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Analytical method development and validation for simultaneous estimation of seven markers in polyherbal formulation JKC by using RP-HPLC

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Abstract

Background The RP-HPLC method has been established to simultaneous estimation of seven markers in polyherbal formulation JKC using the C_{18} (25 × 0.46 cm, i.d,5 µm) column. The mobile phase consisted of methanol: water (80:20) at a flow rate of 1.0 mL/min and observed retention time at 2 to 11 min with sharp points. The marker compounds viz. Andrographolide (AG), Piperine (PP), Picroside-I (P-I), Picroside-II (P-II), α-Cyprone (AC), 6-Shogaol (6S), and 6-Gingerol (6G) were quantified in JKC formulations by HPLC method. Detection was performed at the wavelength (λ) of 229 nm for AG, 343 nm for PP, 279 nm for P-I, 264 nm for P-II, 254 nm for AC, and 280 nm for both 6S and 6G by HPLC–PDA detector.

Results The marker compounds in JKC formulations were observed in different retention times (R_t) i.e. AG at 3.060 ± 0.01 min, PP at 5.460 ± 0.03 min, P-I at 2.789 ± 0.02 min, P-II at 2.553 ± 0.03 min, AC at 10.951 ± 0.02 min, 6S at 6.302 ± 0.03 min, and 6G at 4.111 ± 0.02 min respectively. The proposed method was validated with acceptable linearity (r^2 0.9995–0.9999), precision, robustness, ruggedness, and accuracy (RSD < 2%) under optimum conditions. The limit of detection and quantification of bioactive markers were as: AG (1.386; 4.200 ppm), PP (2.033; 6.161 ppm), P-I (2.822; 8.553 ppm), P-II (2.538; 7.691 ppm), AC (0.269; 0.815 ppm), 6G (0.158; 0.480 ppm), 6S (0.188; 0.569 ppm). The amount (mg/g) of bioactive markers detected and estimated in plants and formulation were as: AG (41.282 ± 0.48; 10.06 ± 0.18), PP (53.81 ± 0.25, 13.82 ± 0.37 in PN, PL; 4.27 ± 0.07), P-I (15.97 ± 0.01; 0.48 ± 0.003), P-II (63.24 ± 0.35; 2.31 ± 0.006), AC (0.42 ± 0.01; 0.36 ± 0.006), 6G (0.71 ± 0.03; 0.16 ± 0.001), and 6S (2.64 ± 0.09; 0.12 ± 0.004) respectively. Method was found to be rugged and robust. The results found for all the validation parameters were within the limits according to ICH guidelines.

Conclusion The proposed method is fast, precise, economic, and specific and used for the simultaneously quantifiable analysis of seven major bioactive markers in the ingredients (herbs) and the JKC formulations.

Keywords Validation, Development, Polyherbal, Ayurveda, ICH guidelines

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Introduction

Fever is a very common problem as well burden to health system. There are various conditions for fever, but drugs are almost very common like paracetamol/ibuprofen/aspirin etc. [1]. In Ayurveda, fever (Jwarahara) is well defined and there are various formulations, including common herbs are elaborated well from viral to dengue fever, and dengue to COVID-19 infection [2, 3]. During COVID-19, an herbal formulation (JKC) was well used in the Kerala region of India with significant control [2–4]. JKC polyherbal formulation renowned for its efficacy in addressing chronic fever, cold, malaria, digestive enhancement, detoxification, immune system fortification, and protection against bacterial infections [2–4]. The quality control of JKC formulation was not done yet, and Central Council for Research in Ayurvedic Sciences, Ministry of AYUSH, Government of India, took the initiative to ensure the quality, safety, and efficacy of the JKC formulation.

The formulation JKC is the combination of seven herbs viz. *Cyperus rotundus* L., *Piper longum* L., *Piper nigrum* L., *Zingiber officinale* Rosc., *Hediotis corymbosa* L., *Andrographis paniculata* Burm. F., *Picrorhiza kurroa* Royle ex. Benth (Table -1). As per Ayurvedic literature, all seven herbs having various pharmacological activities including the treatment of fever and COVID-19 [5–9]. Yadav et al. [9] undertook a comprehensive systematic literature review focused on delineating the pharmacological activities inherent in various species of Piper. The findings of the study robustly affirm the scientifically validated anti-amebic, anthelminthic, anti-tumor, and anti-diabetic properties attributed to Piper species. Nonetheless, in contrast to traditional assertions, the available scientific evidence is deemed inadequate to substantiate the efficacy of Piper species in the treatment of conditions such as insomnia, dementia, epilepsy, rheumatoid arthritis, asthma, spleen disorders, puerperal fever, and leprosy. Nag et al. [10] elucidated that Piperine emerges as a promising therapeutic agent with the capacity to effectively inhibit the mutated spike protein (S), featuring 12 mutations distributed at distinct points, of the SARS-CoV-2 virus. Specifically, the amino acid residue ARG403 within Piperine demonstrates a binding affinity with the mutated S protein, thereby offering the potential to disrupt the intricate interaction between the spike protein and the human angiotensin-converting enzyme 2 (hACE2) complex, as represented by the Protein Data Bank identification 6M0J. Das et al. [11] conducted a comprehensive molecular docking analysis of Andrographolide (AG) with key components associated with the inflammatory pathogenesis of SARS-CoV-2, including furin, TMPRSS-2, ACE-2, Cathepsin L, TLR4-MD2, and IL-6 cytokine. Remarkably, AG exhibited a high binding affinity to these inflammatory regulators, suggesting its potential to attenuate the pathogenic consequences of the SARS-CoV-2-induced 'cytokine storm.' Furthermore, the results from pharmacokinetic and pharmacodynamic studies underscored the safety and efficacy of AG, positioning it as a promising herbal drug for the therapeutic management of COVID-19. Hayati et al. [12] reported that 6G has weak virucidal and significant indirect antiviral activity against chikungunya virus (CHIKV) without showing cell cytotoxicity. The reported results exhibited that 6G inhibits CHIKV infection by suppressing viral replication. Rizvi et al. [13] meticulously assessed the effectiveness of *Picrorhiza kurroa* (*P. kurroa*) against COVID-19, employing a Syrian hamster infection model. The data derived from the hamster challenge experiments compellingly demonstrated the substantial anti-viral and immunomodulatory capacities inherent in *P. kurroa*, thereby underscoring its potential as a promising intervention against COVID-19. Lashgari et al. [14] elucidated that *Zingiber officinale*, commonly known as ginger, emerges as a formidable inhibitor targeting critical signalling pathways including nuclear factor kappa B (NF-κB), signal transducer of activators of transcription (STATs), Nod-like receptor family proteins (NLRPs), toll-like receptors (TLRs), mitogen-activated protein kinase (MAPKs), and the mechanistic target of rapamycin (mTOR). Additionally, ginger demonstrated efficacy in suppressing various pro-inflammatory cytokines, collectively portraying its potent anti-inflammatory properties. Rampogu et al. [15] reported that 6S has antibacterial properties against antibiotic-resistant *staphylococcus aureus* bacteria. Arcusa et al. [16] findings suggest that *Z. Officinale* phytoconstituents 6G and 6S have antioxidant and anti-inflammatory properties that might reduce the inflammation and oxidative stress in neurodegenerative diseases. Angelopoulou et al. [17] also reported that 6G and 6S are promising candidates for preventing and treating Parkinson's disease. Kumar et al. [18] conducted a molecular docking study on phytoconstituents present in *Cyperus rotundus Linn* and reported that β-amyrin and stigmasta-5,22-dien-3-ol could be screened as potential inhibitors of SARS-CoV-2 Mpro. Pundir et al. [19] identifies a potential anti-malarial compound by performing a structure-based screening of 876 phytocompounds derived from essential oils against the OBP4 by molecular docking. The study demonstrated that the two phytocompounds, α-Cyperone and Humulene oxide, have antagonistic activity for the OBP4 receptor. The result revealed that these compounds can minimize the treatment period and the side effects of currently available anti-malarial drugs.

Ensuring the high quality of a polyherbal formulation mandates a meticulous verification of the presence

of each plant within the formulation. Employing a comprehensive strategy that involves both qualitative and quantitative assessment of the markers specific to each individual plant emerges as the optimal approach for the standardization of the polyherbal formulation. The existing challenge inherent in the study of multiple markers lies in the requirement to devise distinct methods for each individual marker. To alleviate the burden associated with employing multiple methods, there arises a crucial need to establish a unified and simultaneous estimation method. Such a method should exhibit precision, accuracy, and cost-effectiveness, thereby facilitating the qualitative and quantitative assessment of multiple markers in a more streamlined and efficient manner. The innovation inherent in the presented research lies in its capability to concurrently estimate seven markers through a singular method within a single analytical run. The meticulously optimized mobile phase, consisting of methanol and water in an 80:20 (v/v) ratio, significantly enhances the resolution of all markers within a brief retention period. The primary goal of this study is to formulate and validate a reversed-phase high-performance liquid chromatography (RP-HPLC) method, enabling the simultaneous estimation of key bioactive markers. This methodology aims to contribute to the standardization of polyherbal formulations by providing a robust and efficient analytical approach. The RP-HPLC method devised in this study stands as a pioneering and distinctive analytical approach, offering expeditious, precise, and accurate simultaneous estimation of seven major bioactive markers. These markers, namely Andrographolide (AG), Piperine (PP), Picroside-I (P-I), Picroside-II (P-II), α-Cyprone (AC), 6-Shogaol (6S), and 6-Gingerol (6G), can be efficiently quantified not only in plant samples but also in various formulations (Fig. 1). The method not only signifies innovation but also encompasses cost-effectiveness, making it an invaluable tool for the comprehensive analysis of these bioactive compounds. The application of this method extends to the realm of quality control analysis for both individual plants and their commercially available polyherbal formulations. Manufacturers seeking to uphold the standards of Ayurvedic/JKC formulations can utilize this method as a robust tool for standardization. Furthermore, this approach serves as a blueprint, paving the way for the development of analogous methods tailored to other markers, thereby facilitating the comprehensive standardization of a diverse array of polyherbal formulations.

Materials and methods

Plant materials and chemicals

The plant materials were sourced from their indigenous habitats and subjected to an authentication process under the expertise of a qualified Botanist at the Institute The plant materials were sourced from their indigenous habitats and subjected to an authentication process under the expertise of a qualified Botanist at the National Ayurveda Research Institute for Panchakarma (NARIP) in Kerala, India. The Jawarhara Kwath Churna (JKC) utilized in the study was acquired from the pharmacy of Institute. These rigorous procurement and authentication procedures ensure the integrity and authenticity of the plant materials and formulated products employed in the research. Authentic standards such as Piperine (PP), Andrographolide (AG), Picroside-I (P-I), Picroside-II (P-II), α-Cyprone (AC), 6 Shogaol (6S), and 6 Gingerol (6G) were procured from the Sigma-Aldrich (USA). The chemicals and solvents utilized throughout the experimentation adhered to the highest quality standards, being of HPLC grade and sourced from Merck. These measures ensure the reliability and accuracy of the materials employed in the experimental procedures.

Qualitative and quantitative determination of bioactive markers by RP-HPLC method

Preparation of test samples for RP-HPLC

The extraction of plant materials and the JKC formulation was carried out using the sonication-assisted extraction (SAE) method. This meticulous process involved subjecting the materials to sonication at a frequency of 50 Hz for a duration of 15 min at a controlled temperature of 40 °C. Two grams of coarse powder from each plant part, as detailed in Table 1, were extracted in 10 mL of methanol. The resulting extracts underwent a filtration process, initially through Whatman no. 41 filter paper and subsequently via 0.22 µ PTFE syringe filtration, ensuring the removal of particulate matter and yielding purified extracts for further analysis. All prepared extracts were standardized by dilution with methanol, establishing a consistent concentration of 1 mg/mL for each *Andrographis paniculata, Piper longum, Piper nigrum*, and *Picrorhiza kurroa* plant parts coarse powder. However, *Cyperus rotundus linn* and *Zingiber officinale* were prepared at a concentration of 10 mg/mL for subsequent RP-HPLC analysis. Simultaneously, a 2 g portion of the JKC powdered sample underwent extraction in 10 mL of methanol. The resulting extract was further diluted with methanol, yielding solutions at concentrations of 10 mg/mL and 50 mg/mL, both stored in a refrigerated environment at 4 °C. These meticulously prepared solutions were reserved for subsequent phytochemical and HPLC analyses.

Preparation of stock solution

A comprehensive stock solution, with a concentration of 1 mg/mL, was prepared for all bioactive markers, namely

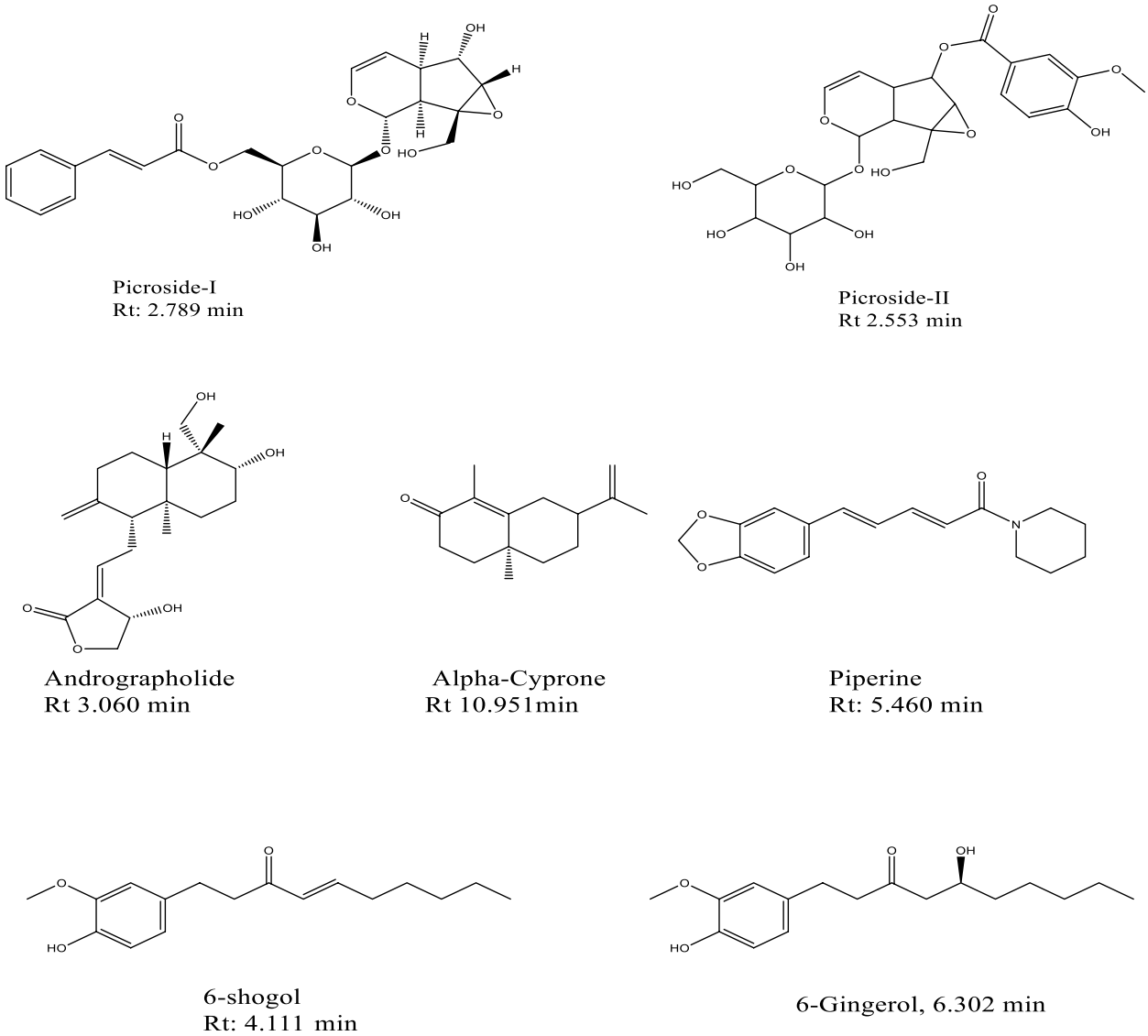


Fig. 1 Bioactive markers present in JKC formulation and its retention time in HPLC chromatogram

Table 1 List of plants and their parts used in polyherbal formulation JKC

S. No	Plant name	Botanical name	Part used	Reference (API)
1	Musthakam	*Cyperus rotundus* Linn	Root tuber	Part-I/Vol-III
2	Pipali	*Piper longum* L	Fruit	Part-I Vol-IV
3	Maricha	*Piper nigrum* L	Fruit	Part-I Vol-III
4	Shunti	*Zingiber officinale* Rosc	Rhizome	Part-I Vol-I
5	Parpata	*Hediotis corymbosa* L	Whole plant	In-house
6	Kalmegha	*Andrographis paniculata* Burm.f	Whole plant	Part-I Vol-VIII
7	Katuku rohini	*Picrorhiza kurroa* Royle ex.Benth	Root/Rhizome	Part-I Vol-II

AG, PP, P-I, P-II, AC, 6S, and 6G, utilizing HPLC-grade methanol. This standardized solution forms the basis for subsequent analyses, ensuring uniformity and precision in the evaluation of these significant bioactive compounds.

Preparation of working standard mixture solution

Stock solutions of AG, PP, P-I, and P-II were diluted with methanol, resulting in the preparation of 1 mL solutions, each attaining a concentration of 700 ppm. In parallel, the stock solutions of AC, 6S, and 6G were similarly diluted with methanol, yielding 1 mL solutions, each standardized at a concentration of 70 ppm for their respective bioactive markers. Subsequently, 1 mL of the prepared solution for each individual marker was methodically combined to achieve a homogenous mixture, resulting in a 7 mL working standard solution. In this composite solution, the final concentrations were harmonized to 100 ppm for AG, PP, P-I, and P-II, while AC, 6S, and 6G attained a final concentration of 10 ppm each. This meticulous process ensures a consistent and well-defined working standard mixture for subsequent analytical procedures.

RP-HPLC instrumentation

The experimental procedures were conducted using the Shimadzu I series RP-HPLC system, wherein a Photodiode Array (PDA) Detector and a C_{18} column featuring a 5 µm particle size and dimensions of 4.6 mm internal diameter × 250 mm length were employed. A gradient mode was utilized for all chromatographic runs. The LC lab solution software played a pivotal role in overseeing equipment control, executing data acquisition, and ensuring seamless integration throughout the analytical processes. This advanced instrumentation and software integration contributed to the precision and reliability of the analytical outcomes.

Optimization of chromatographic conditions

Multiple iterations of experimental runs were carefully conducted, systematically altering the mobile phase composition by varying the methanol-to-water ratio to encompass (50:50, *v/v*), (70:30, *v/v*), and (80:20, *v/v*). Subsequent analysis discerned that the mobile phase featuring methanol and water in an 80:20 (*v/v*) ratio offered optimal conditions for achieving superior separation, precise retention times, and peak purity. Employing this optimized mobile phase, a gradient mode was implemented with a flow rate of 1 mL/min, an injection volume of 10 µL, a column oven temperature set at 35 °C for 15 min runtime, and detection carried out using an HPLC–PDA detector at specific wavelengths: 229 nm for AG, 343 nm for PP, 279 nm for P-I, 264 nm for P-II, 254 nm for AC, 280 nm for 6S, and 6G. These meticulously optimized conditions were designed to streamline the analytical procedure, ensuring optimal peak resolution, reduced run time, and cost-effectiveness.

Method validation

The validity of the present RP-HPLC method was systematically established in accordance with the stringent guidelines outlined by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). The comprehensive validation process encompassed critical parameters, including linearity, limit of detection and quantification, precision and accuracy, robustness, ruggedness, and specificity. This adherence to ICH guidelines ensures the reliability, accuracy, and robustness of the RP-HPLC method, underscoring its suitability for precise and replicable analytical applications [20–22]

Linearity range of bioactive markers

A precisely measured 10 µL of the standard marker mixture, comprising AG, PP, P-I, P-II, AC, 6S, and 6G, was injected into the system and run for 15 min. Calibration curves were constructed within the concentration range of 10–100 ppm for AG, PP, P-I, and P-II, while AC, 6S, and 6G were calibrated in the range of 1–10 ppm, as detailed in Table 2. The ensuing chromatogram analysis was conducted for each standard, and the quantification of the presence of PP, AG, P-I, P-II, AC, 6S, and 6G within the JKC formulation was accomplished through the utilization of the systematically developed calibration plots, illustrated in Supplementary Fig. 1. This approach ensures precise and accurate quantification of the targeted bioactive markers in the tested formulation.

Specificity

The assessment of method specificity in this study involved a comprehensive analysis of the retention time (R_t) values for both reference standards and test samples, encompassing plant samples and the JKC formulation under identical chromatographic conditions (Fig. 2). This rigorous examination ensures the capability of the method to distinctly identify and quantify each bioactive marker. Additionally, the peak purity of AG, PP, P-I, P-II, AC, 6S, and 6G was meticulously evaluated by comparing the spectra at three critical levels: peak start, apex, and peak end of the respective spots, as visually represented in Fig. 3A–G. This multi-level assessment guarantees the specificity of the method in distinguishing and characterizing individual peaks within the chromatogram, enhancing confidence in the reliability of the analytical results.

Table 2 Method validation parameters for quantifying PP, AG, P-I, P-II, AC, 6G, and 6S

Parameters	AG	PP	P-I	P-II	AC	6G	6S
Wavelength (nm)	229	343	279	264	254	280	280
R_t (min)	3.06	5.46	2.789	2.553	10.951	4.111	6.302
Correlation coefficient, r	0.9999	0.9998	0.9995	0.9996	0.9996	0.9998	0.9998
Calibration range (ppm)	10–100	10–100	10–100	10–100	1–10	1–10	1–10
Linear regression equation (y)	10783x – 3642.4	64077x – 37504	23454x + 4640.4	8714.5x – 765.78	2153.9x – 37.564	6351.3x – 311.99	7095x – 20.107
% Recovery	98.43	98.84	98.59	95.98	97.67	92.62	97.65
LOD (ppm)	1.386	2.033	2.822	2.5380	0.269	0.158	0.187
LOQ (ppm)	4.200	6.161	8.552	7.691	0.815	0.480	0.568

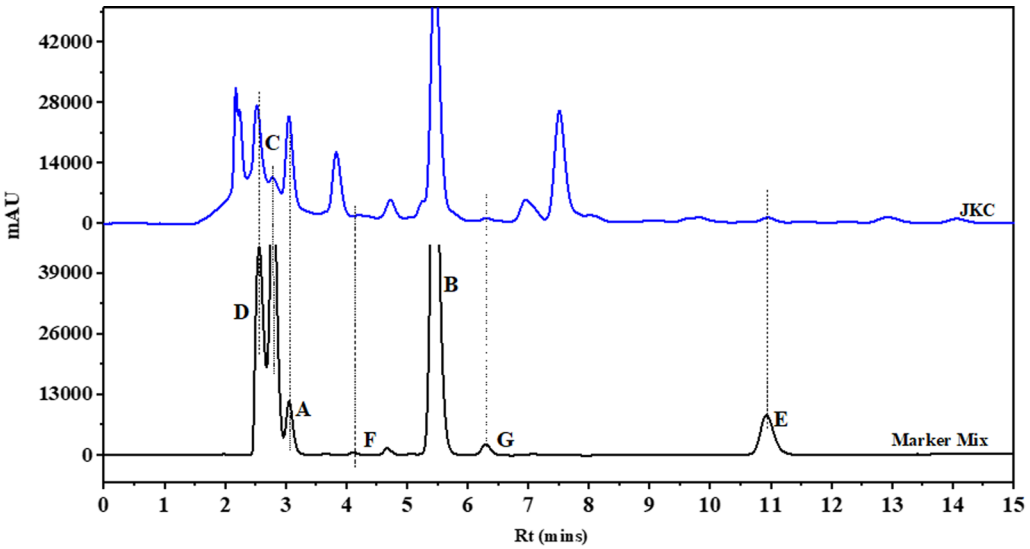


Fig. 2 HPLC Chromatogram of marker compounds mixture of polyherbal formulation JKC (A: Andrographolide, B: Piperine, C: Picroside-I, D: Picroside-II, E: α-Cyprone, F: 6-Gingerol, G: 6-Shagoal) @ 254 nm

Limits of detection and quantification

To ascertain the Limits of Detection (LOD) and Limits of Quantification (LOQ), diverse concentrations of AG, PP, P-I, P-II, AC, 6S, and 6G were systematically introduced alongside methanol as a blank reference. The LOD and LOQ were determined through the application of the response and slope standard deviation method, as outlined in Table 2. The LOD was established as 3.3 times the ratio of the standard deviation (SD) to the slope (S), while the LOQ was set at ten times the same ratio (SD/S). Here, S represents the slope of the calibration curve, and SD signifies the standard deviation associated with the Y-intercept of the regression line. This methodology provides a robust and reliable assessment of the sensitivity and precision of the analytical method for each bioactive marker [23, 24].

Precision- inter-day and intra-day variation

In assessing the precision of the analytical method, intra-day precision pertains to the consistent application of

(See figure on next page.)

Fig. 3 **A** HPLC Chromatogram of polyherbal formulation JKC with Andrographolide (A) and *Andrographis paniculata* (AP) @ 229 nm.**B** HPLC Chromatogram of polyherbal formulation JKC with Piperine (B), *Piper nigrum* (PN) and *Piper longum* (PL) @ 343 nm. **3** HPLC Chromatogram of polyherbal formulation JKC with Picroside-I (C), and *Picrorhiza kurroa* (PK) @ 279 nm.**D** HPLC Chromatogram of polyherbal formulation JKC with Picroside-II (D), and *Picrorhiza kurroa* (PK) @ 264 nm. **E** HPLC Chromatogram of of polyherbal formulation JKC with α-Cyprone (E), and *Cyperus rotundus* (CR) @ 254 nm. **F** HPLC Chromatogram of of polyherbal formulation JKC with 6-Gingerol (F), and *Zingiber officinale* (ZO) @ 280 nm. **G** HPLC Chromatogram of polyherbal formulation JKC with 6-Shogaol (G), and *Zingiber officinale* (ZO) @ 280 nm

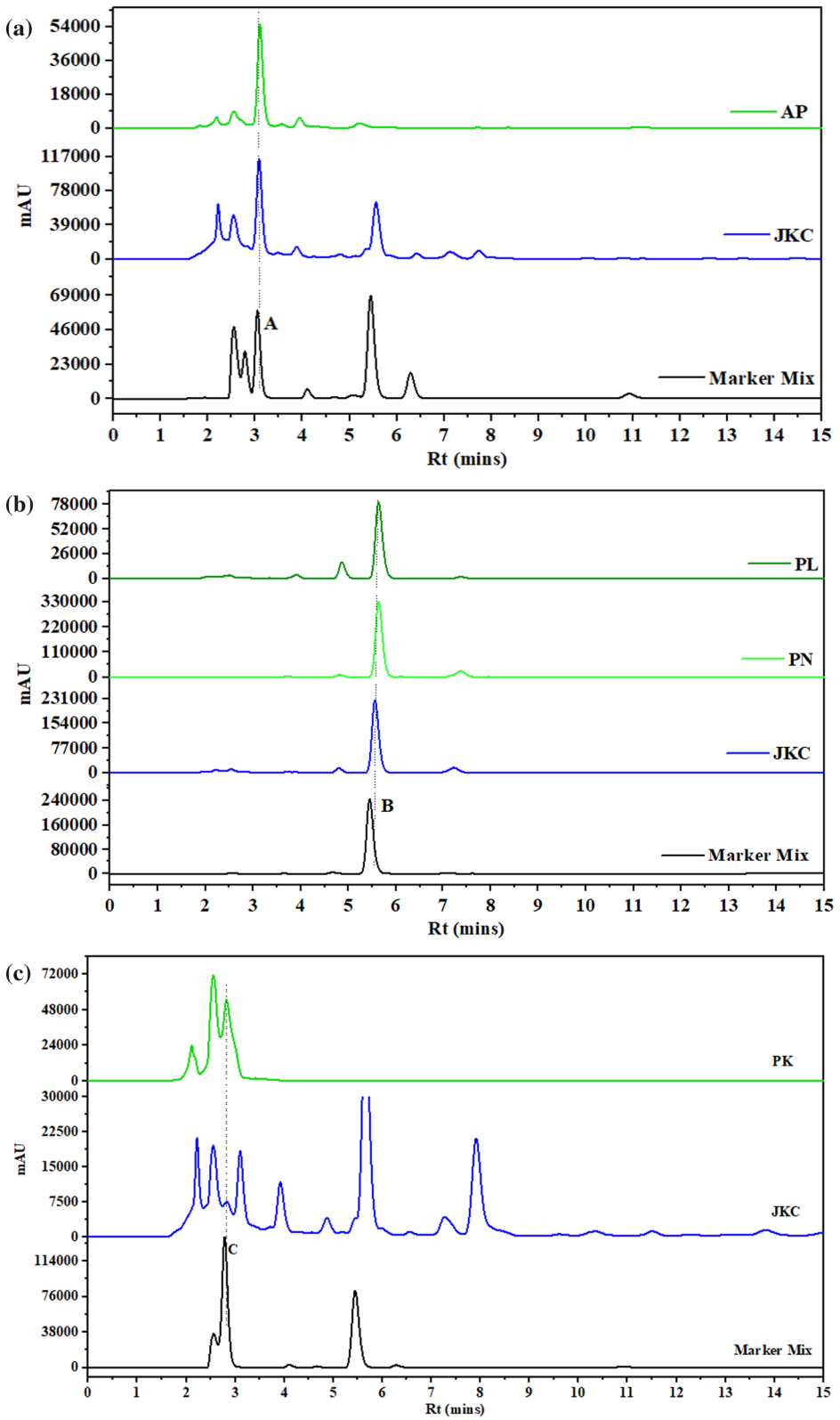
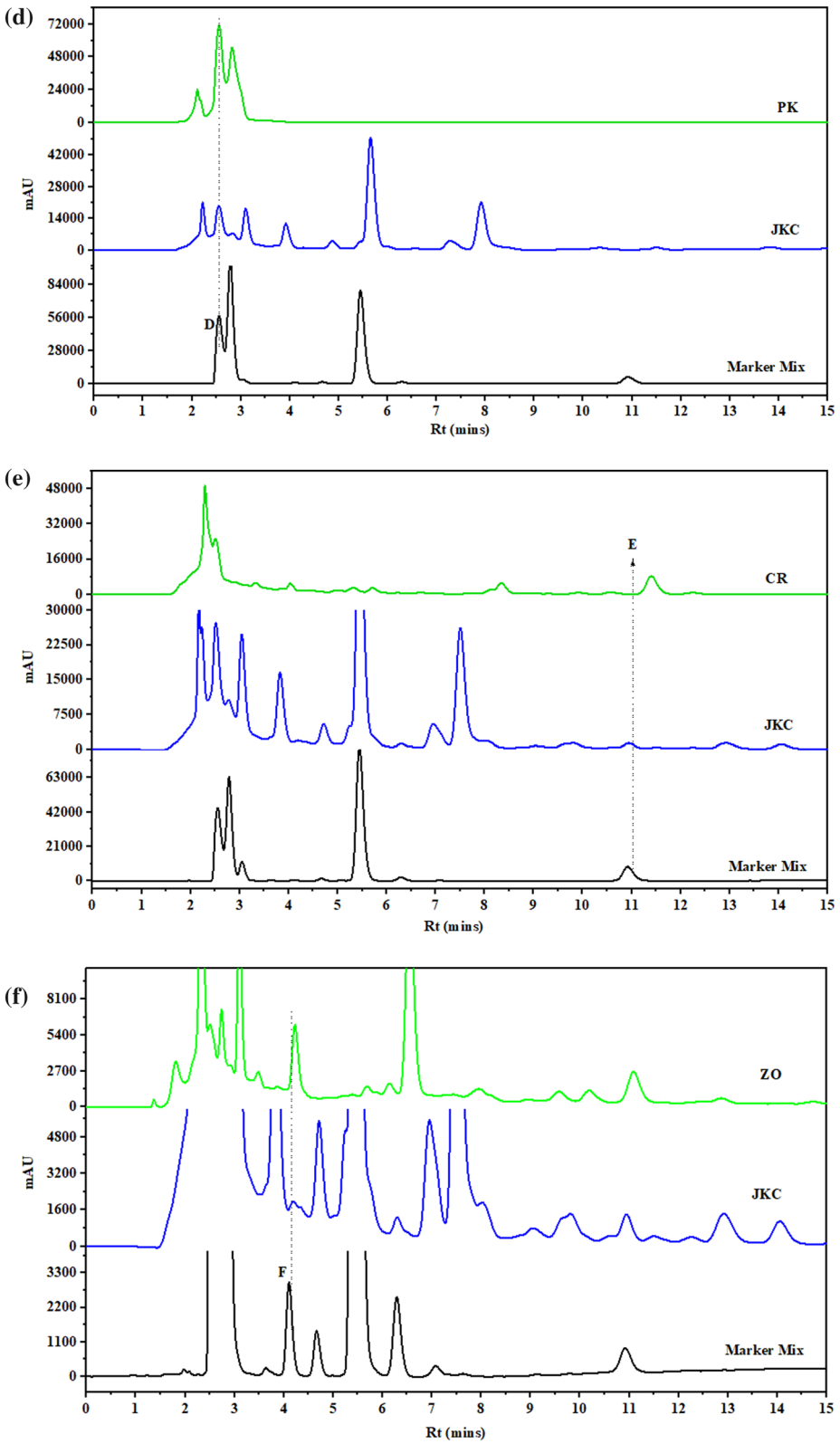


Fig. 3 (See legend on previous page.)



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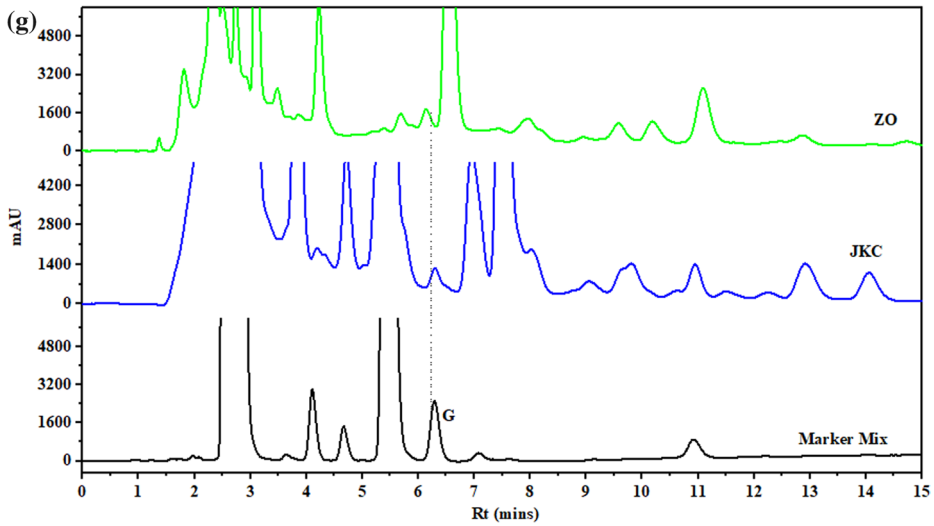

Fig. 3 continued

identical analytical procedures within a laboratory over a brief duration. On the other hand, inter-day precision involves evaluating variations in analyses when the method is applied on different days within the same laboratory. To verify instrument precision, a repeated application of the same concentration under comparable conditions was conducted, with results expressed in terms of relative standard deviation (%RSD). The precision of the method was further scrutinized through the use of three replicates of concentrations, specifically 40, 60, and 80 ppm for AG, PP, P-I, and P-II, as well as 4, 6, and 8 ppm for AC, 6S, and 6G. The method's repeatability was assessed by executing intra-day and inter-day precision at three distinct concentration levels of the bioactive markers, as meticulously detailed in Tables 3 and 4.

Robustness and ruggedness

The ruggedness assessment of the current analytical method involved executing identical experiments under consistent laboratory and chromatographic conditions with the participation of two different analysts, as detailed in Table 4. Meanwhile, the method's robustness was gauged by measuring absorbance at ± 1 nm of the respective wavelengths of the bioactive markers, as outlined in Table 5. Both ruggedness and robustness evaluations were quantified in terms of the %RSD, providing a comprehensive understanding of the method's resilience to variations in analysts and its robust performance under slight alterations in experimental conditions.

Accuracy

The assessment of analytical method accuracy involves evaluating the proximity of test results to the true value of the analyte. In the current study, accuracy was rigorously determined through a recovery study wherein the pre-quantified sample solution (5000 ppm of JKC) was intentionally spiked with three distinct concentrations: 50%, 100%, and 150% (equivalent to 10, 20, and 30 ppm for AG, PP, P-I, P-II, and 1, 2, and 3 ppm for AC, 6G, 6S of the standard marker mixture, as delineated in Table 6. This meticulous approach ensures a robust evaluation of the accuracy of the analytical method across a range of concentrations for each bioactive marker.

Qualitative and quantitative estimation of PP, AG, P-I, P-II, AC, 6S, and 6G in raw drugs and formulations

A carefully prepared solution of each plant sample, with a concentration of 1 mg/mL, was utilized for the purpose of detecting the bioactive markers, namely AG, PP, P-I, P-II, AC, 6G, and 6S. Conversely, for the JKC formulation, a solution with a concentration of 10 mg/mL was employed to specifically detect the presence of AG, PP, P-II, and AC marker compounds. Subsequently, a solution with an elevated concentration of 50 mg/mL was meticulously prepared to detect the presence of P-I, 6G, and 6S markers. These distinct concentrations were chosen with precision to ensure the optimal detection and quantification of each targeted bioactive marker within the diverse sample types.

Results

Optimization of mobile phase

The optimization of the mobile phase and flow rate is pivotal in the RP-HPLC method, influencing the efficacy of the separation process. To achieve the most favourable conditions, a systematic exploration of various

Table 3 Intra-day precision study of Marker compounds of polyherbal formulation JKC

Marker	Conc.(ppm)	Intra-day/day 1/analyst-I								
		Shift 1			Shift 2			Shift 3		
		Mean AUC (n = 3)	SD	%RSD	Mean AUC (n = 3)	SD	%RSD	Mean AUC (n = 3)	SD	%RSD
AG	40	418,899.667	996.474	0.238	434,636	279.378	0.0643	429,989	210.730	0.049
	60	640,014.333	1300.166	0.203	651,787.333	1422.756	0.218	645,811	961.019	0.149
	80	858,045.333	4007.662	0.467	861,714	1959.901	0.227	856,373.667	226.677	0.026
PP	40	2,479,865	2654.992	0.107	2,459,059.333	2449.756	0.099	2,454,725.333	1485.508	0.061
	60	3,815,609	6794.3888	0.178	3,835,538	5729.252	0.149	3,807,503.667	2476.453	0.065
	80	5,150,426.667	22,293.738	0.433	5,189,812	10,662.516	0.205	5,156,717.333	1054.652	0.020
P-I	40	956,478.333	5634.905	0.589	933,882.333	4801.365	0.514	937,504.667	2228.902	0.238
	60	1,454,897	5542.338	0.381	1,474,185	13,945.791	0.946	1,446,134.333	12,798.144	0.885
	80	1,960,468.667	2154.323	0.110	1,968,541	21,612.013	1.098	1,967,262.333	2606.330	0.132
P-II	40	342,167.333	3419.7856	0.999	345,126.333	3482.539	1.009	344,109	2574.283	0.748
	60	523,929.333	3294.906	0.629	525,189.667	2469.930	0.470	524,780	3254.466	0.620
	80	699,900.333	7569.680	1.082	705,007	2729.793	0.387	702,823.333	2661.044	0.379
AC	4	131,542.300	231.528	0.176	130,481.3	722.741	0.554	129,989.7	617.069	0.475
	6	199,585.700	1830.432	0.917	201,119.3	1499.727	0.746	202,653.3	719.650	0.355
	8	272,106.300	1094.469	0.402	274,044	559.452	0.204	274,302	569.260	0.207
6G	4	25,291.333	30.665	0.121	26,365.667	202.179	0.767	26,166.333	164.257	0.628
	6	38,811.667	94.537	0.244	39,638.667	476.708	1.203	39,835.667	374.636	0.940
	8	52,244	191.290	0.366	53,698.667	149.287	0.278	53,217	272.402	0.512
6S	4	27,131.330	356.608	1.314	28,607	325.231	1.137	29,092.670	106.049	0.365
	6	41,709	26.851	0.064	43,979.670	239.678	0.545	43,711.670	240.092	0.549
	8	56,599	190.518	0.337	58,758.670	192.899	0.328	58,254	26.851	0.046

combinations of the mobile phase and flow rate was conducted. This investigation revealed that methanol and water (80:20, *v/v*) and a flow rate of 1 mL/min yielded the optimal separation, characterized by excellent peak purity and resolution. In the current methodology, the chromatogram of the standard marker mixture exhibited retention times (R_t) at 3.060 ± 0.01, 5.460 ± 0.03, 2.789 ± 0.02, 2.553 ± 0.03, 10.951 ± 0.02, 6.302 ± 0.03, and 4.111 ± 0.02 min for AG, PP, P-I, P-II, AC, 6G, and 6S, respectively (Fig. 1). Notably, all markers were consistently observed at the same retention times in the respective plant extracts and the JKC formulation, underscoring the reliability and reproducibility of the analytical method across diverse sample types (Fig. 2).

Method validation

Linearity

The linearity of an analytical method is a crucial indicator of its capacity, within a specified range, to produce test results that are directly proportional to analyte concentration or can be mathematically transformed to exhibit proportionality. Remarkably, a robust linear relationship between peak area and quantity was established across the concentration range of 10–100 ppm for AG, PP, P-I, P-II, and 1–10 ppm for AC, 6G, and 6S. The linear regression data for the calibration plot, expressed as correlation coefficients (r^2), consistently fell within the impressive range of 0.9995–0.9999 for all bioactive markers, as comprehensively detailed in Table 2 and visually depicted in Supplementary Fig. 1. This compelling data attests to the exceptional linear relationship within the studied concentration range, affirming the method's adherence to Beer's law. Such findings underscore the method's suitability for the precise and reliable analysis of AG, PP, P-I, P-II, AC, 6S, and 6G in the JKC formulation.

Specificity

Specificity, a critical attribute of an analytical method, gauges its capability to evaluate the analyte in the presence of a complex sample matrix. The determination of peak purity, as expressed through regression (r^2) values for the bioactive markers, is detailed in Table 2. To further validate the method's specificity, a comprehensive investigation was conducted by comparing the retention time (R_t) values of the markers with those observed in the samples, as visually depicted in Figs. 2 and 3A–G. Remarkably, the R_t values were found to be identical, confirming the method's high specificity. Notably, the

Table 4 Inter-day precision, and ruggedness study of marker compounds of polyherbal formulation JKC

Marker	Conc. (ppm)	Inter-day			Ruggedness		
		Day 2/Analyst-I			Analyst-II		
		Mean AUC (n = 3)	SD	%RSD	Mean AUC (n = 3)	SD	%RSD
AG	40	426,605.667	7233.630	1.696	426,816.333	6255.295	1.466
	60	641,971.333	1743.3170	0.272	638,282.333	7406.736	1.160
	80	851,520.667	2264.506	0.266	852,981.333	2769.883	0.325
PP	40	2,460,900.333	14,225.86413	0.578	2,465,080	14,510.985	0.589
	60	3,854,082.333	23,988.89265	0.622	3,824,663	66,853.234	1.748
	80	5,124,826	24,745.67946	0.483	5,132,626.333	15,850.081	0.309
P-I	40	941,862.333	6524.344744	0.693	940,600.333	13,118.978	1.395
	60	1,452,656.333	11,437.02502	0.787	1,448,779.667	4026.265	0.278
	80	1,953,947	8582.742627	0.439	1,932,094.667	17,834.044	0.923
P-II	40	342,569.667	2002.053279	0.584	343,269	592.717	0.173
	60	532,135.667	5747.8351	1.080	517,609.667	3457.834	0.668
	80	704,215.667	2559.788338	0.363	710,958.333	8883.046	1.249
AC	4	131,427.3	844.3852	0.642	130,240.700	1000.595	0.768
	6	202,901	1398.289	0.689	200,733.700	1534.867	0.765
	8	270,604.3	3598.789	1.329	272,840.300	1361.431	0.499
6G	4	25,381.333	205.0398335	0.808	25,666	353.654	1.378
	6	39,619.667	655.1429869	1.654	38,370.667	62.172	0.162
	8	52,747	549.3423341	1.041	52,449.333	571.546	1.090
s	4	27,229	158.6537	0.583	27,506.670	430.361	1.565
	6	41,670	205.9393	0.494	41,303.330	126.637	0.307
	8	56,404.670	296.3399	0.525	56,089.670	531.068	0.947

absence of degradation products or impurities coexisting with the marker peaks further affirms the method's specificity. This observation underscores the method's ability to discern and quantify the bioactive markers accurately in the presence of complex sample matrices, thereby enhancing confidence in its reliability and suitability for analytical applications.

Limit of detection and quantification

The limits of detection (LOD) and quantification (LOQ) for the bioactive markers are presented in Table 2, showcasing the remarkable sensitivity of the analytical method. Specifically, the LOD and LOQ values for each bioactive marker are as follows: AG with values of 1.386 ppm and 4.200 ppm, PP with values of 2.033 ppm and 6.161 ppm, P-I with values of 2.822 ppm and 8.553 ppm, P-II with values of 2.538 ppm and 7.691 ppm, AC with values of 0.269 ppm and 0.815 ppm, 6- 6G with values of 0.158 ppm and 0.480 ppm, and 6S with values of 0.188 ppm and 0.569 ppm. These low detection and quantification limits highlight the method's capability to precisely identify and quantify minute concentrations of bioactive markers, demonstrating its suitability for rigorous analytical applications.

Robustness and ruggedness

The %RSD of the Area Under the Curve (AUC) was determined and found to be consistently less than 2%, attesting to the high precision and reliability of the developed RP-HPLC method. The method's robustness was further established through a series of deliberately induced small changes, such as variations in wavelength by ± 1 nm, as detailed in Table 5. The consistently low %RSD values obtained under these conditions underscore the method's robust nature. Moreover, the method's ruggedness was affirmed by the lower %RSD values derived from experiments conducted by two different analysts under constant chromatographic conditions, as outlined in Table 4. This resilience of the developed RP-HPLC method against slight variations in wavelengths and the involvement of different analysts demonstrates its suitability and reliability in routine use, ultimately emphasizing the robustness of the method for consistent and dependable analytical results.

Accuracy and precision

The validation of the developed method adheres to the guidelines set forth by the ICH. The %RSD values for accuracy and precision, comprehensively presented in

Table 5 Robustness study analysis data of mixture of marker compounds of polyherbal formulation JKC

Standard Marker	Concentration ppm	Deviation in nm/ Level	Wavelength (nm)	Mean AUC	SD	RSD (%)
AG	60	−1	228	6,44,857	1300	0.203
		0	229	6,40,014	1467	0.227
		+1	230	6,32,335	934	0.148
PP	60	−1	342	38,22,264	6775	0.177
		0	343	38,15,609	6794	0.178
		+1	344	38,02,076	6782	0.178
P-I	60	−1	278	14,47,338	5575	0.385
		0	279	14,54,897	5542	0.381
		+1	280	14,51,036	5545	0.382
P-II	60	−1	263	5,25,638	3419	0.650
		0	264	5,23,929	3295	0.629
		+1	265	5,17,409	3205	0.619
AC	6	−1	253	2,01,758	1873	0.928
		0	254	1,99,586	1830	0.917
		+1	255	1,97,184	1794	0.909
6G	6	−1	279	38,779	81	0.208
		0	280	38,812	95	0.243
		+1	281	38,998	99	0.255
6S	6	−1	279	41,698	34	0.082
		0	280	41,709	27	0.064
		+1	281	41,840	25	0.058

Table 6 Percent Recovery of marker compounds of polyherbal formulation JKC

Standard Marker	Amount of standard present in JKC sample (5kppm)	Amount of standard added (µg)	Theoretical added value	Amount of standard obtained	Recovery (%)	Average Recovery (%)	SD	RSD (%)
AG	48.2005	10.1415	57.248	58.342	98.12	98.4307	0.268053	0.272
	48.2005	19.687	66.898	67.8875	98.54			
	48.2005	30.079	77.203	78.2795	98.62			
PP	20.466	10.005	30.471	30.194	99.09	98.841	0.226783	0.229
	20.466	19.7445	40.2105	39.667	98.65			
	20.466	30.262	50.728	50.111	98.78			
P-I	2.0535	10.1615	12.215	11.851	97.02	98.59154	1.731454	1.756
	2.0535	19.7405	21.794	21.425	98.30			
	2.0535	30.447	32.5005	32.646	100.45			
P-II	10.907	10.2025	21.1095	20.086	95.15	95.97654	1.19944	1.249
	10.907	19.4255	30.3325	28.945	95.43			
	10.907	29.81	40.717	39.639	97.35			
AC	0.335	0.997	1.332	1.308	98.19	97.69658	0.434451	0.444
	0.335	1.97	2.305	2.246	97.44			
	0.335	3.0195	3.3545	3.269	97.45			
6G	1.073	1.0195	2.0925	1.936	92.52	92.62487	0.937163	1.011
	1.073	1.9975	3.0705	2.817	91.74			
	1.073	2.98	4.053	3.794	93.60			
6S	0.8785	1.005	1.8835	1.855	98.49	97.65317	0.757276	0.775
	0.8785	1.979	2.8575	2.772	97.00			
	0.8785	3.0265	3.905	3.806	97.46			

Tables 3 and 4, underscore the robustness and reliability of the method. The intra-day and inter-day experimental studies consistently yielded %RSD values below 2%, affirming the repeatability of the developed method for the precise estimation of bioactive markers in both plant samples and the JKC formulation. Furthermore, the average % recovery for each bioactive marker compound at three different concentration levels demonstrated remarkable accuracy: 98.43% for AG, 98.84% for PP, 98.59% for P-I, 95.57% for P-II, 97.69% for AC, 92.62% for 6G, and 97.65% for 6S. The proximity of % recovery to 100% accentuates the high accuracy inherent in the current analytical method, as detailed in Table 6.

Qualitative and quantitative estimation of PP, AG, P-I, P-II AC, 6S, and 6G in raw drugs and formulations

The quantification of bioactive markers through RP-HPLC method revealed concentrations of 41.28 ± 0.48 mg/g of AG in *Andrographis paniculata*, 53.81 ± 0.25 mg/g of PP in *Piper nigrum*, 13.82 ± 0.37 mg/g of PP in *Piper longum*, 15.97 ± 0.01 mg/g of P-I, and 63.24 ± 0.35 mg/g of P-II in *Picrorhiza kurroa*. Additionally, 0.417 ± 0.01 mg/g of AC in *Cyperus rotundus*, 0.705 ± 0.03 mg/g of 6G, and 2.6356 ± 0.09 mg/g of 6S in *Zingiber officinale* were accurately estimated. The RP-HPLC analysis extended to the JKC formulation, revealing the presence of AG (10.06 ± 0.18 mg/g), PP (4.27 ± 0.07 mg/g), P-I (0.48 ± 0.003 mg/g), P-II (2.31 ± 0.006 mg/g), AC (0.36 ± 0.006 mg/g), 6S (0.12 ± 0.004 mg/g), and 6G (0.16 ± 0.001 mg/g) across three batches (Table 7). The minimal deviation and % RSD observed among batch results underscores the batch-to-batch uniformity of the formulation. The precision and accuracy of the developed method are evident, making it suitable for determining content uniformity during bulk manufacturing of JKC formulations, thereby ensuring the consistent quality of the produced batches.

Discussions

Traditional medicines (TMs), encompassing Ayurvedic medicine (AM) and other classical preparations, are gaining increased recognition for clinical applications. Despite the rich historical prevalence of Herbal medicine as an ancient system for addressing clinical disorders, its widespread application has been hindered by a notable lack of standardization [23, 24]. The imperative need for robust standardization techniques becomes evident in ensuring the quality, quantity, and efficacy of traditional medicines [24, 25]. Implementing proper standardization methodologies is pivotal to unlocking the full potential of these traditional remedies and enhancing their acceptance and utility in contemporary clinical practice [26]. In accordance with the guidelines set forth by the WHO, chromatographic analytical methods emerge as of paramount importance for the quality control and standardization of polyherbal formulations. This recognition underscores the pivotal role that chromatography plays in ensuring the consistency, reliability, and adherence to established quality standards in the manufacturing and assessment of polyherbal formulations [27, 28]. JKC, a polyherbal Ayurvedic medicine renowned for its antimalarial and antipyretic properties in the region of Kerala, India, faces a challenge in terms of a discernible framework for quality control and standardization protocols. The comprehensive analysis of this formulation involves the identification of bio-marker compounds, including AG, PP, P-I, P-II, AC, 6S, and 6G, present in the plants detailed in Table 1, which constitute the fundamental components of the JKC formulation. The elucidation of these bio-marker compounds is pivotal for establishing rigorous quality control measures and standardization protocols essential for the reproducibility and reliability of JKC, ensuring its effectiveness and safety in traditional Ayurvedic practice [28]. Hence, the current investigation was meticulously devised to conduct qualitative and quantitative analyses of the principal bioactive

Table 7 Quantitative estimation of marker compounds of polyherbal formulation JKC (Result n = 3 ± SD)

S.No	Plant	Marker	Marker Quantity in mg/g plant	Quantity in JKC formulation (mg/g)			Average ± SD	%RSD
				B1	B2	B3		
1	*Andrographis paniculata*	AG	41.28 ± 0.48	10.27 ± 0.654	9.97 ± 1.955	9.95 ± 1.214	10.06 ± 0.18	1.79
2	*Piper Nigrum*	PP	53.81 ± 0.25	4.34 ± 0.144	4.25 ± 0.163	4.22 ± 0.081	4.27 ± 0.07	1.53
3	*Piper Longum*	PP	13.82 ± 0.37					
4	*Cyperus Rotundus*	AC	0.42 ± 0.01	0.37 ± 0.097	0.36 ± 0.083	0.36 ± 0.032	0.36 ± 0.006	1.89
5	*Picrorhiza Kurroa*	P-I	15.97 ± 0.01	0.48 ± 0.516	0.48 ± 0.291	0.48 ± 0.676	0.48 ± 0.003	0.62
		P-II	63.24 ± 0.35	2.51 ± 0.169	2.32 ± 0.118	2.32 ± 0.074	2.31 ± 0.006	0.25
6	*Zingiber Officinalle*	6G	0.71 ± 0.03	0.16 ± 0.349	0.16 ± 0.268	0.16 ± 0.069	0.16 ± 0.001	0.52
		6S	2.64 ± 0.09	0.19 ± 0.111	0.19 ± 0.118	0.19 ± 0.589	0.12 ± 0.004	1.79

compounds present in JKC. Within this study, a rigorously validated RP-HPLC method has been innovatively developed and rigorously validated. This method serves the critical purpose of accurately estimating the concentrations of major bioactive markers, namely AG, PP, P-I, P-II, AC, 6S, and 6G within the JKC formulation. The application of this method ensures a robust and reliable analytical framework for the precise quantification of these key bioactive compounds, contributing to the advancement of quality control and standardization protocols for JKC.

The proposed methodology stands out as an uncomplicated, precise, specific, accurate, rapid, and cost-effective approach. Rigorous statistical analysis underscores the inevitability of this method for the comprehensive analysis of AG, PP, P-I, P-II, AC, 6S, and 6G compounds. The developed RP-HPLC method, thus conceived, serves as a valuable tool for manufacturers, facilitating the standardization and quality control processes for both raw materials and the JKC formulation. Polyherbal formulations are complex, combining various phyto-constituents. No single component is solely responsible for overall efficacy. This complexity poses a challenge to conventional quality control standards and standardization approaches [27, 28]. This predicament is further compounded by the multifaceted composition of herbal formulations. The estimation of major bioactive markers from all constituent plants within the JKC formulation emerges as a more precise and accurate method for quality assessment compared to the estimation of only one or two markers [5, 6]. This holistic approach ensures a comprehensive and nuanced understanding of the formulation's quality, reinforcing the significance of the proposed RP-HPLC method in advancing the field of herbal formulation analysis [26–29].

The devised methodology enables the simultaneous estimation of a spectrum of bioactive markers within JKC: AG sourced from *Andrographis paniculata*, AC derived from *Cyperus rotundus linn*, P-I and P-II obtained from *Picrorhiza kurroa*, 6S and 6G extracted from *Zingiber officinale*, while PP is commonly found in *Piper longum* and *Piper nigrum*. Notably, all seven of these bioactive markers are present in significant quantities within the JKC formulation (Fig. 2). The retention time (R_t) and absorption spectra of these seven markers not only mirror the observed characteristics of the plants and formulation but also exemplify the substantial specificity of the method (Fig. 3A–G). Beyond its quantitative applications, this method holds promise for qualitative assessments of bioactive markers in polyherbal formulations. Akowuah et al. [30] previously established an HPLC method to quantify Andrographolide in plant samples, reporting a retention time of approximately 5 min. In contrast, our developed method achieved a retention time of around 3 min for Andrographolide, demonstrating superior accuracy, precision, and sensitivity in the estimation of Andrographolide in both plant samples and formulations (Fig. 3A). Parab et al. [31] developed an RP-HPLC method specifically tailored for the singular marker, Piperine. Their approach encompassed the optimization of experimental conditions, involving a mobile phase composition of Acetonitrile and Water with 0.05% Acetic acid (in a ratio of 70:30), a flow rate set at 1 mL/min, and detection at a wavelength of 342 nm. The reported retention time (R_t) for Piperine was precisely documented at 5.5 min. The newly devised simultaneous analytical method extends its capabilities to encompass the concurrent estimation of Piperine, a significant bioactive compound, at a wavelength of 343 nm. The observed retention time (R_t) for Piperine is precisely delineated at 5.46 min (Fig. 3B). This expanded functionality enhances the method's versatility, providing a comprehensive analytical approach for the concurrent assessment of multiple bioactive compounds, including the crucial marker, Piperine, contributing to a more holistic understanding of the analyzed samples. In the work conducted by Shanbhag et al. [32], a simultaneous method was introduced to quantify P-I and P-II, revealing that these compounds could be detected at a retention time (R_t) falling in the range of 15–20 min. In contrast, the innovative method developed in this study demonstrates a significantly expedited detection, pinpointing P-I and P-II within a mere 3 min (Fig. 3C, D). This substantial reduction in retention time underscores the efficiency and promptness achieved by the proposed methodology in accurately estimating P-I and P-II, showcasing its potential for streamlining and expediting the analytical process. It is imperative to note that previous studies have reported the notable presence of significant amounts of AC in *Cyperus rotundus linn* in plant samples, reinforcing the method's capability to accurately identify and quantify specific phytoconstituents in complex herbal matrices [33]. Similarly, this method also detects AC in plant sample and formulation around 11 min (Fig. 3E). In their pioneering work, Anita et al. quantified 6G utilizing a mobile phase composed of acetonitrile: water: methanol (in a volumetric ratio of 70:20:10) with a flow rate set at 1 mL/min, yielding a reported retention time (R_t) around 4 min [34]. The developed simultaneous method, designed to encompass multiple bioactive compounds, also successfully estimated 6G, exhibiting a retention time of approximately 4 min (Fig. 3F). Notably, the simultaneous method demonstrated similar accuracy in the estimation of 6G compared to the previously reported technique. In the investigation by Kajsongkram et al. [35] the detection of 6G and 6S was accomplished at considerable retention

times of 29 and 41 min, respectively. However, our developed methodology has remarkably improved this aspect, enabling the detection of 6G and 6S within significantly reduced times of 4 and 6 min, as shown in Fig. 3F and G respectively. This efficient identification signifies the absence of interference in the peaks, attesting to the method's ability to mitigate interactions between the bioactive markers and other components within the formulation.

In medicinal plants and plant-based drugs, the quantitative analysis of specific markers is not an easy job [21, 22]. Plant based formulations contained complex matrix and there are various factors like climate, geographical conditions, soil conditions, stress etc. are responsible for the development of secondary metabolites in plants [36–39]. To develop an herbal formulation, the collection time is an important factor, so in current study, the raw drugs were collected and authenticated by the experts. Moreover, good agricultural practices (GAP) and good manufacturing practices (GMP) are the two main pillars to develop an herbal formulation, those were fully adopted in current study [21–26]. All the raw ingredients of polyherbal formulation JKC were collected as suggested in GAP, and formulation was prepared in GMP certified pharmacy. Moreover, storage of herbal formulation is an important issue with special concern of shelf life. In case of polyherbal formulation, if drug stored in proper conditions, there is no chance of marker compounds variations till the expiry of formulation [21–24].

The developed multi-marker approach not only exhibits specificity, precision, and sensitivity but also surpasses the reported method in terms of these crucial analytical parameters. Furthermore, the simultaneous estimation method ensures that the sensitivity of one marker does not compromise the sensitivity of others, exemplifying its robustness and reliability in accurately assessing multiple bioactive components within the formulation.

Conclusion

In this study, the RP-HPLC method has been conceptualized and rigorously validated for the quantitative determination of key bioactive markers present in major plant sources, as well as in the Central Council for Research in Ayurvedic Sciences (CCRAS)-coded formulation, JKC. The outcomes of this investigation unequivocally establish that the developed RP-HPLC analytical method possesses commendable attributes, including cost-effectiveness, expeditiousness, precision, specificity, robustness, and accuracy. This method proves to be exceptionally effective in quantifying AG, PP, P-I, P-II, AC, 6S, and 6G. Moreover, the versatility of the developed RP-HPLC method extends its utility for routine quality control analyses of all seven marker compounds, making it a valuable tool for ensuring the quality and consistency of herbal formulations within the Herbal industry.

The developed RP-HPLC method exhibits commendable efficacy, ensuring robust separation, repeatability, efficiency, and sharp resolution of active compounds. Rigorous validation, adhering to ICH guidelines, encompassed critical parameters such as linearity, accuracy, precision, selectivity, LOD, LOQ, specificity, and robustness. In light of these comprehensive advantages, it is unequivocally concluded that the developed procedure stands as a robust and reliable approach for the simultaneous determination of the seven bioactive markers. This holds significant implications for bolstering quality assurance practices in the realm of Polyherbal formulations. Moreover, the versatility of the RP-HPLC method extends its applicability, positioning it not only for laboratory testing but also for the characterization of bioactive markers in industrial bulk production, thereby fortifying its role in ensuring the quality and consistency of Polyherbal formulations.

Abbreviations

RP-HPLC	Reverse-phase high-performance liquid chromatography
ICH	International Conference on Harmonization
SD	Standard deviation
RSD	Relative standard deviation
LOD	Limit of detection
LOQ	Limit of quantitation
RT	Retention time
R^2	Coefficient of determination
6G	6-gingerol
6S	6-shagol
AC	α-cyperone
AG	Andrographolide
AM	Ayurvedic medicine
AUC	Area under curve
CCRAS	Central Council for Research in Ayurvedic Sciences
ICH	International Council Harmonisation
JKC	Jwarahara Kwatha Choorna
Min	Minute
mL	Mille liter
nm	Nano-meter
PDA	Photo diode array
P-I	Picroside-I
P-II	Picroside-II
PP	Piperine
PPM	Parts per million
PTFE	Poly tetra fluoro ethylene
SAE	Sonication assisted extraction
SARS	CoV-2: severe acute respiratory syndrome coronavirus 2
TMs	Traditional medicines
WHO	World Health Organization

Supplementary Information

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Additional file1.

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References

1. Franceschi F, Saviano A, Carnicelli A, Lorusso C, Novelli A, Candelli M, Ojetti V, Covino M (2023) Treatment of fever and associated symptoms in the emergency department: which drug to choose? Eur Rev Med Pharmacol Sci 27:7362–7369. https://doi.org/10.26355/eurrev_202308_33308
2. Singh R, Goel S, Bourgeade P, Aleya L, Tewari D (2021) Ayurveda Rasayana as antivirals and immunomodulators: potential applications in COVID-19. Environ Sci Pollut Res Int 28(40):55925–55951. https://doi.org/10.1007/s11356-021-16280-5
3. Kumar V, Singh SB, Singh S (2020) COVID-19: environment concern and impact of Indian medicinal system. J Environ Chem Eng 8:104144. https://doi.org/10.1016/j.jece.2020.104144
4. Chavda VP, Kumar A, Banerjee R, Das N (2022) Ayurvedic and other herbal remedies for dengue: an update. Clinic Comp Med Pharmacol 2:100024. https://doi.org/10.1016/j.ccmp.2022.100024
5. Kumar V, Kushwaha V, Charde V, Jagtap C, Gandhi Y, Singh A, Singh R, Srikanth N, Dhiman KS (2022) The validated pharmaceutical standard operating procedure and quality control study of the coded polyherbal tablet formulation AYUSH SG-5. South Afri J Bot 151:319–327. https://doi.org/10.1016/j.sajb.2022.02.038
6. Gandhi Y, Kumar V, Singh G, Prasad SB, Mishra SK, Singh R, Srikanth N, Webster TJ (2024) Chemoprofiling and medicinal potential of underutilized leaves of *Cyperus scariosus*. Sci Rep 14:7263. https://doi.org/10.1038/s41598-024-58041-7
7. Kumar V, Singh SB, Singh S (2021) Pharmacological Perspectives of *Ayurvedic Herbs* viz. *Alstonia scholaris* L., *Picrorhiza kurroa*, *Swertia chirata* and *Caesalpinia crista* against COVID-19: a mini-review. Mini-Rev Org Chem 18:841–849. https://doi.org/10.2174/1570193X17999201102200944
8. Gandhi Y, Mishra SK, Rawat H, Acharya R, Kumar V (2022) Phytomedicines explored under in vitro and in silico studies against coronavirus: an opportunity to develop traditional medicines. South Afri J Bot 151:451–483. https://doi.org/10.1016/j.sajb.2022.04.053
9. Yadav V, Krishnan A, Vohora D (2020) A systematic review on *Piper longum* L.: bridging traditional knowledge and pharmacological evidence for future translational research. J Ethnopharmacol 247:112255. https://doi.org/10.1016/j.jep.2019.112255
10. Nag A, Paul S, Banerjee R, Kundu R (2021) In silico study of some selective phytochemicals against a hypothetical SARS-CoV-2 spike RBD using molecular docking tools. Comp bio med 137:104818. https://doi.org/10.1016/j.compbiomed.2021.104818
11. Das BS, Das NC, Swain SS, Mukherjee S, Bhattacharya D (2022) Andrographolide induces anti-SARS-CoV-2 response through host-directed mechanism: an in silico study. Futur Virol 17:651–673. https://doi.org/10.2217/fvl-2021-0171
12. Hayati RF, Better CD, Denis D, Komarudin AG, Bowolaksono A, Yohan B, Sasmono RT (2021) [6]-Gingerol inhibits chikungunya virus infection by suppressing viral replication. BioMed res Int 2021:6623400. https://doi.org/10.1155/2021/6623400
13. Rizvi ZA, Madan U, Tripathy MR, Goswami S, Mani S (2023) Evaluation of ayush-64 (a polyherbal formulation) and its ingredients in the Syrian hamster model for SARS-CoV-2 infection reveals the preventative potential of *Alstonia scholaris*. Pharmaceutic 16:1333. https://doi.org/10.3390/ph16091333
14. Lashgari NA, Roudsari M, Khayatan D, Shayan M, Momtaz S, Sahebkar A (2022) Ginger and its constituents: role in treatment of inflammatory bowel disease. BioFactors 48:7–21. https://doi.org/10.1002/biof.1808
15. Rampogu S, Baek A, Gajula RG, Zeb A, Bavi RS, Kumar R, Kim Y (2018) Ginger (*Zingiber officinale*) phytochemicals—gingerenone-A and shogaol inhibit SaHPPK: molecular docking, molecular dynamics simulations and in vitro approaches. Annal Clin Microbiol Antimicro 17:1–15. https://doi.org/10.1186/s12941-018-0266-9
16. Arcusa R, Villaño D, Marhuenda J, Cano M, Cerdà B, Zafrilla P (2022) Potential role of ginger (*Zingiber officinale* Roscoe) in the prevention of neurodegenerative diseases. Front nutrition 9:809621. https://doi.org/10.3389/fnut.2022.809621
17. Angelopoulou E, Paudel YN, Papageorgiou SG, Piperi C (2022) Elucidating the beneficial effects of ginger (*Zingiber officinale* Roscoe) in Parkinson's disease. ACS Pharmacol Trans Sci 5:838–848. https://doi.org/10.1021/acsptsci.2c00104
18. Kumar SB, Krishna S, Pradeep S, Mathews DE, Pattabiraman R (2021) Screening of natural compounds from *Cyperus rotundus* Linn against SARS-CoV-2 main protease (Mpro): an integrated computational approach. Comp bio med 134:104524. https://doi.org/10.1016/j.compbiomed.2021.104524
19. Pundir H, Pant M, Joshi T, Bhat S, Pathak R, Bajpai AB (2023) Identification of essential oil phytocompounds as natural inhibitors of Odorant-binding protein to prevent malaria through in silico approach. J Biomol Struct Dyn 4:8323–8333. https://doi.org/10.1080/07391102.2022.2132419
20. Guideline IHT (2005) Validation of analytical procedures: text and methodology. Q2 (R1), 1, 05. https://somatek.com/wp-content/uploads/2014/06/sk140605h.pdf
21. Gandhi Y, Kushwaha V, Kumar V, Rawat H, Charde V (2024) Simultaneously quantification of eight marker compounds by HPLC, and HPTLC analysis for the marker-based shelf-life kinetic study for the standardization of polyherbal drug AYUSH SG-5, medicinal properties and computational studies. Microchem J 199:109958. https://doi.org/10.1016/j.microc.2024.109958
22. Gandhi Y, Rawat H, Kumar V, Thakur A, Mishra SK, Charde V (2023) Identification of two kinds of Colchicum corms by DNA barcoding, physicochemical, chromatographic and chemometric analyses along with pharmacognostic parameters. Microchem J 195:109399. https://doi.org/10.1016/j.microc.2023.109399
23. Kumar V, Singh S, Singh A, Subhose V, Prakash O (2019) Assessment of heavy metal ions, essential metal ions, and antioxidant properties of the most common herbal drugs in Indian Ayurvedic hospital: For ensuring quality assurance of certain Ayurvedic drugs. Biocat Agri Biotech 18:101018. https://doi.org/10.1016/j.bcab.2019.01.056

24. Kumar V, Singh S, Srivastava B, Patial PK, Kondalkar SA, Bharthi V (2019) Volatile and semi-volatile compounds of Tephrosia purpurea and its medicinal activities: experimental and computational studies. Biocat Agri Biotech 20:101222. https://doi.org/10.1016/j.bcab.2019.101222
25. Maurya R, Boini T, Misro L, Radhakrishnan T, Singh R (2023) Review on pharmacognostic, phytochemical and pharmacological characteristics of medicinal plants which can be used to design a novel Ayurvedic formulation to treat hypertension in multidimensional approaches. Curr Hyperten Rev 19:67–78. https://doi.org/10.2174/1573402119666230221084859
26. Maurya R, Thirupataiah B, Misro L, Thulasi R (2022) Different aspects of quality control in the standardization of Ayurvedic formulations. Rec Tren Med Pharm 1:106–120
27. Maurya R, Thirupataiah B, Misro L, Thulasi R (2022) Effect of the solvent polarity and temperature in the isolation of pure andrographolide from *Andrographis paniculata*. Sci Temper 13:243–256
28. Maurya R, Thirupataiah B, Misro L, Thulasi R (2022) Validation of UV method for the standardization of Jwarhara Kwatha Churna. Vignan Prakash Res J Sci Tech. 20:37–48
29. Boini T, Maurya R, Misro L, Radhakrishnan T (2023) Extraction and identification of bioactive compound embelin from embelia ribes fruits. J Med Pharma Allied Sci 12:5571–5581. https://doi.org/10.55522/jmpas.V12I1.4226
30. Akowuah GA, Zhari I, Norhayati I, Mariam A (2006) HPLC and HPTLC densitometric determination of andrographolides and antioxidant potential of Andrographis paniculata. J Food Comp Anal 19:118–126. https://doi.org/10.1016/j.jfca.2005.04.007
31. Parab GV, Mannur VK, Hullatti K (2022) Quality assessment and analytical quality by design-based RP-HPLC method development for quantification of Piperine in *Piper nigrum* L. Futur J Pharmaceutic Sci 8:16. https://doi.org/10.1186/s43094-022-00405-9
32. Shanbhag SV, Bachute MT (2022) Development and validation of RP HPLC method for simultaneous determination of picroside I, picroside II, phyllanthin and boeravinone B in a polyherbal hepatoprotective tablet formulation. Trop J Pharmaceutic Res 21:619–627. https://doi.org/10.4314/tjpr.v21i3.23
33. Shi X, Wang X, Wang D, Geng Y, Liu J (2009) Separation and purification of α-cyperone from *Cyperus rotundus* with supercritical fluid extraction and high-speed counter-current chromatography. Sep Sci Technol 44:712–721. https://doi.org/10.1080/01496390802437081
34. Chando AD, Basudkar V, Gharat S, Momin M, Khan T (2023) Quantitative estimation of 6-gingerol, E-guggulsterone and Z-guggulsterone in a fixed dose combination nanoemulgel by RP-HPLC. Indian Drug 60:121–127. https://doi.org/10.53879/id.60.07.13722
35. Kajsongkram T, Rotamporn S, Limbunruang S, Thubthimthed S (2015) Development and validation of a HPLC method for 6-gingerol and 6-shogaol in joint pain relief gel containing ginger (*Zingiber officinale*). Int J Med Heal Sci 9:813–817
36. Rahul M, Boini T, Lakshminarayana M, Radhakrishnan T, Sudayadas RK (2022) Approaches to improve solubility, stability and the clinical potential of andrographolide: a review. J Young Pharma 14:15–24. https://doi.org/10.5530/jyp.2022.14.3
37. Gandhi Y, Rawat H, Dhanjal DS, Kumar V, Soni H, Charde V (2023) A comparative analysis of phytochemicals, metal ions, volatile metabolites in heart wood, stem bark and leaves of *Salix alba* L. along with invitro antioxidant, antacid, antimicrobial activities for sake of environment conservation by substitution of stem bark with leaf. Chem Biodiver 21:e202310234
38. Kuldeepsingh AK, Narendra AG, Ram PM, Parmeshwar LS, Dipal M, Sinora M, Geetha KA (2021) Assessing suitability of Andrographis paniculata genotypes for rain-fed conditions in semi-arid climates. Info Process Agri 8:359–368. https://doi.org/10.1016/j.inpa.2020.09.003
39. Pholphana N, Rangkadilok N, Saehun J, Ritruechai S, Satayavivad J (2013) Changes in the contents of four active diterpenoids at different growth stages in *Andrographis paniculata* (Burm.f.) Nees (*Chuanxinlian*). Chin Med 15:22–29. https://doi.org/10.1016/j.inpa.2020.09.003

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