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Article

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Quantifying Curcumin, Gallic Acid, and Resveratrol in a Polyherbal Mixture: a Robust HPTLC Method

The need for this study arises from the growing importance of polyherbal formulations in herbal medicine, particularly due to their potential health benefits and synergistic effects. In this research, development and validation of an HPTLC method for simultaneous quantification of curcumin, gallic acid, and resveratrol in a polyherbal mixture are primary focus. Additionally, study assesses the Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and antioxidant potential using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The results reveal that TPC is 212.1 mg gallic acid equivalent per gram of plant material, and the TFC is 133.06 mg rutin equivalent per gram of plant material. Notably, DPPH assay demonstrates mixture's robust antioxidant activity, with an IC_{50} value of 3.83 µg/ml. Furthermore, HPTLC method used a carefully optimized mobile phase, providing excellent resolution for gallic acid, resveratrol, and curcumin with corresponding R_f values of 0.18, 0.43, and 0.53. This method is highly linear over a concentration range of 200–1000 ng/band for all three compounds. Precision studies confirm method's reproducibility with low Relative Standard Deviation values below 2 %. Ultimately, this HPTLC method offers a simple, accurate, precise, and cost-effective solution for routine quality control analysis, shedding light on potential applications of the polyherbal mixture in various therapeutic contexts.

Keywords: HPTLC, curcumin, gallic acid, resveratrol, simultaneous estimation, standardization, anti-oxidant, validation, polyherbal mixture.

Introduction

Since ancient times, herbal drugs have been a blessing to human kind. Worldwide, both single plant extracts and mixtures of herbs are utilized to treat a variety of human disorders [1]. The World Health Organization (WHO) estimates that the usage of herbal remedies is two to three times more frequently than conventional drug globally. Natural compounds contain a variety of chemically active substances which are accountable for their vast pharmacological activity. In recent pharmacology and development of drug, a single chemical substance is accountable for the majority of the beneficial effects of the drug, while in Ayurvedic preparations either a single herb or multiple herb known as polyherbal formulation are responsible for the activity [2]. In polyherbal formulations, the synergistic effects of many medicinal plants contribute to increasing the drug efficacy [3]. These Ayurveda polyherbal formulations have a challenging task involving consistency in quality control, which will guarantee the finished herbal products' therapeutic effectiveness as promised by the accepted Ayurveda texts. The wide varieties of chemical compounds are difficult to analyze, authenticate, and separate fast with appropriate accuracy, precision, and reproducibility [4].

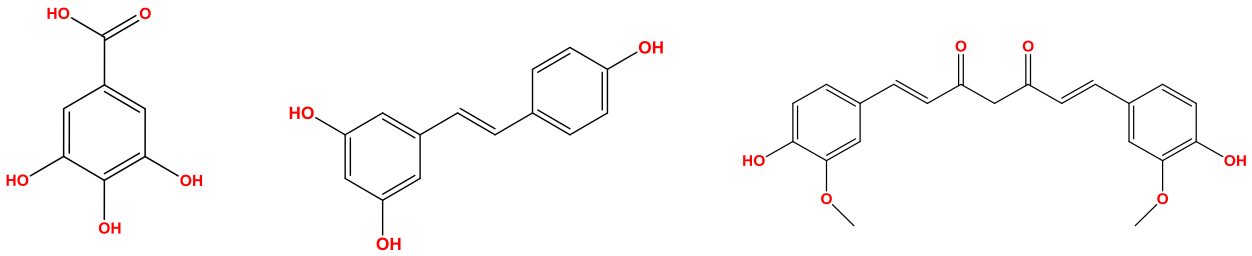
Polyherbal formulation refers to a medicinal or therapeutic preparation that contains a combination of multiple herbs or botanical extracts. These formulations are created by combining different plant-based ingredients known for their therapeutic properties to achieve synergistic effects and enhance the overall efficacy of the formulation [3]. These are typically developed with the intention of providing a synergistic effect by combining the therapeutic properties of various medicinal plants. An in-house polyherbal mixture was prepared which included *Vitis vinifera, Panax ginseng, Withania somnifera, Tinospora chordifolia, Curcuma longa, Zingiber officinale* and *Glycyrrhiza glabra.* The grapevine, or *Vitis vinifera*, contains a number of biologically active components, including anthocyanins, proanthocyanidins, polyphenols, flavonoids and

resveratrol derivative of the stilbene family [5]. *Vitis vinifera* is the most abundant source of resveratrol, a naturally-occurring phytoalexin and polyphenol compound. Resveratrol possesses biological properties that offer health advantages, including safeguarding against conditions like atherosclerosis, coronary heart disease, and cancer [6]. These substances have been proven to exhibit a variety of pharmacological effects, which include antiviral, anti-inflammatory, antimicrobial, and antioxidant properties. These properties have health benefits for human, including reducing lipoproteins of low-density, reducing the risk of heart related disease, cancer, respiratory and gastrointestinal issues, and boosting the immunity [7]. *Panax ginseng* holds a significant position among the tonic treatments used in oriental medicine. *Ginseng* is a high-grade plant with numerous pharmacological properties, including restorative, tonic, nootropic, antiaging, and others [8]. *Curcuma longa* or turmeric is significant aromatic and medicinal plant used as a spice, medicine, cosmetic, and dye [9]. The most significant component of turmeric is curcumin which is responsible for its biological effects. Curcumin offers a numerous beneficial therapeutic benefits, including antineoplastic, antiapoptotic, antiangiogenic, cytotoxic and immunomodulatory [10]. *Glycyrrhiza glabra* is a widely used herb in the ancient Ayurvedic medical tradition, as a medicine and also utilized as a flavouring agent. *Licorice* is used to treat inflammation, liver problems as well as peptic ulcers, arthritic ailments, and gastrointestinal infections. *Licorice* is also used as an antidote to the toxicity of chemotherapy [11].

Standardization of natural substances is a difficult task because of their varied contents. Herbal products must be controlled precisely to ensure reproducible quality. Therefore, standardizing crude drugs poses many challenges. On the other hand, standardization of herbal extract helps in maintaining the quality of the extract and thus its efficacy [12]. To properly identify natural drugs, one should possess an authentic reference standard. A marker is a compound that is unique to the plant being studied and is present in quantifiable level and can be easily separated [13].

HPTLC has been extensively used for the evaluation of herbal drugs and has evolved into standard analytical method owing to its beneficial aspects, including being economical, having a high sampling throughput, and requiring little sample preparation [14–16]. Gallic acid (3,4,5-trihydroxybenzoic acid), resveratrol (3,5,4′-Trihydroxystilbene) and curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione) (Fig 1) have been analyzed by HPLC and HPTLC either singly or with one another but simultaneous estimation of all three is not reported till date [17–20].

The aim of the current study is to develop a simple, precise and reproducible HPTLC method for estimation of gallic acid, resveratrol and curcumin simultaneously in an in-house polyherbal mixture. Developed method is also validated as per ICH Validation Guidelines [21]. Quantitative phytochemical estimation of the mixture for total phenol and flavonoid was also performed along with its anti-oxidant potential.



a) gallic acid b) resveratrol c) curcumin

Figure 1. Objects of the study

Experimental

Materials

A CAMAG HPTLC system comprising semi-automatic sample applicator Linnomat-5,CAMAG TLC plate scanner, CAMAG WINCAT software (version 1.44 CAMAG) and Hamilton Syringe (100 µL) were used during analysis.

Standard resveratrol (Sigma Aldrich), gallic acid (Loba Chemical), 2,2-diphenylpicrylhydrazyl (DPPH) (Sigma Aldrich), rutin (Loba Chemical), ascorbic acid (Sigma Aldrich) and other Analytical Research (AR) Grade chemicals were used.

Methodology

Formulation of polyherbal mixture (PM)

The formulation of a polyherbal mixture involves combining multiple herbal ingredients to create a synergistic blend with desired therapeutic effects. The polyherbal mixture includes *Vitis vinifera, Panax ginseng, Withania somnifera, Tinospora chordifolia, Curcuma longa, Zingiber officinale* and *Glycyrrhiza glabra.* The prepared in-house polyherbal mixture consists of major phytoconstituents as resveratrol, gallic acid and curcumin.

Quantitative phytochemical analysis

Total Phenolic Content (TPC)

The TPC for polyherbal mixture were calculated via the approach of Singleton et al. (1999) with small changes [22]. 0.5 ml of 10 % Folin Ciocalteau Reagent (FCR) was added to 0.2 ml of a test sample, and left for 5 minutes. 2.0 ml of 10 % sodium carbonate was added after 5 minutes. Prepared solution was incubated in dark for 30 minutes. The absorbance was recorded at 765 nm by UV-Visible Spectrophotometer against blank. The TPC content was measured as gallic acid equivalents (GAE)/g of plant material as average of three readings using gallic acid linearity.

Total Flavonoid Content (TFC)

The TFC was evaluated according to the standard aluminum chloride procedure using rutin as the reference standard [23]. 2 ml of plant extract were combined with 0.3 ml of 5% $NaNO_2$. 0.3 ml of 10 % $AlCl_3$ was added after 5 minutes. Then 4 ml of 10% NaOH solution was added to the reaction mixture. Final volume of 10 ml was made using distilled water. Absorbance of the mixture was taken at 510 nm. Rutin standard curve measurements were used to calculate the TFC, which was then reported as rutin equivalent (RU) per g of plant material as average of three readings.

Antioxidant assay by DPPH method

The polyherbal mixture capacity for scavenging free radicals was measured using DPPH radical method as it has capability to donate hydrogen or scavenged radicals [24, 25]. 0.1 mM DPPH solution in methanol was prepared and 2 ml of this solution was added to 2 ml of extract (or standard) solution at varied concentrations (5–50 μg/ml). The absorbance was taken at 517 nm after 30 minutes. Lower absorbance f the reaction mixtures indicated greater free radical scavenging capacity. The following equation was used to determine the capacity to neutralize the DPPH radical:

$$\text{Antioxidant Activity}\left(\%\text{AA}\right)=\frac{\text{absorbance of control}-\text{absorbance oftest}}{\text{absorbance of control}}\times 100$$

The % AA was calculated and average readings were used to plot concentration versus anti-oxidant activity graph.

HPTLC analysis of Resveratrol, Curcumin and Gallic acid

Preparation of standards

All three compounds namely gallic acid, resveratrol and curcumin standard solution were prepared by weighing 1 mg separately and dissolving it in methanol. The volume was made up to 10 ml to obtain a working solution of 100 μg/ml.

Preparation of sample (polyherbal mixture solution)

The sample was prepared by weighing 100 mg of polyherbal mixture and the volume was made up to 10ml with methanol and was sonicated for 30 minutes. The filtrate was used for spotting on the TLC plate.

Chromatographic conditions

The samples were spotted as 6 mm wide band using a Hamilton microliter syringe of 100μL through a regulated nitrogen stream using a Camag Linomat V semi-automatic sample applicator on Merck precoated silica gel aluminium plate $60F_{254}$ (10×10 cm) with 250 μm thickness. Prior to applying the sample, the precoated TLC plate was prewashed with methanol and activated for 10 minutes at 110 °C. Development of method was done in a Camag twin trough chamber (10×10 cm) saturated previously with the mobile phase for 15 minutes. The optimized composition of mobile phase was Toluene: Ethyl acetate: Methanol: Formic acid (7:2:1:0.5 v/v/v/v). The process of densitometric scanning was carried out using the Camag TLC Scanner III, which was operated using the WinCATS software (version 1.44 CAMAG), at 269 nm wavelength.

Validation of HPTLC method

Linearity

Linearity was carried out by using working solution of gallic acid, resveratrol and curcumin separately. The working solution volume 2, 4, 6, 8, 10 µL were spotted on the HPTLC plate to get concentration in the range of 200–1000 ng/spot for gallic acid, resveratrol and curcumin respectively.

Accuracy

To evaluate the precision of the technique, the recovery of gallic acid, resveratrol, and curcumin in a mixture were determined using the standard addition method. In order to assess accuracy, predetermined amounts of gallic acid, resveratrol, and curcumin standard solutions were separately added at levels of 80 %, 100 %, and 120 % to the sample solution with a known quantity. The peak areas were measured, and the percent recovery and relative standard deviation were calculated.

Precision

Reproducibility of test application and determination of area of peak were performed using repetitions of similar concentration (600 ng/band of gallic acid, resveratrol and Curcumin) and were described using the relative standard deviation (%RSD). The intra- and inter-day variation for the determination of gallic acid, resveratrol and curcumin were carried out at concentration levels of 600 ng/band.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD of a specific analytical method is the smallest quantity of analyte within a sample, which can be identified and not certainly quantified as an accurate value. LOD was determined as:

$$LOD = \frac{3.3 \times \text{standard deviation of } y \text{ intercept}}{\text{slope of calibration curve}}$$

The LOQ of as specific analytical method is the smallest quantity of analyte within a sample, which can be quantitatively assessed using adequate precision and accuracy. LOQ was determined as:

$$LOQ = \frac{10 \times \text{standard deviation of } y \text{ intercept}}{\text{slope of calibration curve}}$$

Robustness

By making small changes in the mobile phase volume and saturation time, the effect on R_f and area was studied. The robustness of the technique was examined at a concentration level of gallic acid, resveratrol and curcumin of 600 ng/spot.

Results and Discussion

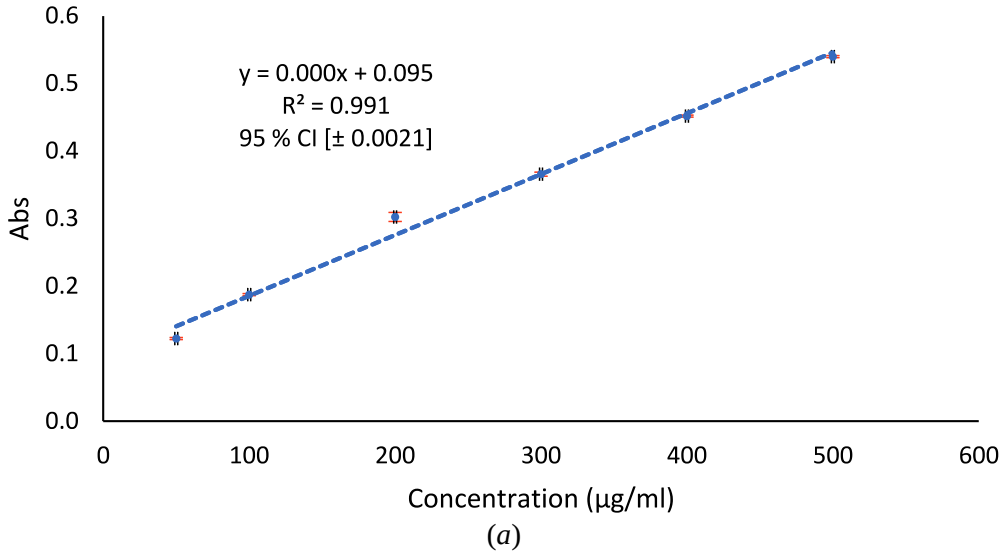
Formulation of polyherbal mixture

In-house polyherbal mixture (PM) was prepared by mixing *Vitis vinifera, Panax ginseng, Withania somnifera, Tinospora chordifolia, Curcuma longa, Zingiber officinale* and *Glycyrrhiza glabra* extracts.

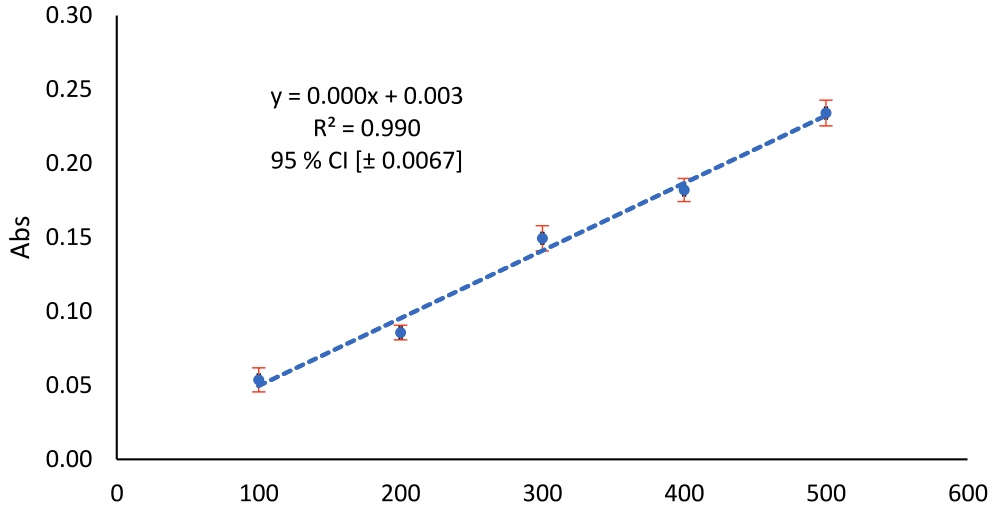
Quantitative phytochemical screening

The quantitative phytochemical analysis of polyherbal mixture was done by total phenolic content (TPC) and total flavonoid content (TFC). The total phenolic content in the PM was derived from a standard curve of gallic acid ranging from 50 to 500 µg/ml and using the equation $y = 0.009x + 0.0957$, 95 % CI [±0.00073] with correlation coefficient $r^2 = 0.9911$ (Fig. 2*a*). The average total phenolic content of the polyherbal mixture was found to be 212.1±7.407 mg of GAE/g of plant material. The total flavonoid content in the PM was calculated from a standard curve of rutin ranging from 100 to 500 µg/ml and using the equation $y = 0.0005x + 0.0038$, 95 % CI [±0.0067] with correlation coefficient $r^2 = 0.9904$ (Fig. 2*b*). The average total flavonoid content of the polyherbal mixture was found to be 133.06±1.333 mg of RU/g of plant material.

The activity to scavenge DPPH radical was evaluated by the reduction in the absorbance at 517 nm, which is dependent on the concentration ($n = 3$). Percent anti-oxidant activity for 50 µg/ml of polyherbal mixture was found to be 97.22 % which was higher than standard ascorbic acid of same concentration. The IC_{50} value of the polyherbal mixture was found to be 3.83 µg/ml which indicate that it has greater antioxidant properties than the standard ascorbic acid (Fig. 3).



(*a*)



(*b*)

Figure 2. Determination of (*a*) Total Phenolic Content (TPC), (*b*) Total Flavonoid Content (TFC)

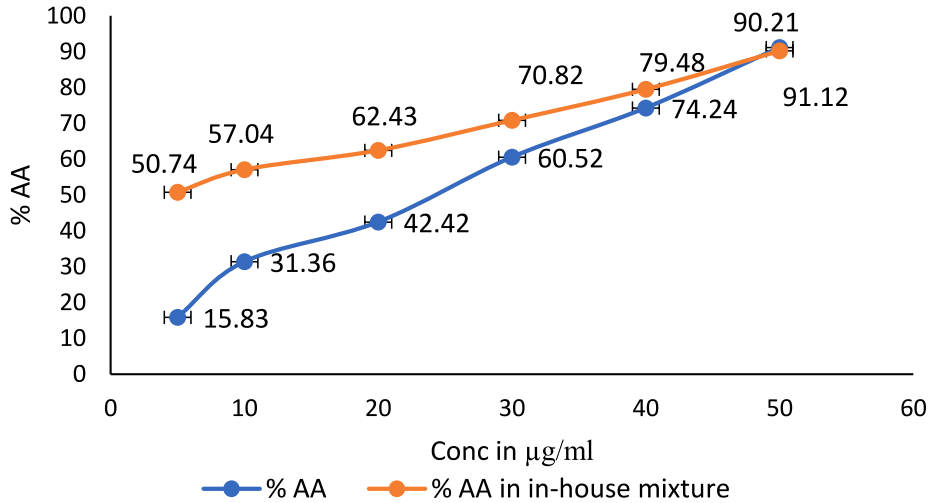
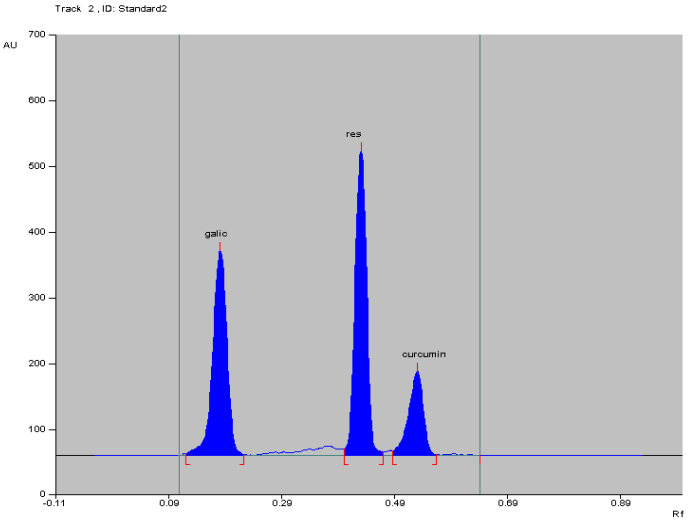


Figure 3 Determination of percentage anti-oxidant activity (% AA)

HPTLC analysis of resveratrol, curcumin and gallic acid

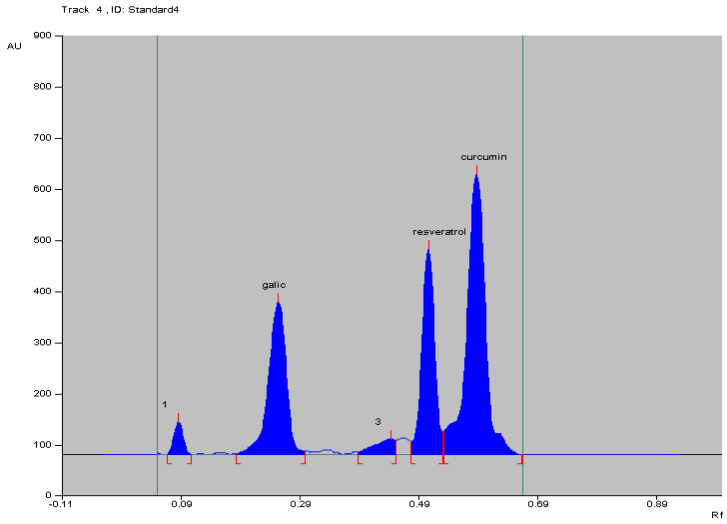
The HPTLC procedure was optimized to quantify curcumin, gallic acid and resveratrol in the polyherbal mixture. The mobile phase toluene: ethyl acetate: methanol: formic acid (7:2:1:0.5 v/v/v/v) gave good resolution with R_f = 0.18 for gallic acid, R_f = 0.43 for resveratrol and R_f = 0.53 for curcumin (Fig. 4*a*). Under the optimized chromatographic condition, the polyherbal mixture showed sharp peaks and good separation when scanned at 269 nm (Fig. 4*b*). Overlay UV spectra of matching R_f values of standard curcumin, gallic acid and resveratrol and peaks of polyherbal mixture confirmed their presence in polyherbal mixture (Fig. 5).



Track 2, ID: Standard2

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.12 Rf	3.1 AU	0.18 Rf	311.8 AU	34.52 %	0.22 Rf	1.5 AU	6573.0 AU	37.86 %	galic
2	0.40 Rf	9.3 AU	0.43 Rf	463.7 AU	51.33 %	0.47 Rf	5.4 AU	7991.3 AU	46.03 %	res
3	0.49 Rf	7.4 AU	0.53 Rf	127.8 AU	14.15 %	0.56 Rf	1.1 AU	2796.3 AU	16.11 %	curcumin

(*a*) Densitogram of standard curcumin, gallic acid and resveratrol

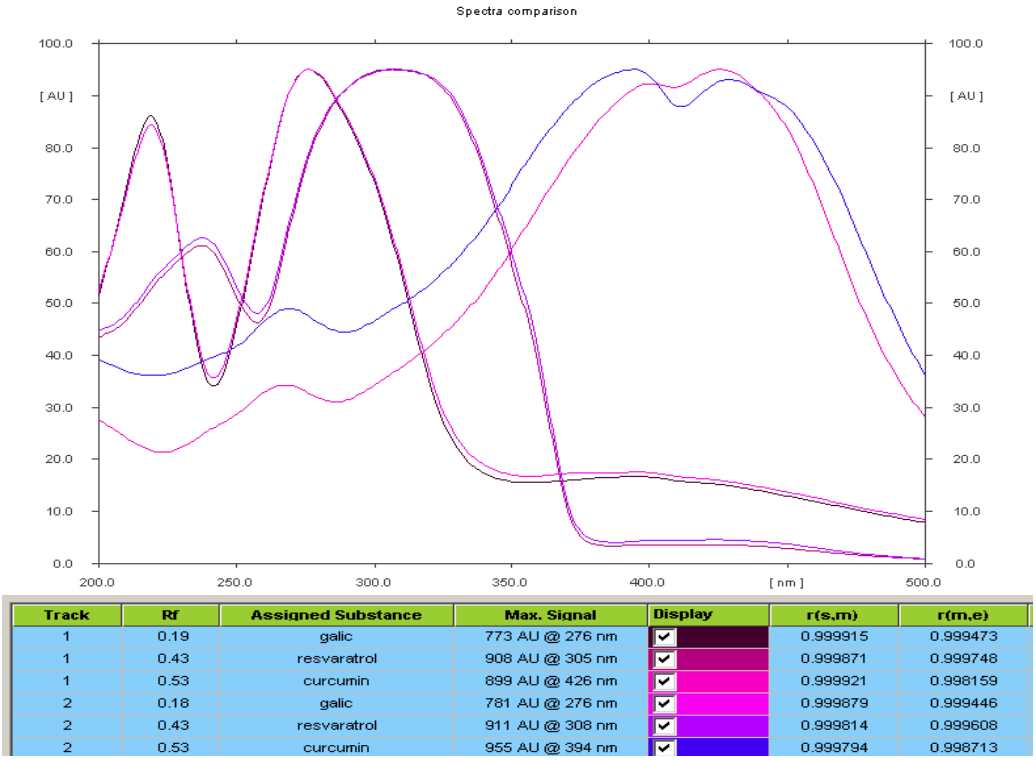


Track 4, ID: Standard3

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.15 Rf	2.1 AU	0.22 Rf	566.5 AU	37.57 %	0.26 Rf	9.5 AU	15472.1 AU	42.82 %
2	0.37 Rf	6.7 AU	0.40 Rf	16.5 AU	1.10 %	0.40 Rf	15.3 AU	319.7 AU	0.88 %
3	0.40 Rf	15.4 AU	0.46 Rf	388.2 AU	25.74 %	0.48 Rf	38.7 AU	7529.3 AU	20.84 %
4	0.48 Rf	39.4 AU	0.54 Rf	536.5 AU	35.59 %	0.57 Rf	6.0 AU	12814.9 AU	35.46 %

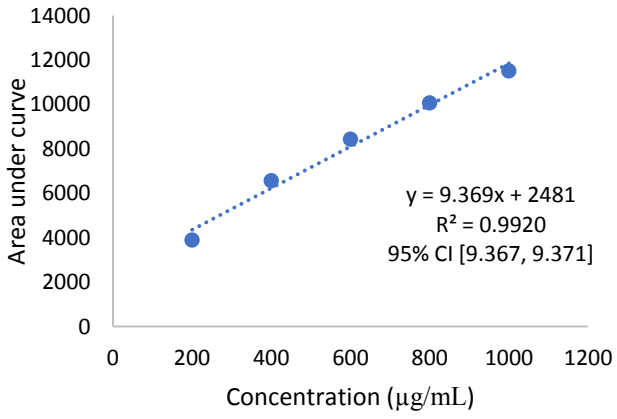
(*b*) Densitogram of polyherbal mixture indicating curcumin, gallic acid and resveratrol

Figure 4.

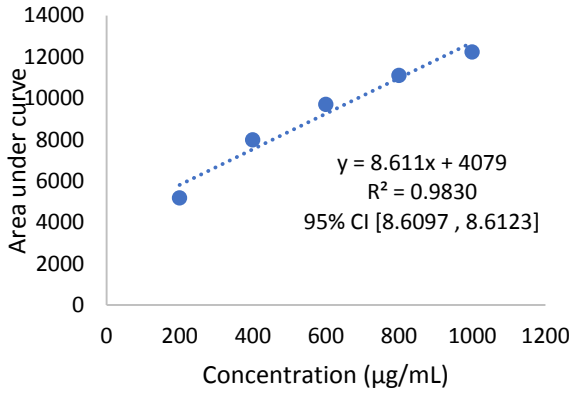


Track	Rf	Assigned Substance	Max. Signal	Display	r(s,m)	r(m,e)
1	0.19	galic	773 AU @ 276 nm		0.999915	0.999473
1	0.43	resvaratrol	908 AU @ 305 nm		0.999871	0.999748
1	0.53	curcumin	899 AU @ 426 nm		0.999921	0.998159
2	0.18	galic	781 AU @ 276 nm		0.999879	0.999446
2	0.43	resvaratrol	911 AU @ 308 nm		0.999814	0.999608
2	0.53	curcumin	955 AU @ 394 nm		0.999794	0.998713

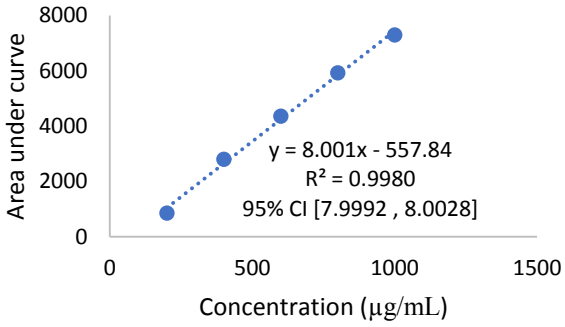
Figure 5. Overlay spectra of gallic acid, resveratrol and curcumin



(*a*) gallic acid



(*b*) resveratrol



(*c*) curcumin

Figure 6. Calibration curve of standards

Validation of developed HPTLC method

The linearity graph was plotted using different concentrations of gallic acid, resveratrol and curcumin. The regression coefficient value (r^2) was observed to be 0.99209 for gallic acid, 0.98300 for resveratrol and 0.99802 for curcumin from the graph (Table, Fig. 6).

The proposed HPTLC techniques have been verified for intra- and inter-day variations. The percent relative standard deviations (%RSDs) values were determined to be 0.0073, 0.0076, 0.0161 (for intra-day) and 0.0062, 0.0058, 0.0151 (for inter-day) for gallic acid, resveratrol and curcumin respectively. This indicates that the method was precise. Recovery tests were conducted at three distinct concentration levels 80, 100, and 120% to examine the method's accuracy. The lowest concentration at which the standards solution can be consistently identified was recorded as 0.30 μg/spot, 0.26 μg/spot, 0.24 μg/spot and the lowest analyte concentration at which it can be consistently measured was found to be 0.91 μg/band, 0.79 μg/band, 0.75 μg/band for gallic acid, resveratrol and curcumin respectively. By making minor adjustments to the mobile phase volume and saturation duration, the robustness of the new method was tested, where % RSD was found to be in the limit < 2 %.

T a b l e

Validation parameters of the developed HPTLC method

Parameters	**Results**		
	Gallic acid	**Resveratrol**	**Curcumin**
Linearity	Y = 9.369x +2481	Y = 8.611x + 4079	Y = 8.001x – 557.8
Slope	9.369	8.611	8.001
Y-intercept	2481	4079	-557.8
Correlation Coefficient	0.99209	0.98300	0.99802
R_f	0.18	0.43	0.53
Level of accuracy	**% Recovery**		
80 %	100.1604 ± 0.01	100.0249 ± 0.06	101.1639 ± 1.55
100 %	100.0004 ± 0.01	99.99715 ± 0.002	100.4646 ± 0.588
120 %	99.93298 ± 0.02	99.99556 ± 0.01	100.1058 ± 0.06
Precision	**%RSD**		
Intra day	0.0073 ± 0.61	0.0076 ± 0.74	0.0161 ± 0.69
Inter day	0.0062±0.51	0.0058 ± 0.56	0.0151 ± 0.65
LOD (ng/band)	0.30 ± 0.05	0.26 ± 0.04	0.24 ± 0.053
LOQ (ng/band)	0.91 ± 0.09	0.79 ± 0.05	0.75 ± 0.08
Robustness	**%RSD**		
Mobile phase volume (±1 ml)	0.0031 ± 0.26	0.0037 ± 0.36	0.0080 ± 0.34
Saturation time (±5 min)	0.0088 ± 0.74	0.0033 ± 0.32	0.0060 ± 0.26

Conclusions

This research focused on the development and validation of an HPTLC method for the simultaneous quantification of curcumin, gallic acid, and resveratrol in a polyherbal mixture, along with assessing its Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant potential using the DPPH assay. The findings indicate that the polyherbal mixture is rich in TPC, measuring 212.1 ± 7.407 mg gallic acid equivalent (GAE) per gram of plant material, and TFC, which was found to be 133.06 ± 1.333 rutin equivalent (RU) per gram of plant material. The DPPH assay demonstrated strong antioxidant activity with an IC_{50} value of 3.83 μg/ml.

Furthermore, the HPTLC method was meticulously optimized, providing excellent resolution for gallic acid, resveratrol, and curcumin, with corresponding R_f values of 0.18, 0.43, and 0.53. The method exhibited strong linearity ($r^2 > 0.99$) over a concentration range of 200–1000 ng/band for all three compounds. Precision studies confirmed the method's reproducibility with low Relative Standard Deviation (RSD) values below 2 % for both intra-day and inter-day analyses.

This HPTLC method stands out for its simplicity, accuracy, precision, and cost-effectiveness, making it suitable for routine quality control analysis. These results shed light on the potential applications of the polyherbal mixture in various therapeutic contexts, underscoring its robust phytochemical profile and potent

antioxidant properties, further supporting its use in herbal medicine and quality control assessments. This research contributes to the growing importance of polyherbal formulations in the field of herbal medicine.

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**The authors' names are presented in the following order: First Name, Middle Name and Last Name*

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Sejal Prakash Gandhi** conceptualization, data curation, validation, visualization, manuscript writing; **Gresi Devidas Mate** data curation, formal analysis, investigation, methodology, visualization; **Dheeraj Nagore** data curation, formal analysis, manuscript writing & editing; **Sohan Satyanarayan Chitlange** conceptualization, resources, supervision, validation, manuscript editing.

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Conflicts of Interest

The authors declare no conflict of interest.

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