Analytical characterization of biomarkers in an optimized novel antidiabetic polyherbal formulation using high-performance thin-layer chromatography and liquid chromatography with tandem mass spectrometry

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Background
Diabetes mellitus is a chronic health issue that requires novel approaches to treatment and a multimodal approach to prevention. In the treatment of diabetes, a polyherbal formulation is the finest alternative medicine. A polyherbal formulation was developed in-house and evaluated for its antidiabetic potential on streptozotocin-induced diabetes rat. The same extract was now characterized analytically utilizing a variety of methods.

Objective
The goal of this study was to quantify the biomarkers in a novel antidiabetic polyherbal formulation made in-house with *Cinnamonum zeylanicium* bark, *Eugenia jambolana* seeds, *Vinca rosea* whole plant, and *Gymnema sylvestre* (GS) leaves, using high-performance thin-layer chromatography (HPTLC) and liquid chromatography with tandem mass spectrometry (LC–MS/MS).

Materials and methods
Cinnamaldehyde (CIN), gallic acid (GLA), vincristine (VC), vinblastine (VB), and gymnemic acid (GYA) were identified as bioactive components of polyherbal formulation hydroalcoholic extract utilizing HPTLC and LC–MS/MS. Acetonitrile, methanol, and 0.1 percent formic acid was used as mobile phase, chromatographic separation was accomplished in 30 min using a gradient system and a SUNFIRE C18, 250×4.6, 5-μm analytical column with a flow rate of 1.0 ml/min in LC–MS/MS research. Scanned in a positive mode with a scan speed of 100–2000 AMU/s over a mass range of 20–1974 Da. The electron-spray ionization mode was used, with a source temperature of 150°C and a desolvation temperature of 350°C. The HPTLC separation was performed using ethyl acetate/acetonitrile/water/formic acid/N-dimethyl formamide 5.5 : 2.5 : 0.5 : 1.0 : 0.5 (v/v) as the mobile phase on precoated silica gel 60 GF254 plates. At room temperature, the plates were developed to a distance of 9.0 cm. CIN, GLA, VC, VB, and GYA plates were scanned and measured at wavelengths of maximum absorption of 259, 287, 342, 355, and 387 nm, respectively. Band size, chamber-saturation duration, migration of the solvent front, slit width, and other experimental parameters were carefully examined, and the optimized chromatographic conditions were chosen.

Results
LC–MS analysis of the hydroalcoholic extract of the polyherbal formulation revealed the presence of all the five bioactive chemical constituents, CIN, GLA, VC, VB, and GYA. Similarly, the drug samples were satisfactorily resolved with Rf 1.81±0.01, 0.05±0.01, 0.02±0.01, 0.09±0.01, and 0.04±0.01 for CIN, GLA, VC, VB, and GYA respectively, using HPTLC.

Conclusion
The importance of combining Ayurvedic formulations with contemporary high-throughput screening techniques will spark new interest in more powerful biocompatible drug leads. The findings of this study lend scientific credence to the therapeutic applications of the polyherbal formulation.

Keywords:
cinnamaldehyde, gallic acid, high-performance thin-layer chromatography, liquid chromatography with tandem mass spectrometry, vinblastine and gymnemic acid, vincristine

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Introduction

Ayurveda is an Indian medical system [1] and is an ancient system of traditional medicine that has been used on the Indian peninsula from 5000 BC to provide natural solutions to cure ailments and improve health

[2,3]. Single active ingredients, herbal extracts, and herbal mixtures have all played a role in the prevention and treatment of diseases, particularly in the case of severe chronic disorders [4]. Natural products, although being the oldest kind of medication, have shown to be excellent sources of therapeutically effective medications. Plants are the most significant suppliers of medicines among them [5]. Traditional remedies were popular in every area of the globe where plants were the most significant and readily available resources. Exceptional advances in synthetic drug design have resulted in the delivery of medicines that are far more potent than their natural counterparts. Plant-based chemicals, on the other hand, have kept their place, most likely owing to safety concerns. As a result, a study into plant products and the chemicals found in them, as well as their supposed historic usage as medicines, is seen as just as essential as contemporary techniques of manufacturing substances [6,7].

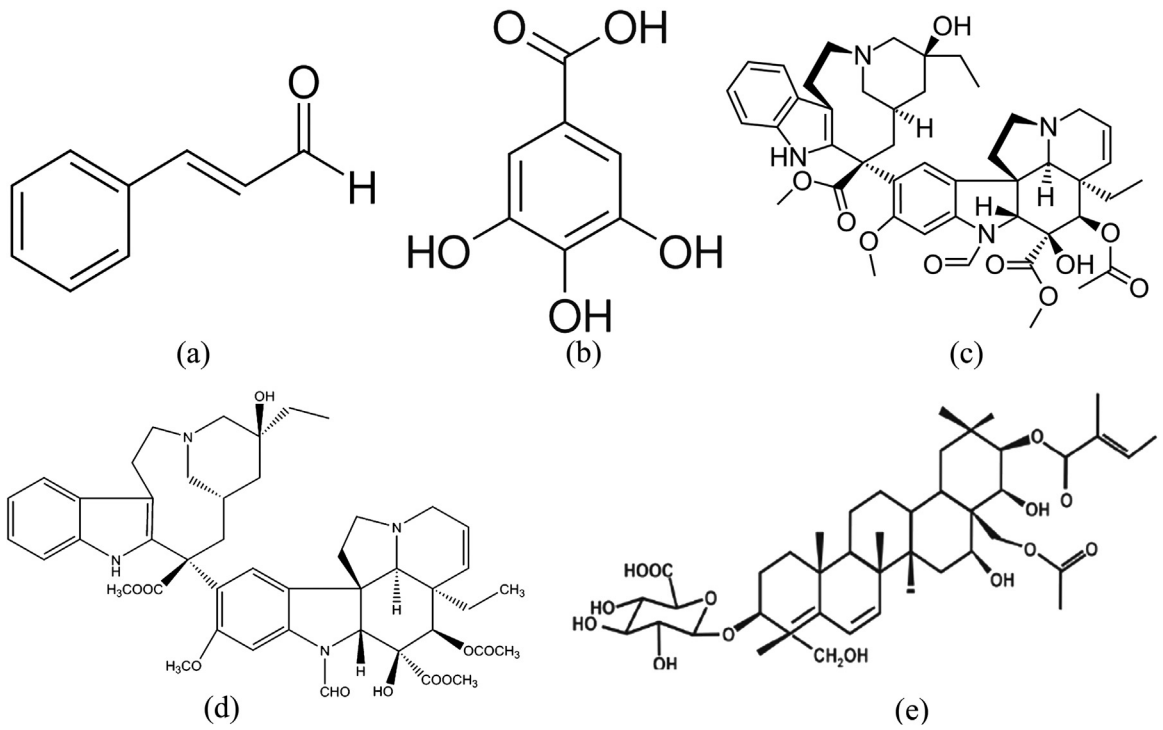
Herbal preparations are made up of a variety of pharmacologically active ingredients. Within the human body, they work together in a synergistic manner. The presence of phytoconstituents contributes to the herbal preparation's potency and efficacy [8–10]. Herbal medicines' effectiveness is influenced by their quality. Quantity, quality, stability, and pharmacological effects of herbal drugs are determined by a variety of factors such as plant origin, growth conditions, harvest time, manufacturing process and adulteration in crude drugs, environmental conditions, and chemical reactions such as oxidation, decomposition, hydrolysis, and racemization [11,12]. The qualitative and quantitative assessments required for quality control, pharmacological, toxicological, and clinical investigations of these medicines rely heavily on bioactive components and/or chemical markers [13]. According to FDA standards [14] and the European Agency for the Evaluation of Medicinal Products [15], a plant drug's chemical fingerprints of biologically active molecule (marker compound) must be established before it can be legally sold [15,16]. Because of the complexity of conventional herbal medications and the lack of information about their active ingredients, the FDA may rely on a mix of tests and controls to verify the drugs' identification, purity, quality, strength, potency, and consistency. As a result, natural medicine quality control is the need of the hour. Quality issues (lack of consistency, safety, and efficacy) appear to be overshadowing the potential genuine health benefits of various herbal products at the moment, and a major cause of these issues appears to be the lack of simple and reliable analytical techniques and methodologies for the chemical analysis of herbal materials [17]. In a number of resolutions, the WHO stressed the need of utilizing contemporary technology and implementing appropriate standards to guarantee quality control of these therapeutic plant products [18].

Various herbs have been claimed for their hypoglycemic benefits in the Ayurvedic system of medicine and are still used today [19–23]. For the production of polyherbal formulation, four promising antidiabetic plants (listed below) were chosen based on the literature. *Cinnamonum zeylanicium* bark, *Eugenia jambolana* seeds, *Vinca rosea* entire plant, and *Gymnema sylvestre* leaves were the plants and their medicinal components.

Several researches have shown that the aforementioned plants have antidiabetic efficacy in various antidiabetic models. In streptozotocin (STZ) and alloxan-induced diabetic rats, *C. zeylanicium* extract reduced blood glucose levels, serum lipid profiles, and bodyweight to normal levels [24–27]. In STZ-induced diabetic mice, dried seeds of *E. jambolana* considerably reduced blood glucose levels in a time-dependent manner compared with conventional drugs like glibenclamide and metformin [28–32]. In STZ-induced diabetic mice, administration of an ethanolic extract of *V. rosea* reduced fasting and postprandial blood glucose levels while also increasing bodyweight, lowering lipid levels, and improving hepatic and renal function [33–37]. In diabetic mice, the dried leaf extract of *Gymnema sylvestre* decreased blood glucose, glycosylated hemoglobin, serum lipid levels, and enhanced insulin action and glycosylated plasma proteins [38–41]. In continuation to our previous research, a polyherbal extract was prepared and evaluated for its antidiabetic activity [42], The current research work aims at analytical characterization of the extracted bioactive phytoconstituents, namely cinnamaldehyde (CIN) in *C. zeylanicium*, gallic acid (GLA) in *E. jambolana*, vincristine (VC), vinblastine (VB) in *V. rosea*, and gymnemic acid (GYA) in *G. sylvestre*. In the literature, high-performance thin-layer chromatography (HPTLC) [43–46], high-performance liquid chromatography (HPLC)–ultraviolet (UV) [47–50], gas chromatography-mass spectrometry (GC–MS) [51–54], and liquid chromatography with tandem mass spectrometry (LC–MS/MS) [55–59] have been described for qualitative and quantitative analysis of CIN, GLA, VC, VB, and GYA. Figure 1 shows the chemical structures in various biological matrices.

In pharmaceutical analysis, HPLC with UV is a highly useful analytical technology strongly recommended by the WHO. HPLC is also widely utilized in the

Figure 1



Chemical structures of (a) cinnamaldehyde, (b) gallic acid, (c) vincristine, (d) vinblastine, and (e) gymnemic acid.

pharmaceutical industry for research and development as well as quality control [60]. However, using UV detection at the conclusion of an HPLC separation necessitates meticulous setup and calibration, particularly for extract analysis [61]. Developing an HPLC method for estimation/separation of the desired single natural molecule present in a plant-extract sample contaminated with complex molecules or similar chemical structure and physicochemical properties or degradation product produced during storage is also a difficult task for the herbal industries [62]. For the study of metabolites in plant extracts, tandem MS as well as HPTLC are more sensitive and selective [63]. They are more accurate in measuring phytoconstituents at microgram and even nanogram levels, have a low operating cost, and have a high sample throughput. These methods require little sample preparation, resulting in shorter analysis times and lower per-analysis costs. UV radiation is impossible to penetrate many components in complicated plant preparations (e.g. sugar). After derivatization, these substances can be analyzed qualitatively and quantitatively. HPTLC, like HPLC and GC, may be used to generate chromatographic fingerprints and determine and identify complicated herbal extracts with the use of a data analysis system and optimum experimental conditions. In comparison with HPLC and GC, HPTLC allowed for the simultaneous quantification of many compounds in multiple samples on a single plate [64]. Furthermore, the colorful images provide HPTLC with additional understandable visible color and/or fluorescence characteristics.

To the best of our knowledge, such repeatable, accurate, and cost-effective LC–MS/MS and HPTLC methods for the simultaneous determination of CIN, GLA, VC, VB, and GYA in polyherbal formulations or other biological materials have not yet been published. We developed LC–MS/MS and HPTLC methods for the simultaneous quantification of CIN, GLA, VC, VB, and GYA in a polyherbal formulation, keeping in mind the medicinal importance of these bioactive phytoconstituents as well as the advantages of this method over sophisticated techniques (HPLC, GC–MS/MS, etc.).

Materials and methods

Materials

The plants were collected in and around the Nallamala forest region in Srisailam, Kurnool District, and Seshachalam forest area near Tirupathi, Andhra Pradesh, India, in their native habitats. The plants were certified by Dr K. Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateshwara University, Tirupati, Andhra Pradesh. In the shade, plant portions were dried and defatted using petroleum

Table 1 Physical characteristics and % yield of extracts

Plants	% Yield (w/w)	Moisture content (%w/w)	Ash value (%w/w)			Foaming index	Swelling index (ml)
			Total	Acid insoluble	Water insoluble		
Cinnamonum zeylanicium bark	12.50	2.38±0.13	2.77±0.21	0.13±0.04	0.11±0.02	<100	3.73±0.34
Eugenia jambolana seeds	10.78	2.18±0.90	3.52±1.01	0.25±0.12	0.24±0.02	<100	2.77±0.73
VR whole plant	11.34	2.11±0.19	4.38±1.33	0.67±0.03	0.31±0.02	<100	4.88±0.34
GS leaves	10.86	1.83±0.22	3.71±0.44	0.30±0.14	0.21±0.08	<100	3.90±0.89

All values are expressed in mean±SD, n=3.

ether. The defatted material was extracted with 70% ethanol and then vacuum-dried using a soxhlet machine. All of the chemicals utilized were of the highest quality. Merck, India, provided ethyl acetate, acetonitrile, water, formic acid, N-dimethyl formamide, methanol, and HPTLC plates covered with silica gel, G 60 F254. Sigma-Aldrich, India, provided the CIN, GLA, VC, VB, and GYA standards.

Table 2 Optimized Gradient mobile phase system for liquid chromatography

Time	A (5.0 ACN)	B (MeOH)	C (95.0, 0.1% formic acid)
0 min	95	1.5	1
1 min	95	1.5	6
6 min	70	1.5	6
16 min	40	1.5	6
24 min	20	1.5	6
30 min	95	1.5	6

Preparation of extracts

Continuous soxhlet extraction with ethanol was used to extract *C. zeylanicium* bark, *E. jambolana* seeds, *V. rosea* entire plant, and *G. sylvestre* leaves. All plant components (1 kg) were air-dried and roughly pulverized in a separate mixer. About 500 g of each crude drug powder was properly weighed and stored in a soxhlet system at a temperature between 60 and 70°C for 10 h before being extracted with 70% ethanol. The extraction was kept going, until the solvent was completely evaporated. Extracts ranging from dark brown to black were obtained. These extracts were filtered after cooling to eliminate the residue. On a rotary evaporator, the extracts were concentrated under reduced pressure and then dried to produce a powder. The extracts were kept in amber-glass containers (refrigerated) once their % extract was calculated. For the identification of different phytoconstituents in the extracts, preliminary phytochemical assays were performed. In the amount necessary for the investigation, the dry powder was diluted with 0.5 percent carboxymethylcellulose used as a vehicle. Table 1 shows the physical properties of the extracts as well as their percent yield.

Optimization of liquid chromatography with tandem mass spectrometry conditions

The HPLC–electron-spray ionization (ESI)–MS/MS analysis was carried out using a Waters Alliance 2695 HPLC interface and an Ab-Sciex Q-TOF mass spectrophotometer with an ESI source. The binary solvent manager, sample manager, column oven, and PDA detector were all included in the Waters Alliance 2695. Data were collected and processed using Ab Sciex Analyst software, version 1.5.1. With a gradient system in varied proportions, chromatographic separation was performed on a SUNFIRE C18, 250×4.6, 5-µm analytical column using solution A: acetonitrile, solution B: methanol, and solution C: 0.1% formic acid as mobile phases. Table 2 shows the optimized-gradient mobile-phase system for separation of analytes. One liter of solvents were fed into the LC–MS at a flow rate of 1.0 ml/min. Scanned in a positive and negative mode with a scan speed of 100–2000 AMU/s over a mass range of 20–1974 Da. About 5 µl was injected into LC. The ESI mode was used, with a source temperature of 150°C and a desolvation temperature of 350°C.

Quantitative analysis of poly herbal preparation (PHP) using high-performance thin-layer chromatography

Instrumentation and conditions

Linomat V Automatic Sample Spotter (CAMAG, Muttenz, Switzerland), 100 ml of syringe (Hamilton, Bonaduz, Switzerland), glass twin-trough chamber (20 cm×10 cm×4 cm) (CAMAG), TLC Scanner 3 linked to Win Cats software (CAMAG), 0.2-mm thickness precoated with silica gel, and G 60 F254 (Merck) were used in this study. The experiment was carried out under the conditions with temperature of 25±2°C and relative humidity of 40%.

Preparation of standards

The standard solutions of CIN, GLA, VC, VB, and GYA were prepared containing known concentrations of 0.4, 0.1, 0.5, 0.5, and 0.4 mg/ml, respectively, in 10 ml of diluent.

Sample preparation for high-performance thin-layer chromatography

In a 100-ml conical flask, 40.0 g of polyherbal extract (equal quantities of all plants) was precisely weighed, and 30 ml of water was carefully added and mixed. In a 250-ml separating funnel, the resultant solution was transferred. In the separating funnel, 50 ml of chloroform was added and shaken gently for 3 min to extract the solution. After allowing the layers to fully separate, the lower chloroformic layer was filtered through a paper filter using anhydrous sodium sulfate (~10 g) in a 250-ml conical flask. An additional 50 ml of chloroform was used to remove the top water layer. The extraction was repeated with 50 ml of chloroform parts (five times in total). Under vacuum, the extract was evaporated to dryness. The dry residue was dissolved in 5 ml of ethanol and poured into a 10-ml volumetric flask quantitatively. The volume of the solution was increased to the required level. The filtrate was used as a sample after it was filtered with Whatman filter paper no. 44.

Procedure

The analysis was carried out using HPTLC silica gel G60 F254 plates with fluorescent indicator, measuring 20 cm×10 cm. Before beginning the study, HPTLC plates were cleaned using an escalating technique of predevelopment with methanol. The HPTLC plate was submerged in a CAMAG glass chamber (20 cm×10 cm) with 30 ml of HPLC-grade methanol. The compartment was sealed with a glass lid and left to develop the plate with methanol, until it reached the top. The plate was taken from the TLC glass chamber after full development and dried in an oven at 85°C for 5 min. Using a CAMAG Linomat 5, five spots of 10 ml of standard preparation and a spot of 10 ml of sample preparation (PHP) were applied as bands on the same plate (in the form of bands). Automatic spray-on applicator with a 100-ml syringe with settings of 6-mm band length, 15-mm spacing between bands, 15-mm distance from the plate-side edge, and 15-mm distance from the plate bottom. The plate was created by submerging a sample HPTLC plate in a CAMAG glass chamber (20 cm×10 cm) with ethyl acetate/acetonitrile/water/formic acid/N-dimethyl formamide 5.5 : 2.5 : 0.5 : 1.0 : 0.5 (v/v/v/v/v) as the solvent system. After full development, the plate was allowed to dry in a fume cupboard for 10 min before being placed in a 105°C hot-air oven for 5 min. Utilizing a TLC Scanner III CAMAG with a D2 source, the plate was scanned in the densitometer using linear scanning at 259, 287, 342, 355, and 387 nm for CIN, GLA, VC, VB, and GYA, respectively, and the area of the spots corresponding to the respective standards was integrated.The amount of analytes in polyherbal preparation was calculated by the following formula:.

$$\frac{A_{SMP} \times W_{STD} \times f \times \text{Dilution of Smp} \times \text{Application vol. of sample} \times P \times D \times 10}{A_{STD} \times \text{Dilution of Std} \times W_{SMP} \times \text{Application of vol. of standard} \times 100}$$

where A_{SMP} is average area of sample; A_{STD} is the average area of the standard; W_{STD} is the weight of the standard, mg; W_{SMP} is the weight of the sample, g; dilution of Smp is dilution of sample, ml; dilution of Std is dilution of standard, ml; P is percent purity of standard; f is conversion factor; D is density sample, mg/ml.

Validation of high-performance thin-layer chromatography method

The developed HPTLC method was further validated as per ICH guidelines [65].

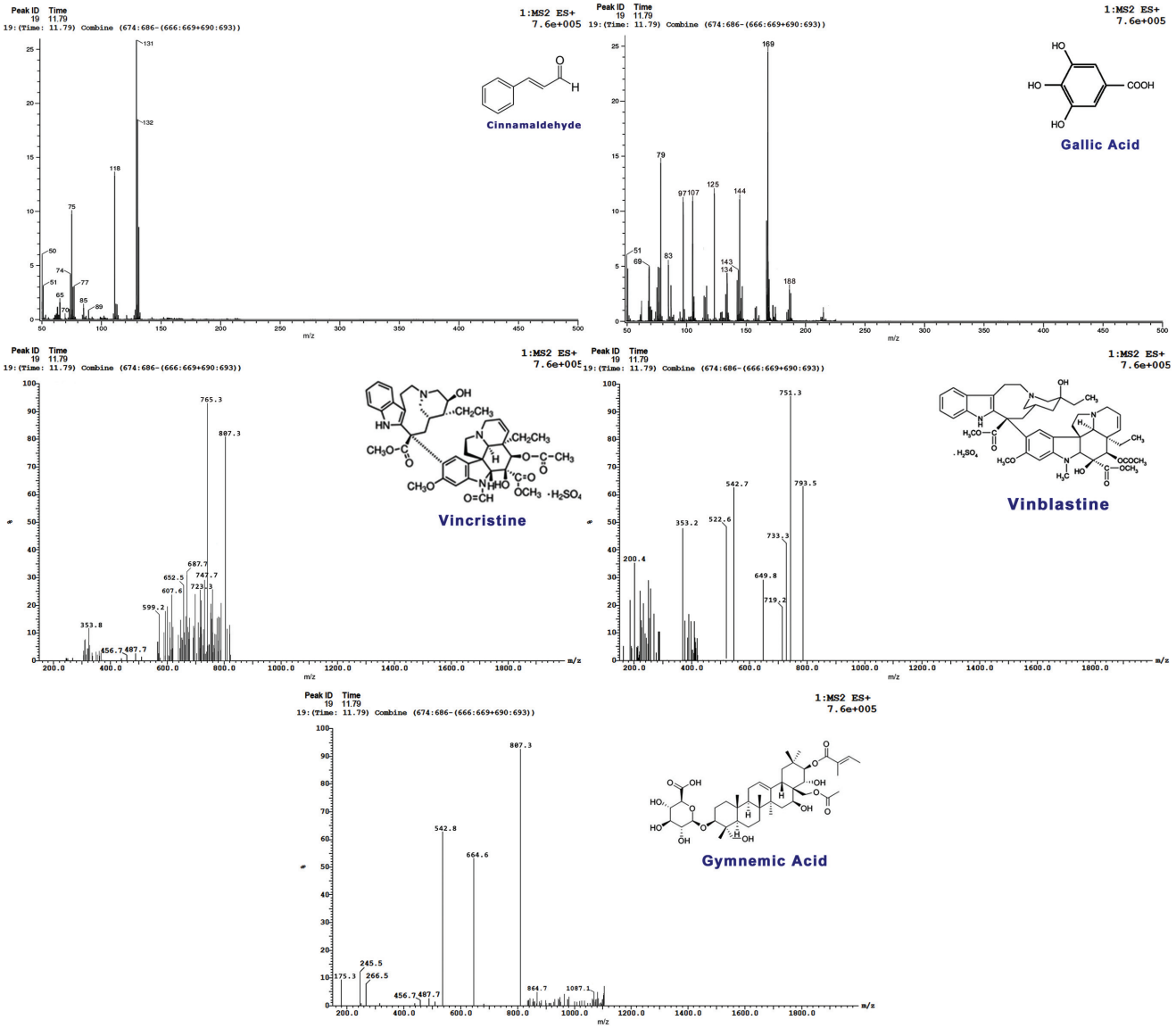
Results and discussion

For assessing the small-molecule components of cellular metabolism, LC–MS and HPTLC have emerged as the method of choice. The simultaneous investigation of dozens to hundreds of chemical species is possible with LC–MS and HPTLC. LC–MS is a high-tech detection technology that uses high-capacity chromatographic separation and high-sensitivity MS to analyze and detect trace components. Both of these approaches may be used to accurately analyze bioactive components on a qualitative and quantitative level. Using HPTLC and LC–MS/MS, the presence of the components in the polyherbal formulation was confirmed.

Liquid chromatography with tandem mass spectrometry analysis

The present study is the first to investigate and identify phytochemical compounds of polyherbal extract from *C. zeylanicium* bark, *E. jambolana* seeds, *V. rosea* whole plant, and *G. sylvestre* leaves. The predominant compounds of the above-said herbs were identified and confirmed by LC and MS/MS. Figures 2 and 3 depict the MS spectra and the LC chromatograms of the analytes, respectively, the data are discussed below:

Figure 2



MS/MS spectra of polyherbal preparation containing CIN, GLA, VC, VB, and GYA. CIN, cinnamaldehyde; GLA, gallic acid; MS/MS, mass spectrometry; VB, vinblastine; VC, vincristine.

(1) CIN (MW 132 g/mol) – HPLC: Rt=21.41 min, ESI–MS (positive): MS, m/z 132 (M+H)+, tandem MS/MS (precursor ion at m/z 132 [C_9H_8O]), m/z 133 [C_9H_9O]+, m/z 131 [C_9H_6O]+, m/z 118 [C_8H_6O]+.

(2) GLA (MW 169) HPLC: Rt=16.54, ESI–MS (negative): MS, m/z 169 (M+H)+, tandem MS/MS (precursor ion at m/z 125[$C_6H_6O_3$]), m/z 107 [$C_6H_3O_2$]+, m/z 97 [$C_5H_6O_2$]+.

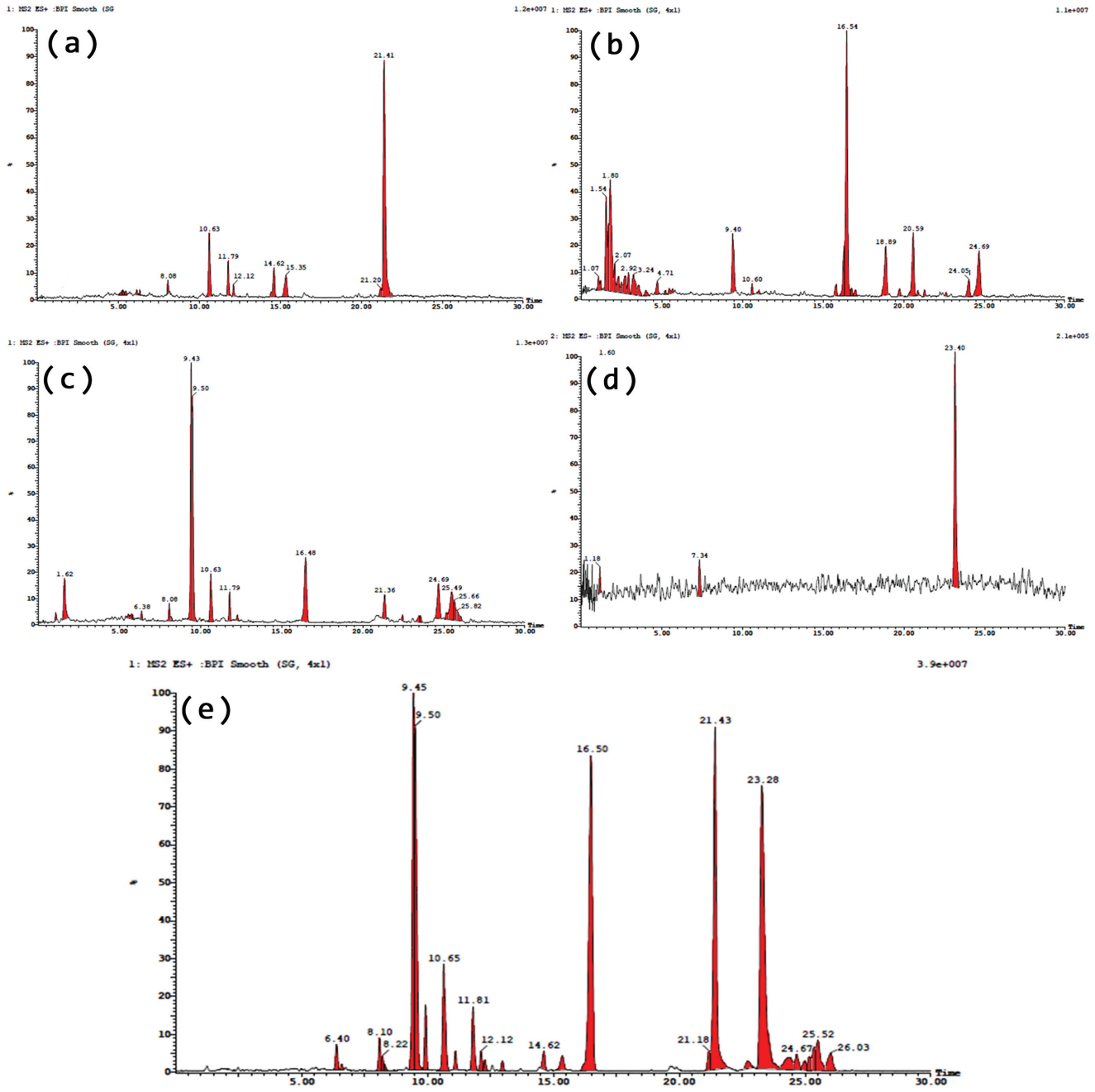
(3) VC (MW 825) HPLC: Rt=9.43, ESI–MS (positive): MS, m/z 807 (M+H)+, tandem MS/MS (fragment ion at m/z 765 [$C_{44}H_{53}O_8N_4$], m/z 747 [$C_{44}H_{51}O_7N_4$]+, m/z 723 [$C_{42}H_{51}O_7N_4$]+, m/z 687 [$C_{42}H_{47}O_5N_4$]+, m/z 599 [$C_{34}H_{39}O_6N_4$]+, m/z 353 [$C_{21}H_{25}O_3N_2$]+.

(4) VB (MW 810) HPLC: Rt=9.50, ESI–MS (positive): MS, m/z 793 [$C_{46}H_{57}O_8N_4$], m/z 751 [$C_{44}H_{54}O_7N_4$]+, m/z 733 [$C_{44}H_{53}O_6N_4$]+, m/z 719 [$C_{43}H_{51}O_6N_4$]+, m/z 649 [$C_{40}H_{49}O_4N_4$]+, m/z 542 [$C_{33}H_{40}O_4N_3$]+, m/z 522 [$C_{33}H_{36}O_3N_3$]+, m/z 353 [$C_{21}H_{25}O_3N_2$]+.

(5) GYA (MW 807) HPLC: Rt=23.40, EMIMS (negative): MS, m/s 807 [$C_{43}H_{66}O_{14}$] (M)+, tandem MS/MS (fragment ion at m/z 664 [$C_{35}H_{52}O_{12}$]+, m/z 542 [$C_{31}H_{42}O_8$]+, m/z 487 [$C_{28}H_{39}O_7$]+, m/z 175 [$C_{10}H_{23}O_2$]+, m/z 113 [$C_7H_{13}O$]+.

The retention durations of standard CIN, GLA, VC, VB, and GYA in LC chromatograms are similar to the phytoconstituents in a polyherbal preparation's LC chromatogram. Polyherbal preparation comprises CIN, GLA, VC, VB, and GYA, according to the LC–MS/MS results. Table 3 compares the retention

Figure 3



LC chromatograms of reference-marker compounds: (a) cinnamaldehyde, (b) gallic acid, (c) vincristine (VC) and vinblastine, (d) gymnemic acid analytes, and (e) polyherbal preparation. LC, liquid chromatography.

periods of analytes in standard and polyherbal preparations, indicating that CIN, GLA, VC, VB, and GYA are present in polyherbal preparation.

High-performance thin-layer chromatography analysis

The current work used HPTLC to perform quantitative assessment of biomarkers CIN, GLA, VC, VB, and GYA in a polyherbal formulation. All phytoconstituents were highly resolved and quantitatively assessed, as seen in the chromatogram (Fig. 4). To achieve good separation, several mobile-phase compositions were used to optimize the technique. For the detection of the above-mentioned herbal analytes, different solvent systems were used, with the solvent system containing ethyl acetate/acetonitrile/water/formic acid/N-dimethyl formamide 5.5 : 2.5 : 0.5 : 1.0 : 0.5 (v/v/v/v/v), resulting in good resolution of all the compounds in the presence of other herbal extract constituents.

Under UV light, a TLC plate was examined for the presence of CIN, GLA, VC, VB, and GYA, which were identified by a conspicuous colored spot. Under UV light at 259, 287, 342, 355, and 387 nm for CIN, GLA, VC, VB, and GYA, the Rf value in both sample

Table 3 m/z values of marker compounds with ccomparison between the retention times of analytes in standard and polyherbal preparation

S. No	Phyto-constituent	m/z	Retention times (min)	
			Standard	In polyherbal
1	Cinnamaldehyde	132	21.41	21.43
2	Gallic acid	169	16.54	16.50
3	Vincristine	825	9.43	9.45
4	Vinblastine	810	9.50	9.50
5	Gymnemic acid	807	23.40	23.28

and reference standard (Fig. 4) was found to be similar. A HPTLC technique for quantitative assessment of biomarkers contained in the PHP has been devised that is accurate, simple, and specific. For CIN, GLA, VC, VB, and GYA, the technique used in this investigation resulted in satisfactory peak shape. The other PHP components did not cause any problems. Specific physiologically active CIN, GLA, VC, VB, and GYA components were discovered in the polyherbal formulation, resulting in the establishment of a standard for validation of those compounds.

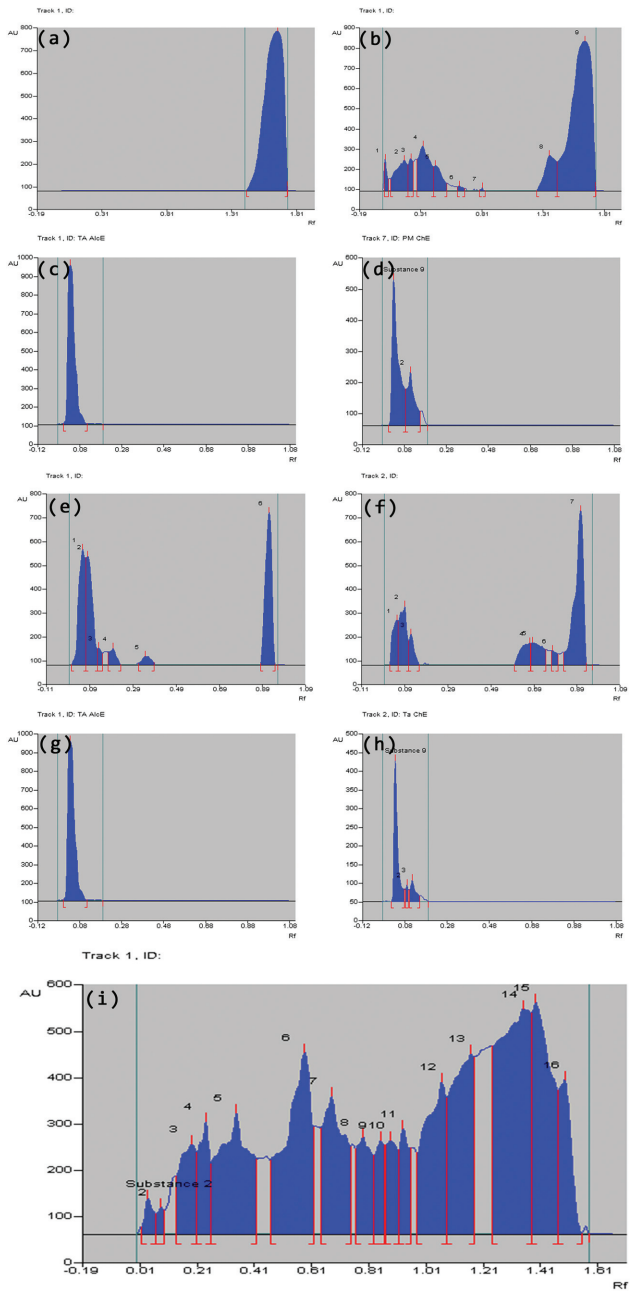
Comparison of the spectral characteristics of peaks for standards and sample revealed that the identity of standards that are similar HPTLC fingerprints were obtained for the bioactive compounds extracted from the respective source that gives reliable indications of the sample and standard. Figure 4a–i is the illustration of HPTLC-scanned chromatograms of biomarkers and PHP. The remaining validation and other analytical parameters are discussed in Table 4.

Conclusion

A qualitative and quantitative determination of bioactive secondary metabolites of a PHP, including CIN, GLA, VC, VB, and GYA, was carried out in this study. Isolation, identification, and characterization of bioactive metabolites were the study's main goals. All of the chemical components were separated and identified by physicochemical tests, and HPTLC and LC–MS/MS were used to quantify them. The devised HPTLC technique for quantifying biomarkers contained in PHP was proven to be simple, accurate, and sensitive. The method employed in the current study resulted in good peak shape of the biomarkers. The present standardization provides a specific and rapid tool in the herbal research, permitting to set quality specifications for identity, transparency, and reproducibility of biomarkers.

Financial support and sponsorship
Nil.

Figure 4



(a) Typical HPTLC chromatogram obtained for reference compound of CIN at λ259 nm, (b) typical HPTLC chromatogram obtained for CIN extracted from *Cinnamonum zeylanicium* bark at λ259 nm, (c) typical HPTLC chromatogram obtained for reference compound of GLA at λ287 nm, (d) typical HPTLC chromatogram obtained for GLA extracted from *Eugenia jambolana* seeds at λ287 nm, (e) typical HPTLC chromatogram obtained for reference compound of VC and VB at λ342 and 355 nm, respectively, (f) typical HPTLC chromatogram obtained for VC and VB extracted from VR whole plant at λ342 and 355 nm, respectively, (g) typical HPTLC chromatogram obtained for reference compound of GYA at λ387 nm, (h) typical HPTLC chromatogram obtained for GYA extracted from GS leaves at λ387 nm, and (i) typical HPTLC chromatogram obtained for PHP at its respective wavelengths. CIN, cinnamaldehyde; GLA, gallic acid; HPTLC, high-performance thin-layer chromatography; PHP, poly herbal preparation; VB, vinblastine; VC, vincristine.

Conflicts of interest
There are no conflicts of interest.

Table 4 Validation and other analytical data of high-performance thin-layer chromatography method

Parameters	Result				
	Cinnamaldehyde	Gallic acid	Vincristine	Vinblastine	Gymnemic acid
Max Rf in standard (min)	1.81	0.08	0.10	0.89	0.09
Max Rf in PHP (min)	1.81	0.08	0.11	0.88	0.09
Linear range (μg/ml)	10–100	6–18	10–30	10–30	5–15
Regression equation	Y=255.8x+5011	Y=5723.3x+7822	Y=723.2x+9370	Y=263.2x+6372	Y=6282.2x+383
Correlation coefficient (r^2)	0.999	0.998	0.998	0.999	0.997
LOD (μg/ml)	0.42	0.54	0.56	0.48	0.04
LOQ (μg/ml)	1.49	1.38	2.44	1.64	0.12
Concentration (μg/ml) in PHP	13.44	9.33	11.22	14.88	9.63

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