a	1.2750 ^a
RSD (%)	1.1517	0.0287	0.1451	0.3477	0.0012	0.8191

Notes: Mean of values (n=3).

The Duncan test was used for analysis ($P < 0.05$). Values followed by the same letter in a column are not significantly different.**PBR**

The HPLC chromatograms of *P. lanceolata* and *P. major* showed the presence of apigenin, catalpol, and gallic acid at the Rt of around 2.5, 3.7, and 5.7 min, and the RSD% of the Rt of 1.88, 0.88, and 0.76%, respectively. Other studies have been found to determine these compounds separately in two *Plantago* species by HPLC,

such as a peak for catalpol around 4.35 min [36], 5 min [37], 13.9 min [38], and for gallic acid around 4.40 min [39] and 4.6 min in HPLC assay of *P. lanceolata* [40].

In our earlier research, we used the HPLC method to measure the levels of flavonoid, phenolic, and iridoid

glycoside compounds in methanolic and 80% aqueous-methanol extracts of different parts of *P. lanceolata* and *P. major*. Statistical analysis of the crude extracts revealed that the aerial parts of both species contained higher levels of apigenin and gallic acid than the root parts. However, the aerial parts of *P. lanceolata* and the root parts of *P. major* had more catalpol content [5, 14, 41, 42]. Despite these findings, the methods used were not optimized, and only crude extracts were used, indicating a need for more detailed research.

As a highly polar compound, glycoside iridoid catalpol easily dissolves in water. Tong et al. (2015) investigated the typical polar biphasic solvent systems, including ethyl acetate–n-butanol–water and n-butanol–water in different amounts using thin-layer chromatography. This technique is used for the separation of a non-volatile mixture. Their study showed that a large amount of catalpol was partitioned into the aqueous phase due to its high polarity. Combining the mentioned solvents (ethyl acetate–n-butanol–water, 2:1:3, V/V/V) provided a good fraction system for separating catalpol from the purified crude extract [43]. The present study also stated that the aqueous extract of *P. lanceolata* leaves and *P. major* roots contained more catalpol content. The researchers reported that aucubin, a well-known marker of *Plantago* species, was present in all samples, and the highest amounts were found in *P. media* and *P. argentea* (44.27 and 39.93 mg/g DW, respectively); however, catalpol was detected only in *P. altissima*, *P. argentea*, and *P. lanceolata* [44, 45]. However, in the present paper, catalpol was also seen in lower amounts in *P. major*. Another research has shown the high content of phenolic acids in *P. lanceolata* aqueous-methanolic extracts and indicated that the benzoic acid derivatives hydroxybenzoate and 3,4,5-trihydroxybenzoate (gallic acid) are highly represented [46]. In the current work, the highest amount of gallic acid was obtained in aqueous, n-butanol, and methanolic fractions, indicating better solubility in polar solvents. On the other hand, the biological applications of flavonoids are limited due to low bioavailability as well as low aqueous solubility [47]. Dichloromethane fraction showed the presence of flavonoid apigenin in *Orbignya peciosa* Mart. leaves [48]. Similarly, dichloromethane extract also showed the highest apigenin content in *Plantago* species. The yield of secondary metabolites, such as flavonoid, phenol, and iridoid glycoside in the *Plantago* species varies under the influence of many factors, including the pattern of climatic temperature during the plant growing season, collection area, harvest time, drying method, environmental stresses, and extraction methods [41]. The data revealed the good precision and accuracy of all the proposed procedures. This

protocol has advantages, including economic extraction projects, quick accuracy, ease, high sensitivity, a short chromatographic run time, and reproducibility.

Conclusions

In this study, the determination of optimum conditions for extracting these medicinal compounds, including apigenin, catalpol, and gallic acid, was developed in two species of *Plantago*. As far as we know, this is the first report on the simultaneous evaluation of apigenin, catalpol, and gallic acid contents in leaf and root parts of two species of this genus with various solvents. Among the important medicinal compounds in *Plantago* species extracts, the iridoid glycosides aucubin and catalpol can be utilized as analytical markers to evaluate the quality of different extracts due to being iridoid-containing sources. Research on available species with medicinal properties is important. In this regard, knowledge about the composition present in the plants and the most economical and simple method to measure them is essential.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Project administration, investigation, formal analysis, writing original draft and revising: Samaneh Rahamouz-Haghighi; Funding and supervision: Khadijeh Bagheri and Ali Sharafi.

Conflict of interest

The authors declared no conflicts of interest.

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