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RESEARCH ARTICLE

Estimation of Biomarkers in an Antianaemic Polyherbal Oral Liquid Formulation using HPTLC and Simultaneous Estimation of Ascorbic Acid, Gallic Acid and Resveratrol Using the RP-HPLC Method

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Abstract

Background: Anaemia affects over two billion people globally, and there is growing interest in natural, polyherbal remedies as safe and effective alternatives to conventional therapies. Standardisation of such formulations is essential for ensuring quality, safety, and efficacy. This study aims to develop and standardise a polyherbal oral liquid formulation with antianaemic potential and to quantify the key bioactive markers ascorbic acid, gallic acid, and resveratrol using validated High Performance Thin Layer Chromatography (HPTLC) and Reverse Phase- High Performance Liquid Chromatography (RP-HPLC) methods. **Methods:** Plant materials (*Punica granatum*, *Boerhaavia diffusa*, *Vitis vinifera*, and *Terminalia belerica*) were collected, authenticated, and extracted using hydroalcoholic and freeze-drying techniques. A polyherbal oral liquid formulation was developed and subjected to macroscopic, physicochemical, and phytochemical evaluations. Total phenolic content was estimated via the Folin-Ciocalteu method. Biomarkers were quantified using HPTLC and simultaneously estimated by RP-HPLC, followed by method validation for precision, linearity, accuracy, Limit of Detection (LOD), Limit of Quantification (LOQ) and robustness as per International Council on Harmonisation (ICH) guidelines. **Results:** The HPTLC analysis revealed the presence of ascorbic acid (0.021%), catechin (0.09%), gallic acid (0.21%), and resveratrol (0.004%). RP-HPLC quantification in the formulation showed ascorbic acid (1.12 ± 0.214 mg/g), gallic acid (2.25 ± 0.114 mg/g), and resveratrol (0.042 ± 0.314 mg/g). The analytical method showed high linearity ($R^2 > 0.99$), precision (RSD < 2%), and acceptable LOD and LOQ values. **Conclusion:** The developed polyherbal oral liquid formulation was successfully standardised and validated. The study confirmed the presence and quantity of key antioxidant and hematinic biomarkers, supporting its potential as a safe, natural antianaemic therapy.

Major Findings: The polyherbal oral liquid formulation was standardised and validated using HPTLC and RP-HPLC. Quantification confirmed significant levels of gallic acid (2.25 mg/g), ascorbic acid (1.12 mg/g), and trace resveratrol (0.042 mg/g), supporting its antianaemic potential.

Keywords: Antianaemic Activity, Ascorbic Acid, Phenolic Content, Polyherbal Formulation, Resveratrol Analysis, RP-HPLC Method Validation

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1. Introduction

Traditional medicinal systems, such as *Ayurveda, Siddha*, and *Unani*, have long utilised herbal formulations for the prevention and treatment of diseases, including anaemia, a global health issue affecting over two billion people, particularly in developing countries[1]. Anaemia, characterised by decreased haemoglobin levels and red blood cell count, leads to fatigue, weakness, and impaired immune response. While synthetic iron supplements are commonly used, they often cause gastrointestinal disturbances and poor patient compliance[1]. As a result, the search for safe, effective, and natural alternatives has intensified. Polyherbal formulations have gained prominence due to their synergistic therapeutic effects, minimal side effects, and broad-spectrum action. Specifically, medicinal plants such as *Punica granatum*[2], *Vitis vinifera*[3], *Boerhaavia diffusa*[4], and *Terminalia belerica*[5] are known to be rich in phenolic compounds, flavonoids, and other bioactive constituents that exhibit antioxidant, immunomodulatory, and hematinic properties[5-7]. These bioactives, particularly ascorbic acid, gallic acid, catechin, and resveratrol, contribute significantly to red blood cell production, iron absorption, and oxidative stress reduction[8]. Despite the popularity of such formulations, there is limited literature on the standardisation and simultaneous quantitative estimation of these key biomarkers in a combined polyherbal matrix using advanced chromatographic techniques. Thus, scientific validation of the active constituents in such formulations is critical to ensure therapeutic consistency, safety, and efficacy. Although numerous studies have reported the individual pharmacological benefits of *Punica granatum*, *Vitis vinifera*, *Boerhaavia diffusa*, and *Terminalia belerica,* there is a notable lack of comprehensive standardisation data for a combined polyherbal oral liquid formulation targeting anaemia. Additionally, the simultaneous quantification of major antioxidant and hematinic phytoconstituents such as ascorbic acid, gallic acid, and resveratrol using validated HPTLC and RP-HPLC techniques remains unexplored. Most previous works focus on single herbs or crude extracts without robust analytical validation or dosage form development. The primary aim of this study was to develop and validate a polyherbal oral liquid formulation containing extracts of *Punica granatum*, *Vitis vinifera*, *Boerhaavia diffusa*, and *Terminalia belerica* for potential antianemic use. The formulation was evaluated for its phytochemical content, particularly total phenolics, followed by the standardisation and simultaneous quantitative estimation of key biomarkers ascorbic acid, gallic acid, and resveratrol using HPTLC and RP-HPLC. The methods were validated under ICH guidelines to ensure accuracy, precision, repeatability, linearity, LOD/LOQ, and robustness. This research contributes to the scientific standardisation of herbal formulations, ensuring reproducibility, regulatory compliance, and improved therapeutic efficacy.

2. Materials and Methods

2.1 Authentication, Macroscopic Analysis, and Physicochemical Parameters

Medicinal plant materials used in the formulation were procured from local herbal markets in Chennai and Villuppuram, Tamil Nadu. These included *Punica granatum*, *Boerhaavia diffusa*, *Vitis vinifera*, and *Terminalia belerica*. Authentication of the plant materials was carried out by the Siddha Central Research Institute, Arumbakkam, Chennai, Tamil Nadu, India. *P. granatum* (P15052401G), *B. diffusa* (B15252404D), *V. vinifera* (V15052402V), and *T. belerica* (T15052403D). Organoleptic and macroscopic characteristics, including colour, odour, taste, texture, and morphology, were observed for each plant sample. Data were recorded following standard pharmacognostical parameters. Physicochemical analysis was performed on individual plant powders and the final Polyherbal Formulation (PHF). Parameters such as total ash, acid-insoluble ash, loss on drying, pH, alcohol-soluble and water-soluble extractive values were determined using World Health Organisation (WHO) guidelines for crude drug standardisation[9].

2.2 Hydroalcoholic Extraction

Coarsely powdered plant materials *(B. diffusa* root, *T. belerica* fruit, *V. vinifera* fruit) were subjected to Soxhlet extraction using a 1:1 ethanol: water mixture. For each extraction, 100g of material was extracted for 8 hours, repeated to obtain a sufficient yield. The extract was concentrated under reduced pressure using a rotary evaporator. Fresh juice of *P. granatum* fruit pulp was

freeze-dried at –8°C with a coolant circulation of 3000 mL/min. The operation ran for 20 minutes, yielding an 8% Brix concentrate[10].

2.3 Development of Polyherbal Oral Liquid Formulation

A polyherbal liquid formulation was prepared using standard industrial mixing equipment, including an SS mixing vessel, a Remi mechanical stirrer, and a colloidal mill. The formulation included the plant extracts along with excipients: Sorbitol 70%, glycerin, potassium sorbate, hydroxyethyl cellulose, sucralose, citric acid, polyoxyl 40 hydrogenated castor oil, disodium EDTA, grape flavour, purified water, and *Kassishaka Suddha* (Ferrous sulfate).

2.4 Preliminary Phytochemical Evaluation and Estimation of Total Phenolic Content

Qualitative phytochemical screening was performed to detect the presence of glycosides, alkaloids, flavonoids, phenols, tannins, saponins, carbohydrates, and proteins using standard methods. Total Phenolic Content (TPC) was quantified using the Folin-Ciocalteu method. Each sample (2.5mL) was mixed with diluted Folin reagent and sodium bicarbonate, incubated, and absorbance was read at 765nm. TPC was calculated in terms of mg of Gallic Acid Equivalents (GAE) per gram of extract[11].

2.5 HPTLC Method for Quantification and Estimation of Biomarkers

Standard Preparation Standards (gallic acid, ascorbic acid, catechin, and resveratrol) were prepared at 100 µg/mL in methanol and filtered through 0.45µm PVDC filters. Sample Preparation 5mL of PHF was extracted with ethyl acetate for 3 hours. The solvent layer was concentrated, dissolved in methanol, and filtered through a 0.45µm Polyvinylidene Chloride (PVDC) filter. Samples were spotted on precoated silica gel aluminium plates (10x20cm, 250µm) using a Camag autosampler. Plates were activated at 110°C for 10 minutes. Mobile phase: toluene: ethyl acetate: formic acid: dichloromethane (6:4:0.9:0.5). Scanning was performed at 250 and 366nm using a TLC scanner with WinCATS software. Ascorbic acid, gallic acid, and resveratrol (analytical grade), HPLC-grade acetonitrile, and potassium dihydrogen phosphate buffer were used. RP-HPLC was performed using Waters Alliance e2695 with a Photodiode Array (PDA) detector (2998 model). Separation was done on a Phenomenex C18 column (250×4.6mm, 5µm) using a mobile phase of potassium dihydrogen phosphate buffer: acetonitrile (800:100), flow rate 1 mL/min, 40-minute runtime, dual wavelength detection. Stock solutions (10 mg/mL) were prepared in methanol and filtered through 0.45µm Polyvinylidene Difluoride (PVDF) membranes. Standard curves were plotted for 10-200 µg/mL.

2.6 Method Validation

It was carried out according to the ICH guidelines. System suitability was evaluated by injecting six replicates of the standard solution and observing key parameters such as retention time, tailing factor, and theoretical plates to ensure optimal chromatographic performance. Linearity was assessed for each biomarker across the concentration range of 0–200 µg/mL, with calibration curves plotted and regression data calculated to determine the correlation coefficients. LOD and LOQ were calculated based on the standard deviation of the response and the slope of the calibration curve. System precision was evaluated by injecting the standard solution six times to determine reproducibility. Method precision was assessed through intra-day and inter-day analyses of test samples to evaluate consistency over time. Accuracy was determined by performing recovery studies at three levels (80%, 100%, and 120% of the label claim) using the standard addition method. Robustness was verified by making deliberate variations in method parameters, including flow rate (±0.1 mL/min), detection wavelength (±1 nm), and column temperature (±1°C), to ensure the reliability of the method under small changes in conditions.

3. Results and Discussion

3.1 Pharmacognostic and Physicochemical Evaluation of Selected Indian Medicinal Plants

The macroscopical evaluation provides a comparative analysis of the external morphological features of four selected medicinal plants *Punica granatum*, *Boerhaavia diffusa*, *Vitis vinifera*, and *Terminalia belerica*. The parts used vary across the plants: fruits and seeds for *P. granatum* and *V. vinifera*, root for *B. diffusa*, and fruit for *T. belerica*. In terms of colour,

P. granatum exhibits a yellowish-brown to red hue with whitish-pink seeds, while *B. diffusa* is yellowish-brown to brown, *V. vinifera* is dark brown to black, and *T. belerica* is greyish brown with a wrinkled surface. Odour is mostly indistinct in all samples except for *V. vinifera*, which has a sweet and pleasant aroma. The taste profile ranges from sour-sweet in *P. granatum* to slightly bitter in *B. diffusa*, sweet in *V. vinifera*, and astringent in *T. belerica*. Regarding shape, the fruits and roots vary: *P. granatum* is rounded and hexagonal, *B. diffusa* cylindrical, *V. vinifera* oblong, and *T. belerica* spherical. The texture also differs significantly. *P. granatum* is coriaceous with hard seeds, *B. diffusa* is rough due to longitudinal striations, *V. vinifera* is smooth, and *T. belerica* has a pubescent (hairy) surface. These distinct macroscopical characteristics help in the proper identification and quality assessment of the crude drug materials as described in Table 1. The proximate analysis is used to assess the quality and purity of pharmaceutical plant materials in their crude form. Impurities such as carbonates, oxalates, and silicates are identified through ash value determination. Total ash, comprising both physiological and non-physiological components, reflects the presence of extraneous matter. Adulteration in plant drugs is typically detected through extractive values. The physicochemical analysis of the selected Indian medicinal plants included evaluation of extractive values, Loss on Drying (LOD), ash values, and pH, with all data presented in Table 2. The percentage yields from plant extractions were found to be hydro-alcoholic extract of *Boerhaavia diffusa* root – 9.3%, *Terminalia belerica* fruit – 17.3%, *Vitis vinifera* – 8.6%, and freeze-dried fruit juice of *Punica granatum* – 7.3%.

3.2 Formulation and Evaluation of Herbal Syrup Containing *Kassishaka Suddha*

The process involves transferring a non-crystallising solution of sorbitol 70% to a mixing vessel. Potassium sorbate is then dissolved in purified water and added to the sorbitol solution. Next, hydroxyl ethyl cellulose is dispersed in glycerin and added to the mixture, followed by the addition of purified water. The mixture is thoroughly stirred until it is swollen. The hydroxyethyl cellulose solution is then transferred to a mixing jar that contains sorbitol. The *Kassishaka Suddha* (Ferrous Sulphate) was dissolved in purified water with citric acid. The resulting solution was then transferred to a mixing vessel. Next, the individual extract was dissolved in purified water along with polyoxyl 40 hydrogenated castor oil, and this mixture was also transferred to the mixing vessel. Finally, sucralose was dissolved in purified water and transferred to the mixing vessel. Each ingredient is added and combined for approximately 10 minutes. The mixture is then processed in a colloidal mill at a speed of 10000 rpm for 10 minutes. Grape flavour is then added, and the volume is adjusted with purified water. The physical properties of the syrup formulation were assessed, and the following observations were made: viscosity, ranging from 120 to 150 centipoise (cps), pH - 5.1, specific gravity - 1.150.

3.3 Phytochemical Screening and Estimation of Total Phenolic Content in Individual Extracts and Polyherbal Formulation

The preliminary phytochemical evaluation revealed the presence of various bioactive constituents across the tested plant extracts: *Punica granatum* (PG), *Boerhavia diffusa* (PD), *Vitis vinifera* (VV), *Terminalia*

Table 1. Macroscopical evaluation of selected medicinal plants

Parameters	*Punica granatum*	*Boerhaavia diffusa*	*Vitis vinifera*	*Terminalia belerica*
Parts Used	Fruit, Seed	Root	Fruit, Seed	Fruit
Colour	Yellowish brown to red and seed whitish-pink	Yellowish brown to brown colour	Dark brown to black	Greyish brown with a wrinkled appearance
Odour	Not distinct	No distinct odour	Sweet pleasant	No distinct odour
Taste	Sour to sweet, and seeds are astringent	Slightly bitter	Sweet	Astringent
Shape	Rounded hexagonal shape	Cylindrical	Oblong	Spherical
Texture	Coriaceous and seed hard	Rough due to minute longitudinal striation	Smooth	Pubescent surface

Table 2. Physiochemical analysis of selected medicinal plants for polyherbal syrup

Ingredients	*Punica granatum**	*Boerhaavia diffusa*	*Vitis vinifera*	*Terminalia belerica*
Total Ash %	1.02 ± 0.251	6.80 ±0.300	1.251 ±0.452	2.16 ± 0.173
Acid Insoluble Ash %	0.12 ±0.025	0.46 ± 0.060	0.080±0.021	0.21 ±0.030
Foreign Matter %	Nil	1.13 ± 0.306	1.25 ± 0.106	0.20 ±0.100
Loss on drying %	3.012 ± 0.256	4.03 ± 0.208	11.21± 0.508	6.56 ±0.603
Alcohol Soluble Extractive %	15.01 ± 0.526	21.33 ± 1.528	26.25± 0.586	18.60 ± 0.706
Water Soluble Extractive %	75.02 ±0.245	35.33 ± 0.577	73.56 ± 0.977	36.46 ± 0 461

*Physicochemical analysis was done for fruit juice powder. The values are provided as a mean with standard deviation (n=3)

belerica (TB), and a Polyherbal Formulation (PH). All samples showed the presence of glycosides, flavonoids, carbohydrates, phenols, and proteins and amino acids, with the polyherbal extract exhibiting the most comprehensive phytochemical profile. Notably, phenols were strongly present in all except PD, while flavonoids were particularly abundant in VV. Alkaloids and saponins were strongly present in PD and TB, suggesting potential anti-inflammatory or antimicrobial properties. Gums and mucilage were absent in PG and PD but present in the other extracts listed in Table 3. Volatile oils were absent across all samples. The strong presence of multiple bioactive groups in the polyherbal extract supports its potential for synergistic therapeutic activity, especially for antioxidant and anti-inflammatory applications. An analysis was conducted on the hydro alcoholic extract of each drug and polyherbal formulation to evaluate the concentration of total phenols. The findings of this analysis were presented. From the estimation, the total phenolic compounds were found in individual extracts *Punica granatum* 28.45 ± 2.35, *Boerhaavia diffusa* 22.135 ± 4.25, *Vitis vinifera* 36.128 ± 5.23, *Terminalia belerica* 32.813 ±4.35 and *Polyherbal formulation* 35.014 ±2.35 and the standard calibration curve was plotted against absorbance on the y-axis and concentration at the x-axis and the linear regression R^2 0.992 (Figure 1)

Figure 1 represents the standard calibration curve constructed for the quantification of total phenolic content using a spectrophotometric method. The x-axis shows the concentration of the standard (in mg/gm), while the y-axis denotes the corresponding absorbance values. The calibration curve exhibits a strong linear relationship with the regression equation y = 1.2248x + 0.0026 and a correlation coefficient (R^2 = 0.9928), indicating excellent linearity and reliability of the method. This curve serves as a reference for estimating the phenolic content in plant extracts or formulations by interpolating absorbance values of unknown samples.

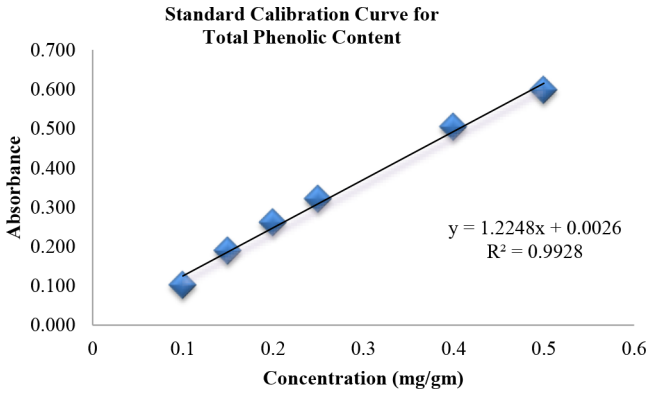


Figure 1. Standard calibration curve for the estimation of total phenolic content.

3.4 HPTLC Method Development and Quantification of Bioactive Markers in Polyherbal Formulation

The HPTLC method was optimised to quantify the ascorbic acid, catechin, gallic acid, and resveratrol in the polyherbal formulation (Table 4 and Figure 2). The mobile phase Toluene: ethyl acetate: acetic acid: dichloromethane (6:4:0.9:0.5 v/v/v/v) gave good resolution with R_f = 0.10 of ascorbic acid, R_f = 0.34 of catechin, R_f = 0.41 of gallic acid, and R_f = 0.64 of resveratrol. Under the optimised chromatographic conditions, the individual plant extract and polyherbal formulation showed sharp peaks and good separation when scanned at 254nm and 360nm.

Figure 2 presents a comprehensive HPTLC profile of various medicinal plant extracts and their polyherbal formulation, visualised at 360nm under Ultraviolet (UV) light. The top and bottom rows

Table 3. Preliminary phytochemical evaluation

Test of Constituents	Glyco sides	Ster oids	Flavon oids	Alkal oids	Proteins and Amino Acids	Carbo hydrates	Gums and Mucilage	Fats and fixed oils	Volatile Oils	Sapo nins	Phe nols
PG	+	+	+	-	++	++	-	++	-	-	++
PD	++	+	+	++	+	+	-	+	-	++	+
VV	+	-	++	-	++	++	+	+	-	+	++
TB	++	+	+	++	++	++	+	+	-	-	++
PH	++	+	+	+	++	++	+	+	-	+	++

(+)- Present; (++) - Strongly present; (-) - Absence, PG-*Punica granatum*, BD - *Boerhavia diffusa*, VV - *Vitis vinifera*, PG - *Punica granatum*, PH-Polyherbal extract

Table 4. HPTLC quantification of biomarker in developed formulation

Marker Name	Mobile Phase	Derivatisation	Wave Length	R_f Value	Quantification
Ascorbic acid	Toluene: Ethyl acetate: Acetic Acid: Dichloromethane (6:4:0.9:0.5)	Anisaldehyde Sulphuric acid reagent	254 nm	0.10	0.021 %
Catechin			254 nm	0.38	0.09 %
Gallic acid			366 nm	0.44	0.21%
Resveratrol			366 nm	0.67	0.004 %

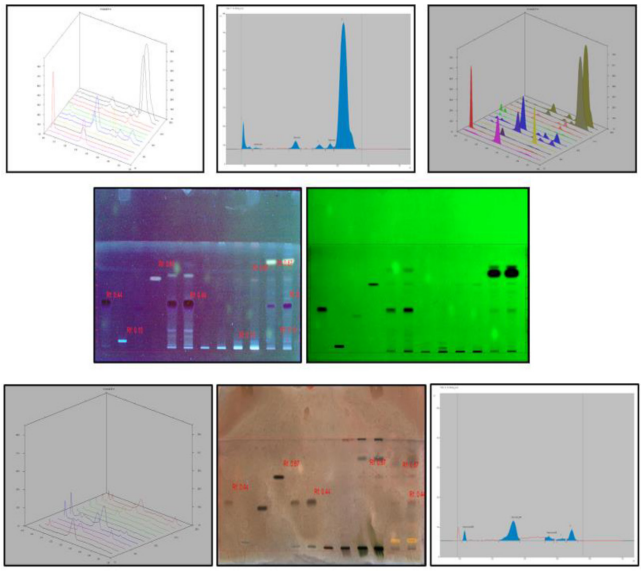


Figure 2. HPTLC fingerprint analysis and chromato gram.

include 3D densitometric chromatograms and 2D chromatographic peaks, demonstrating compound separation and intensity profiles. The central images capture the developed TLC plates under UV light at 360nm (left) and post-derivatisation or visualisation under different illumination (right), showcasing the resolved bands corresponding to phytochemical constituents. Clear differences in band intensity and Rf values indicate the presence and relative concentration of bioactive compounds in the individual and combined extracts. These chromatographic patterns serve as unique fingerprints for the standardisation and authentication of the tested samples.

3.5 RP-HPLC-Based Quantitative Estimation and Validation of Biomarkers in Polyherbal Formulation

The RP-HPLC method was employed to separate and quantify three compounds such as Ascorbic acid was eluted at 3.605 minutes, and its concentration in the sample was 1.12 mg/g of dry weight, with a variability of ±0.214 mg/g, indicating a moderate presence and reliable quantification. Gallic acid appeared at 6.483 minutes and was found to be 2.25 mg/g ± 0.114, the highest concentration among the three compounds, suggesting it is the major phenolic constituent in the sample. Resveratrol had a retention time of 16.480 minutes and was present at a very low level of 0.042 mg/g ± 0.314. The high standard deviation compared to the mean suggests variability or detection near the quantification limit, which may affect the reliability of this value presented in Table 5 and Figure 3. The analysis indicates that Gallic acid is the predominant compound in the sample, followed by Ascorbic acid, while Resveratrol is present in trace amounts with less quantifiable precision. The retention times confirm good chromatographic separation. The data presented

in Table 6 demonstrate the validation parameters LOD, LOQ, linearity, and regression equations for ascorbic acid, gallic acid, and resveratrol using the proposed RP-HPLC method. The concentration range for all three compounds was 0-200 µg/mL, indicating the method's wide applicability for quantitative analysis across a broad spectrum. The LOD and LOQ values indicate the method's high sensitivity, particularly for resveratrol (LOD: 0.64 µg/mL, LOQ: 2.25 µg/mL) and gallic acid (LOD: 0.82 µg/mL, LOQ: 2.68 µg/mL), making it suitable for detecting even trace amounts of these compounds. The regression equations and correlation coefficients (R^2) confirm excellent linearity, such as Ascorbic acid: $R^2 = 0.996$; Gallic acid: $R^2 = 0.998$; Resveratrol: $R^2 = 0.999$. These values (>0.99) reflect a strong linear relationship between concentration and peak area, essential for accurate quantification. The proposed RP-HPLC method is proven to be sensitive, linear, and reliable for the estimation of ascorbic acid, gallic acid, and resveratrol in the polyherbal formulation. Every compound had a relative standard deviation (RSD) of less than 2%, which suggests a high degree of repeatability mentioned in Table 7. For ascorbic acid, the percentage recovery ranged from 99.561% to 99.960%, with RSD values below 1.05%, indicating excellent accuracy and consistency across all levels. Gallic acid showed recovery rates between 99.816% and 100.205%, with slightly higher RSDs at some levels but still within acceptable limits (≤1.35%), confirming good method reproducibility. In the case of resveratrol, recovery values were 99.569% to 101.230%, with RSD values ranging from 0.536% to 1.545%, showing that the method is sufficiently accurate and reliable for low concentration markers as well. Overall, the results confirm that the method is accurate, precise, and robust, with percentage recovery rates close to 100% and RSD values below 2%, thereby validating the reliability of the method for quantitative estimation of the selected biomarkers in the formulation.

Figure 3 illustrates the High-Performance Liquid Chromatography (HPLC) chromatogram of the developed polyherbal formulation, showing the retention times and peak intensities of key phytoconstituents. Prominent peaks are observed at

Table 5. Quantification value by RP-HPLC

Compounds	Retention Time (min)	Quantified Value (mg/gm)
Ascorbic Acid	3.605	1.12 ± 0.214
Gallic Acid	6.483	2.25 ± 0.114
Resveratrol	16.480	0.042 ± 0.314

The values are provided as a mean with standard deviation (n=3)

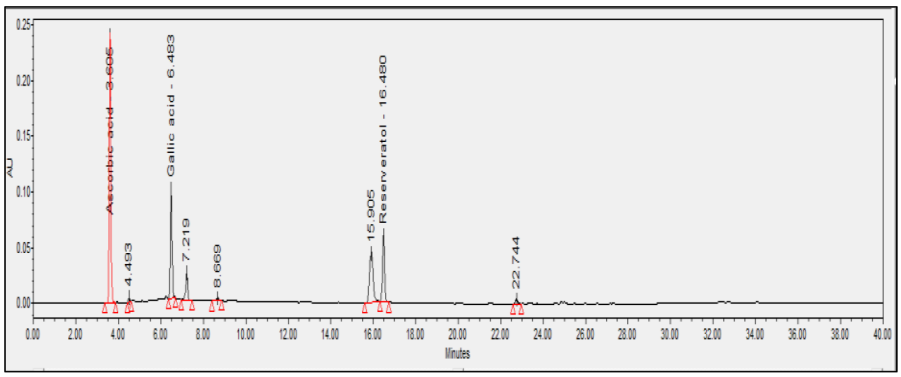


Figure 3. HPLC chromatograph of the developed formulation.

Table 6. Limit of detection (LOD), regression parameter, limit of quantification (LOQ), and linearity of the proposed RP-HPLC method

Compounds	Concentration Range (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	Regression Equation	R^2
Ascorbic acid	0-200	10.12	39.85	y=10288x-62210	0.996
Gallic acid	0-200	0.82	2.68	y=5900.x+60436	0.998
Resveratrol	0-200	0.64	2.25	y=92166x-1E+06	0.999

The values are provided as a mean with standard deviation (n=3)

Table 7. Recovery study of ascorbic acid, gallic acid, and resveratrol in developed polyherbal formulation

Com pounds	Percentage of Standard added in placebo (%)	Percentage Recovery Rate	RSD (%)
Ascorbic acid	80	99.921 ± 0.458	1.045 ±0.002
	100	99.960 ± 0.356	0.645 ±0.012
	120	99.561 ± 1.253	0.445 ±0.008
Gallic acid	80	100.205 ± 0.218	1.345 ±0.013
	100	99.862 ± 0.658	0.565 ±0.005
	120	99.816 ± 0.895	1.245 ±0.011
Resve ratrol	80	99.569 ± 0.265	0.656 ±0.008
	100	101.230 ± 0.758	1.545 ±0.035
	120	100.233 ± 0.325	0.536 ±0.004

The values are provided as a mean with standard deviation (n=3)

4.438 minutes for Ascorbic acid, 6.483 minutes for Gallic acid, and 16.480 minutes for Resveratrol, indicating their successful identification and quantification within the formulation. The presence of these bioactive compounds confirms the formulation's phytochemical richness. Minor peaks at other retention times suggest the presence of additional phytoconstituents or matrix components. The sharp and well-resolved peaks demonstrate good chromatographic separation and method specificity, supporting the reliability of the developed RP-HPLC method for quality control and standardisation purposes.

4. Conclusion

The present study successfully formulated and standardised a polyherbal oral liquid containing extracts of *P. granatum*, *B. diffusa*, *V. vinifera*, and *T. belerica*, targeting the management of anaemia. Comprehensive qualitative and quantitative analyses were conducted to evaluate its phytochemical profile and antioxidant potential. The HPTLC method enabled simultaneous identification and quantification of key biomarkers ascorbic acid, catechin, gallic acid, and resveratrol in both individual plant extracts and the final formulation. Additionally, a validated RP-HPLC method was developed for accurate, reproducible, and robust estimation of ascorbic acid, gallic acid, and resveratrol. The method demonstrated excellent linearity, sensitivity, precision, and recovery within acceptable ICH guidelines. The presence of high levels of phenolic and flavonoid content confirms the formulation's therapeutic potential, particularly due to its antioxidant and hematinic constituents. This study lays the foundation for further pharmacological and clinical investigations of the formulation as a standardised, natural antianaemic remedy.

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6. Author Contribution

R. Kopperundevi contributed to the conceptualisation, design of the study, and draft. Dr. K. Shalini was involved in data collection, formal analysis, and manuscript review. Dr. N. Damodharan supervised the overall work, performed validation, and served as the corresponding author. All authors have read and agreed to the published version of the manuscript.

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