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Original Article

RP-HPLC Method Development and Validation for Simultaneous Quantification of Ascorbic and Gallic Acids in Natural Extract-Based Granules

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ABSTRACT

Background: Ascorbic acid and gallic acid are known for immune-boosting and antioxidant properties. **Objectives:** To develop a straightforward, sensitive, validated, and economical reverse phase liquid chromatographic technique for the simultaneous measurement of ascorbic and gallic acid in granule formulation and natural extracts (Amla and Chyavanprash). These compounds are essential for their therapeutic benefits. **Materials and Methods:** Ascorbic and Gallic acid were separated from the complex mixture of various polyphenols present in natural extract. At 30°C, these two compounds were separated using a Thermo C18 analytical column with an isocratic program consisting of two solvents-A & B. The ratios of Solvent- A (0.3% orthophosphoric acid in HPLC grade water) and Solvent-B (80% acetonitrile in HPLC grade water) were 85:15 v/v, and the flow rate is 0.5 mL/min. using a UV-PDA detector at a single wavelength of 250 nm. **Results:** In accordance with ICH guidelines, the developed method was validated for linearity, precision, accuracy, robustness, Limit of Detection (LOD), & Limit of Quantification (LOQ). The granule formulation has 0.261% gallic acid and 0.079% ascorbic acid. **Conclusion:** Ascorbic acid and gallic acid in natural extracts and granule samples were successfully quantified and validated using the RP-HPLC method. The method proved it's simple, precise, sensitive, rapid and robust.

Keywords: Amla Extract, Ascorbic acid, Chyavanprash, Gallic acid, Method development, RP-HPLC, Validation.

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INTRODUCTION

Major source of Ascorbic Acid (Figure 1) is Citrus fruits, in which polyphenols is another important constituent. Phenolic compounds possess antioxidant activity which helps as scavengers of free radicals, also helps in cardiovascular diseases (Yussif, 2019; Singh *et al*., 2008; Raghu *et al*., 2007; Oliveira *et al*., 2020). The fruit of Amla (*Emblica officinalis*) is an excellent source of Vitamin C and various polyphenols, including Gallic acid (Figure 2) and Tannic acid. A very common and well-liked traditional Ayurvedic remedy in India is Chyavanprash. It is classified as a Rasayana medicine, which is renowned for its capacity to strengthen immunity and fend off illness. The phrase "Rasayana" refers to the route that "Rasa" took (Rasa: plasma; Ayana: path). Antioxidant-rich rasayana medicines are well-known for their ability to modulate the immune system and protect the liver. Due to its widespread use and commercial success, Chyavanprash is the subject of numerous studies focusing on its ethnopharmacological and biological activities. These studies aim to correlate its traditional uses with its pharmacological activities, as well as to investigate its chemical composition and quality (Raghavan *et al*., 2023). Ideally using a spectrophotometric method, total polyphenols can be measured. To separate out polyphenols, HPLC is the preferred technique because of its greater adaptability, accuracy, and precision as well as its relatively low cost (Sawant *et al*., 2010; Singh *et al*., 2012). In Amla extract and Chyavanprash extract number of polyphenols are present and for the same selective HPLC method requires which can separate them with good resolution.

In pandemic, immunity plays an important role, Ascorbic Acid and Gallic acid have supportive ability to boost immunity. Products containing same are commonly seen in the market as immunity-strengthening products, for the same selective HPLC method which can separate them with good resolution. Separation and Quantitatively estimation of both the compounds by use of RP-HPLC method, which have been reported

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previously such as HPLC/DAD, HPLC-MS/MS, and RP-HPLC/ELSD. However, these methods still face certain challenges. Some struggle to effectively quantify and separate both compounds into complex natural extracts. Techniques that involve derivatization or solid-phase extraction can be time-consuming and add complexity. Additionally, methods using buffers as mobile phase components often lead to longer processing times and reduced column lifespans (Sawant *et al*., 2023; Sharma and Singh, 2022; Wianowska and Olszowy-Tomczyk, 2023; Patel and Mehta, 2021; Kumar and Gupta, 2020; Lee and Kim, 2023; Zhang and Wang, 2022; Silva and Oliveira, 2021; Rao and Reddy, 2020; Singh and Verma, 2021; Gupta and Sharma, 2022; Kumar and Singh, 2023). While HPLC-MS/MS is known for its high sensitivity and reliability, its high cost remains a significant consideration for routine analysis.

The market demand for polyphenols that boost immunity is growing day by day, so it's important to have a simple way to estimate ascorbic and gallic acid that can overcome the aforementioned constraints. Therefore, the purpose of this study was to ensure the presence of both active components in granules containing natural extracts by developing and validating a quick, simple, selective, repeatable, and economical RP-HPLC method.

Research Gap

There are still several difficulties, especially when it comes to natural extracts like Chyavanprash, even with the notable improvements in analytical methods for measuring ascorbic and gallic acids using High-Performance Liquid Chromatography with Reversed-Phase (HPLC-RP). Achieving a satisfactory resolution of these active compounds is challenging due to the sample matrix's complexity which includes many herbs. Furthermore, some of the current methods use complex mobile phases to elute the compounds, which can take up to 35 min (Raghavan *et al*., 2023). A significant challenge is to shorten the runtime without sacrificing or enhancing resolution. Derivatization is one of the intricate sample preparation procedures used in many modern techniques, and it can be labor-intensive and time-consuming (Singh *et al*., 2008). Progress in this field depends on streamlining these processes without sacrificing analytical performance.

To address all these challenges this research aims to develop the method, which is simple, sensitive, rapid, robust and capable for simultaneous estimation of ascorbic and gallic acid in natural extract granule formulation and natural extracts.

MATERIALS AND METHODS

Gallic acid and ascorbic acid are naturally present in the finished product from amla and chyavanprash extracts. For these extracts, Konark Herbal Health Care in Vapi was the source. We purchased standard gallic and ascorbic acids from Amoli Organics Pvt. and NBRI Lucknow. Ltd., Methanol and acetonitrile of HPLC grade were purchased from Qualigens Pharma Pvt. Ltd., whereas Merck Specialty Private Ltd, Mumbai, India supplied the orthophosphoric acid. We used an in-house Milli-Q machine (Merck-Millipore) to produce HPLC-grade water.

Preparation of Granules formulation containing Natural extracts

Amla extract, Chyavanprash extract, and *Sphaeranthus indicus* extract were among the natural extracts that were carefully weighed as per (Table 1) and sieved through a 200-mesh screen. Because of their hygroscopic properties, these extracts were ground into a fine powder and combined with a precisely measured quantity of sweetener, colloidal silicon dioxide, and lactose anhydrous to create a dry mixture. A clear granulating solution was made by dissolving Polyvinylpyrrolidone (PVP) K-30 in water after it had been weighed. This solution was then combined with other ingredients, such as croscarmellose sodium, to form a moist, coherent paste. The resulting paste was passed through a #10 mesh sieve to produce granules. These granules were dried in an oven at 40-45°C until completely dry. Once dried, the granules were sieved through a #22 mesh sieve placed over a #44 mesh sieve to separate the fines from the granules (Shah *et al*., 2024).

Chromatography

The HPLC system used was a Shimadzu (Japan) model (LC-2030C 3D) with a variable wavelength detector, vacuum degasser, thermostat column, standard auto-sampler, and quaternary pump. A BDS Hypersil™ C18 column (250 mm × 4.6 mm, 5 µm) made of stainless steel and silica was used to carry out the separation. Solvent A (0.3% orthophosphoric acid in HPLC grade water) and Solvent B (80% acetonitrile in HPLC grade water) were selected as the mobile phase with the flow rate was 0.5 mL/min. A:B solvent (85:15 v/v) was the ideal solvent ratio for the mobile phase. A PDA detector was used to measure the absorption of gallic and ascorbic acids at 250 nm. Shimadzu Corporation's Lab

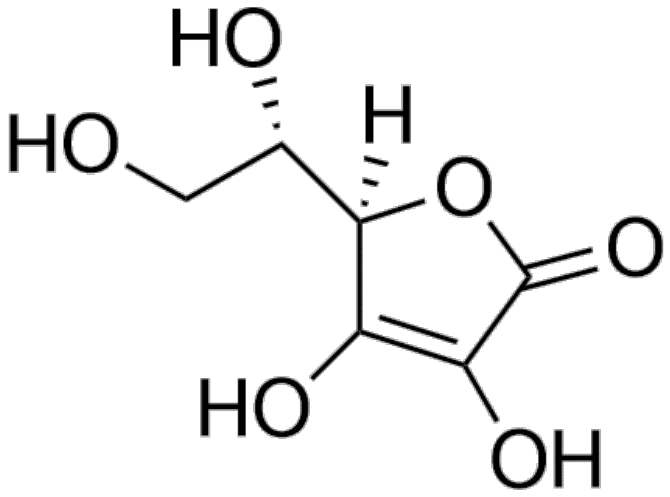

Figure 1: Ascorbic acid.

Table 1: Optimized formula of Granules formulation containing Natural extracts.

Formulation	% Content (w/w)
Amla extract	58.82
Chyavanprash extract	18.82
Gorakhmundi dry extract (*Sphaeranthus indicus*)	11.76
Crosscarmellose Sodium	2.35
Colloidal silicone dioxide	0.70
PVP K-30	1.411
Lactose Anhydrous	6.05
Sweetener	0.0588
Total	100

Solution software was used to acquired, monitored and process chromatographic data.

Preparation of Standard

Preparation of Standard Solution

10.82 mg of standard gallic acid and 10.75 mg of standard ascorbic acid were weighed and put into a 10 mL volumetric flask to create the stock solution. HPLC-grade methanol (about 7-8 mL) was added, and the mixture was shaken and sonicated for 2 min. After that, the same solvent was used to adjust the volume to the desired level. Following extensive mixing, working standards were made from the stock solution using serial dilution, with concentrations for both compounds ranging from 0.001 to 1 mg/mL. These standards were then used for HPLC analysis. To identify individual peaks of ascorbic and gallic acids individual standards were injected in HPLC.

Preparation of sample solution

Natural extracts Samples (Amla Extract and Chyavanprash Extract)

Samples of Amla and Chyavanprash natural extracts were each weighed to about 1 g and put into different 100 mL volumetric flasks. As a diluent, 50 mL of HPLC-grade methanol were added to each flask. After that, the samples were sonicated for 2 min. Following sonication, the volume was set at 100 mL, and the solution was then filtered into a glass vial using a 0.45 µm PVDF syringe filter. The concentrations of ascorbic acid and gallic acid were then measured independently by injecting the filtered samples into a High-Performance Liquid Chromatography (HPLC) instrument.

Finished product (Granules) sample preparation

The granules containing natural extracts of Amla and Chyavanprash were ground into a fine powder using a pestle and mortar. Approximately 1 g of this powdered sample was transferred into a 100 mL volumetric flask. To this flask, 50 mL of HPLC grade methanol was added as a diluent. The mixture was sonicated for 2 min to ensure thorough mixing. After sonication, the volume was adjusted to 100 mL. After that, the solution was collected in a glass vial after passing through a 0.45 µm PVDF syringe filter. High-Performance Liquid Chromatography (HPLC) was used to inject these filtered samples in order to measure the concentration of ascorbic acid and gallic acid.

Method validation

The validation of the analytical method was conducted in compliance with the International Conference on Harmonization's (ICH, 1996) guidelines.

Range, linearity, precision, specificity, accuracy, Limit of Detection (LOD), Limit of Quantitation (LOQ), and robustness were among the parameters evaluated during the validation process.

Linearity

Standard ascorbic acid and gallic acid solutions with concentrations ranging from 0.001 mg/mL to 1 mg/mL were used to evaluate linearity. Both the active compounds' concentrations in the samples fall within this range. The samples' linearity was assessed between 2.5 mg/mL and 15.0 mg/mL. Plotting the peak area against the standard solutions' concentration produced a calibration curve.

Precision

According to the ICH guideline for precision, the analytical procedure evaluates how closely multiple measurements from different samplings of sample agree under specified conditions. Precision is classified into intraday, interday, and repeatability. By injecting samples six times and comparing the results with standard, precision can be calculated, with Relative Standard Deviation (RSD) of the six injections being calculated.

Intra-day precision is shown by variations within a single day, indicating results that are closely aligned from multiple samplings. Repeatability, which reflects precision under the same conditions, is achieved by injecting the standard and samples six times. Intermediate precision is demonstrated by performing the experiment in a different lab, by a different person, and at a different time. Using different instruments for the same experiment shows the method's robustness. The acceptance criterion for all measurements was a maximum RSD of 2.0%.

Accuracy

The accuracy of an analytical method is determined by how closely the measured value matches the true value. To assess accuracy, samples were spiked with 50%, 100%, and 150% of a known standard. The percentage recovery of ascorbic and gallic acid was then calculated. Three sets of measurements were taken for unspiked samples, 50% spiked samples, 100% spiked samples,

and 150% spiked samples to determine the final Relative Standard Deviation (RSD) and percentage recovery. The acceptance criteria for accuracy were set at 90-110% recovery with a maximum RSD of 2.0%.

Detection Limit

The lowest concentration of an analyte in a sample that can be identified but not precisely measured is known as the detection limit of a given analytical technique. Various methods can be used to determine the detection limit, including visual inspection, signal-to-noise ratio, and plotting standard error on a calibration curve.

$$\text{Detection limit} = \frac{3.3 \text{ X } \sigma}{S}$$

Where,

'σ' is Standard deviation,

'S' is Slope derived from calibration graph,

(ICH Q2 R1 guidelines).

Quantification Limit

The quantification limit of a particular analytical method is the minimum concentration of an analyte in a sample that can be measured quantitatively with adequate accuracy and precision. Various methods can be used to determine the quantification limit, including visual inspection, signal-to-noise ratio, and plotting standard error on a calibration curve. This is particularly useful for detecting contaminants and degradation products at low concentrations in sample matrices.

$$\text{Quantification limit} = \frac{10 \text{ X } \sigma}{S}$$

Where,

'σ' is Standard deviation,

'S' is Slope derived from calibration graph,

(ICH Q2 R1 guidelines).

Robustness

Robustness indicates the reliability of an analytical method under normal operating conditions, reflecting its ability to withstand small, intentional variations in method parameters. In this instance, we evaluated the method's robustness by varying the wavelength and flow rate for the vitamin. Despite changes in the retention time, the method consistently produced the same results.

Ruggedness

Ruggedness can be assessed by making minimal changes to the main procedure without altering the solvent or analyte.

System suitability

During the development stage, system suitability tests are conducted to ensure that the system can perform the proposed method for identifying specified standards and samples. The capacity factor, theoretical plates, tailing factor and resolution are important factors for system suitability.

Specificity

The aim of the specificity test is to measure the analyte of interest amidst other expected components like the matrix, degradants, and contaminants. The method's specificity is assessed using a placebo and solvent.

RESULTS

Chromatography

The elution of gallic acid and ascorbic acid under the same conditions took 10 min. The retention periods and UV spectra of the ascorbic acid and gallic acid in the sample were compared with those of the standard compounds in order to identify the peaks in the HPLC chromatogram of the granule sample. Peak was found to be more than 99% pure. Figure 3 shows the chromatogram of the standards for ascorbic acid and gallic acid, and Figure 4 shows the chromatogram of the granule sample based on ascorbic acid and gallic acid at 250 nm.

Method validation was carried out for various parameters including linearity (Calibration Curve), specificity, accuracy, range, precision, Detection limit, Quantification limit and robustness. Linearity curve of ascorbic and gallic acids standard was carried out in the range of 0.001 - 1.0 mg/mL, having

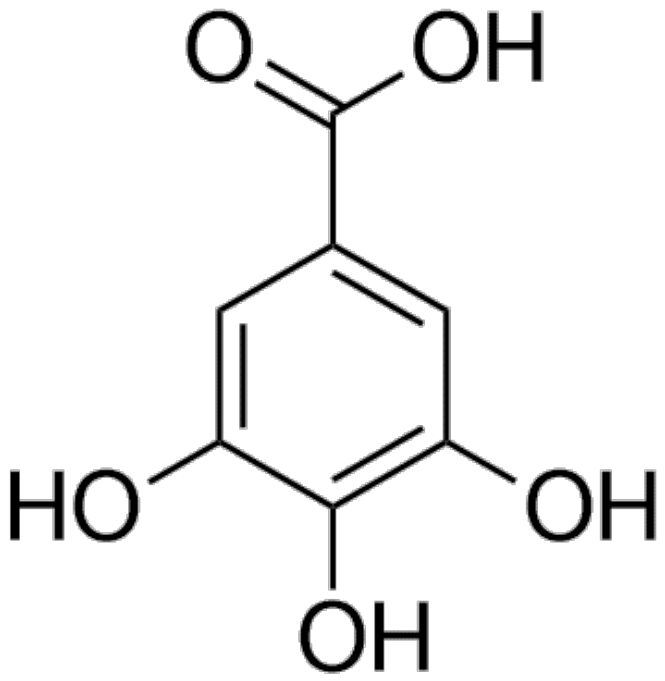

Figure 2: Gallic acid.

correlation coefficients value (r^2) for Ascorbic acid was 0.9947 and for Gallic acid 0.9932 (Table 2). The linearity of the sample was evaluated between 2.559 and 15.06 mg/mL, and the resulting correlation coefficients (r2) were 0.995 (Ascorbic Acid) and 0.9916 (Gallic Acid) as shown in (Table 3).

Inter-day and intra-day precision of both the actives in the sample are shown in (Table 3). It demonstrated that the method has acceptable precision, with RSD values well below 2.0%. Recovery rates at 3 different levels (50, 100 and 150%) for Ascorbic acid were 99.59%, 100.18%, and 101.31%, and for Gallic acid were 98.35%, 97.77%, and 99.10% (Table 3), indicating the method's accuracy. Detection limit and Quantification limit for both the actives were 0.01 ppm and 0.03 ppm, respectively (Table 2), reflecting the method's high sensitivity, calculated using the below formula:

LOD = 3.3 σ/S and LOQ = 10 σ/S.

Where,

σ- Standard deviation,

S-slope of the calibration graph.

Minor modifications to the mobile phase ratio (83:17 v/v and 87:13 v/v) and flow rate (0.4 mL/min and 0.6 mL/min) were made in order to assess the method's robustness, as indicated in Table 3. The technique proved resilient to even small changes in the chromatographic conditions.

Ascorbic acid, Gallic acid content in the Amla extract sample, Chyavanprash (obtained from Konark Herbals & HealthCare Pvt. Ltd., Malpur, Baddi) and in the granule formulation was analysed using this newly developed method (HPLC), as detailed in (Table 4). Individual peaks for both the actives of the sample chromatograms were identified by comparing them with the standards and their respective retention times.

DISCUSSION

Ascorbic and Gallic Acid act as antioxidants and play an important role in boosting immunity. These are commonly used in granules as antioxidants. These ingredients were selected for study. For the simultaneous identification, quantification, and assay of ascorbic and gallic acids, an HPLC method that is quick, easy, reliable, and validated has been developed. The developed method demonstrated selectivity, reproducibility, and robustness for the selected compounds, achieving good resolution between the peaks. This method is easily adoptable for routine analysis in quality control labs for products containing these actives. This RP-HPLC method was also found to be linear, sensitive, precise, robust, and accurate after successfully

Table 2: Ascorbic acid and Gallic acid standards validation parameter results.

Parameters	Std Ascorbic acid	Std Gallic acid
Range (mg/mL)	0.001075 - 1.075	0.001082 - 1.082
Linearity	y = 526038x + 6236.6	y = 292479x - 2054
Correlation coefficient (r^2)	0.9947	0.9932
Detection Limit (ppm)	0.01	0.01
Quantification Limit (ppm)	0.03	0.03

Note: Where, "x" is concentration (mg/mL) of Ascorbic acid/Gallic acid in and "y" is area of peak at 250 nm.

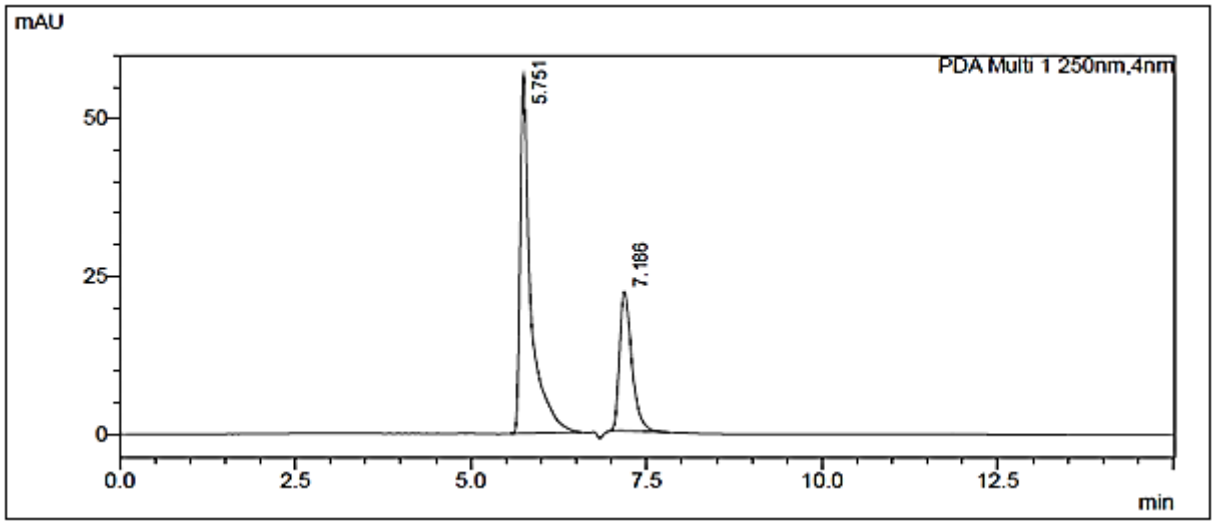

Figure 3: Standard Ascorbic Acid (RT-5.75 min) and Gallic Acid (RT-7.18 min)-HPLC Chromatogram.

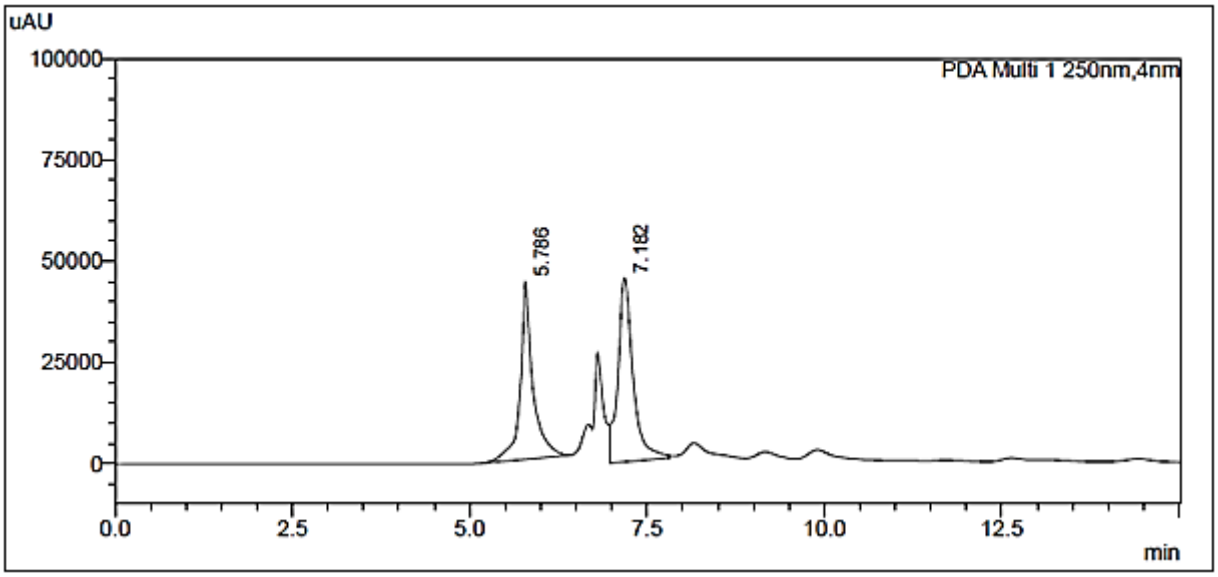

Figure 4: Ascorbic Acid (RT-5.78 min) and Gallic Acid (RT-7.18 min) in Granules sample-HPLC Chromatogram.

Table 3: Ascorbic and Gallic Acid in Granules sample -validation parameters results.

Parameters	Ascorbic acid	Gallic acid	Acceptance-criteria
Linearity Range in (mg/mL)	2.559 - 15.06	2.559 - 15.06	(r^2) 0.99
Regression	y = 52134x + 20511	y = 41045x + 16795	
Correlation coefficient (r^2)	0.995	0.9916	
Precision (RSD%)	1.11	0.76	RSD ≤ 2.0
a. Intra-day			RSD-(Relative standard deviation)
b. Inter-day	1.1	1.43	
Robustness			RSD ≤ 2.0
1. Change in ratio of Mobile phase ratio			
a) Mobile phase Ratio A: B 83:17 v/v	1.148	1.552	
b) Mobile phase Ratio A: B 87:13v/v	0.393	0.772	
2. Change in Flow rate (mL/min)			
a) Flow rate change -0.40	1.437	1.238	
b) Flow rate change - 0.60	1.151	0.47	
Accuracy			90-110%
50% spiked level (% recovery)	99.59%±0.511	98.35%±0.47	
100% Spiked level (% recovery)	100.34%±0.60	97.77%±1.26	
150% Spiked level (% Recovery)	101.31%±0.43	99.10%±0.30	

being validated in compliance with ICH Q2 (R1) guidelines. The mobile phase was optimized to be a simple isocratic system, making it easier to compare with previously reported complex gradient systems. The sample preparation method was simple with no derivatization and complex preparation requirement, the run time for this method was optimised to 15 min, which is shorter than previously reported methods. Due to the shorter run time, solvent consumption is reduced in comparison to traditional HPLC method, thus lowering the analysis cost. This validated method is able to quantify and separate both ascrobic and gallic acids with good resolution in complex natural extract as well as in granules formulation. In addition to facilitating quick product development, the data covered in this study ensures the product's quality and safety.

Table 4: The content of Ascorbic and Gallic Acid in extracts and Granules formulation.

Sl. No.	Sample	% Ascorbic acid	% Expected	% Gallic acid	% Expected
1	Amla extract	0.085	-	0.14	-
2	Chyavanprash extract	0.162	-	1.03	-
3	Granules F06	0.079	0.072-0.088	0.261	0.248-0.303

CONCLUSION

A robust RP-HPLC method was developed and validated for the quantification of ascorbic and gallic acids. The method adhered to ICH guidelines, ensuring accuracy, precision, linearity, and specificity. It demonstrated excellent resolution for both compounds within a shorter analysis time using a simple isocratic mobile phase, making it suitable for routine analysis and high-throughput applications. This method holds potential for increased utility in the food and herbal pharmaceutical industries, with further optimization for more complex matrices.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

R&D: Research and development**; UV:** Ultraviolet, **RP:** Reversed Phase; **HPLC:** High Performance Liquid Chromatography; **ICH:** International Council for Harmonisation; **C18:** Carbon-18; **mm:** Millimetre, **mg:** Milligram; **mL:** Millilitre**, nm:** Nanometre; **µL:** Microliter.

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