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RESEARCH ARTICLE

A Novel Approach for the Simultaneous Determination of Hecogenin and Stigmasterol in *Chlorophytum borivilianum* Extract Using High-Performance Liquid Chromatography

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Abstract

Background: A reliable technique to determine stigmasterol and hecogenin simultaneously in *Chlorophytum borivilianum* root extract is essential for their analysis in pharmaceutical formulations. **Aim:** To establish and validate a High-Performance Liquid Chromatography (HPLC) technique to determine stigmasterol and hecogenin simultaneously in *C. borivilianum* root extracts. **Methods:** To accomplish chromatographic separation, a Shimadzu Prominence-I LC-2030C plus HPLC system with a Shimpak GIST C8 column (150mm × 4.6mm, 5µm) was used. The mobile phase was made up of water and acetonitrile (10:90 %v/v). The wavelength for detection was set at 210nm for Hecogenin and 240nm for Stigmasterol. Method validation followed Q2(R1) guidelines for ICH, assessing precision, accuracy, linearity, specificity and robustness. **Results:** Linearity was confirmed within the 10–60 µg/mL concentration range for both the analytes, yielding coefficients of correlation of 0.9981 for Hecogenin and 0.9997 for Stigmasterol. Hecogenin and stigmasterol had limits of detection (LOD) of 0.1373 µg/mL and 0.3521 µg/mL, respectively, while the limits of quantification (LOQ) were 0.4161 µg/mL and 1.0670 µg/mL. Recovery rates ranged from 98.66% to 101.73%, and both the intra-day and inter-day precision % RSD values were less than 2%. The assay of the *C. borivilianum* root extracts identified Hecogenin at 8.83% w/w and Stigmasterol at 2.94% w/w. **Conclusion:** The HPLC method is developed, validated and suitable for routine analysis of Hecogenin and Stigmasterol in plant-based formulations. **Major Findings:** The study developed and validated a novel HPLC method for simultaneous quantification of Hecogenin and Stigmasterol in *Chlorophytum borivilianum* extracts. The method demonstrated high sensitivity (LOD: 0.1373–0.3521 µg/mL), accuracy (98.66%–101.73% recovery), and precision (%RSD < 2%), confirming its reliability for routine phytochemical analysis.

Keywords: *Chlorophytum borivilianum*, Hecogenin, HPLC, ICH Q2 Guidelines, Method Validation, Stigmasterol

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1. Introduction

Several plant saponins have been historically used to treat a variety of illnesses in traditional medicine, including infertility[1-16]. The primary compounds found in *C. borivilianum* are saponins, specifically stigmasterol and hecogenin[17-21]. These compounds are accountable for the antioxidant, immune-boosting and aphrodisiac properties of *C. borivilianum*[22-23]. Stigmasterol exhibits structural similarities to testosterone and has an important impact on the aphrodisiac effects of *C. borivilianum*. Hecogenin contributes to the synthesis of anabolic hormones in a concurrent manner. Reports indicate enhanced synthesis of the medicinally significant compounds hecogenin and stigmasterol using *in vitro* root cultures of *C. borivilianum*[24-25]. Researchers conducted HPTLC and HPLC studies to qualitatively analyse saponins and establish a standard for the saponin of *C. borivilianum*[26-29].

There is no simultaneous method reported on these important phytoconstituents of the *C. borivilianum* roots. An attempt has been made to develop a new HPLC technique for simultaneous estimation of hecogenin and stigmasterol for quality control of extract and formulation containing *C. borivilianum* roots.

2. Materials and Methods

2.1 Reagents and Chemicals

Hecogenin and Stigmasterol were obtained from Yucca Enterprises, India with high-purity standards. HPLC-grade solvents, including Acetonitrile (ACN) water and methanol, were sourced from Rankem Laboratory Chemicals India. Every chemical used was of analytical or HPLC quality to ensure the highest analytical precision.

2.2 Chromatographic Conditions and Instrumentation

A Shimadzu Prominence-I LC-2030C plus HPLC system with a quaternary pump, degasser, autosampler and Photodiode Array (PDA) detector was used to perform the chromatographic study. A Shimpak GIST C8 column (150mm × 4.6mm, 5µm) was used for the separation. The mobile phase had a flow rate of 1.0 mL/min and was composed of water: ACN (10:90%v/v). Hecogenin was detected at 210nm, while stigmasterol was detected at 240nm. The injection volume was 20µL, and the column was kept at ambient temperature.

2.3 Standard Solutions and Calibration

Stock solutions containing 250 µg/mL of hecogenin and stigmasterol were prepared in methanol. Working solutions were obtained by appropriate dilution of stock solutions with the mobile phase. A calibration curve was constructed for both analytes by plotting peak areas against concentrations, ranging from 10 to 60 µg/mL for Hecogenin and Stigmasterol.

2.4 Method Validation

Specificity, linearity, accuracy, precision, Limit of Detection (LOD), Limit of Quantitation (LOQ), and robustness were all assessed by ICH Q2(R1) recommendations. Specificity was determined by analysing blank, standard and test solutions to ensure no interference at the retention times of Hecogenin and Stigmasterol. Linearity was assessed by constructing calibration curves and precision was evaluated by determining the intra-day and inter-day % RSD. The accuracy was estimated through recovery experiments at three concentration levels that are 50%, 100%, and 150%.

2.5 Assay

A total of 50mg of the ethanolic extract of the plant containing Hecogenin and Stigmasterol was precisely measured and placed into a volumetric flask of 50mL. To ensure complete dissolution, 40 mL of methanol was added, and the solution was sonicated for 10 to 15 minutes. After cooling, the solution was passed through a 0.45µm membrane filter to eliminate particulate matter and then diluted to 50 mL with methanol. 1.2mL of the stock solution was transferred into a 10mL volumetric flask and diluted to a final volume of 10mL with methanol. The final solution was checked for clarity and injected for analysis into the HPLC system.

3. Results

3.1 Chromatographic Separation

The optimised HPLC method successfully separated Hecogenin and Stigmasterol with clear and well-defined

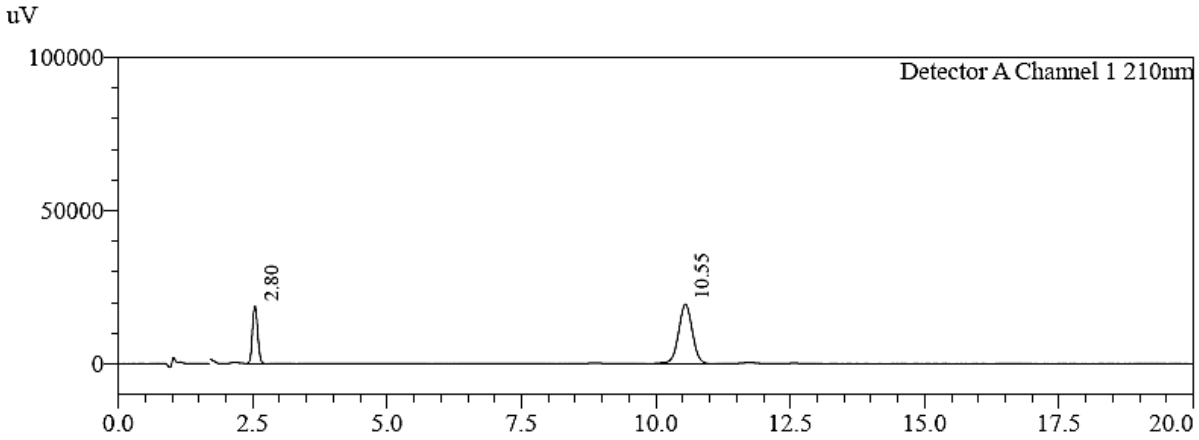


Figure 1. Chromatogram of standard Hecogenin and Stigmasterol.

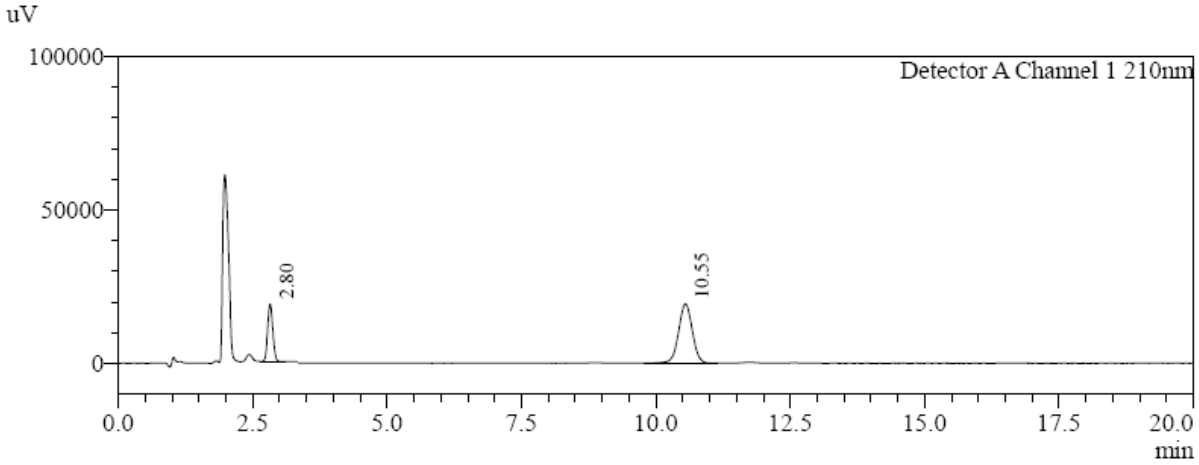


Figure 2. Chromatogram of sample Hecogenin and Stigmasterol.

peaks. Hecogenin and stigmasterol exhibited retention times of 2.80 and 10.52 minutes, respectively. No significant interference from the extract was observed, confirming the specificity of the method (Figures 1 and 2).

3.2 Linearity

With the coefficients of correlation (R^2) for Hecogenin and Stigmasterol exceeding 0.9981 and 0.9997, respectively, the technique demonstrated excellent linearity for both compounds within the studied concentration ranges. The results of linearity have been shown in Table 1. A Chromatogram showing the linearity of Hecogenin and Stigmasterol has been shown in Figure 3. Calibration curves for Hecogenin and Stigmasterol have been shown in Figures 4 and 5 respectively.

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

The LOD and LOQ were determined to be 0.1373 µg/mL and 0.4161 µg/mL for Hecogenin and 0.3521 µg/mL and 1.0670 µg/mL for Stigmasterol, respectively. These values demonstrate the method's high sensitivity for both analytes.

3.4 Accuracy

Accuracy was performed by spiking at 15, 30 and 45 µg/mL (50, 100 and 150%) of both Hecogenin and Stigmasterol solutions. The recovery of Hecogenin and Stigmasterol was found to be between 98.66% and 101.73% at all three spiking levels, confirming the

Table 1. Results of linearity for Hecogenin and Stigmasterol

Analyte	Conc.(ppm)	Mean Peak Area	SD	%RSD	Coefficients of correlation (R^2)
Hecogenin	10	32487.00	15.0000	0.0462	0.9981
	20	66232.00	100.0000	0.1510	
	30	98517.00	85.0000	0.0863	
	40	124378.00	228.9607	0.1841	
	50	163856.00	100.0000	0.0610	
	60	194894.00	300.0000	0.1539	
Stigmasterol	10	84394.00	998.0000	1.1849	0.9997
	20	174055.00	1001.0000	0.5745	
	30	261586.00	1207.0000	0.4587	
	40	341823.00	1805.0000	0.5266	
	50	426679.00	1999.0000	0.4685	
	60	510422.00	2835.0000	0.5554	

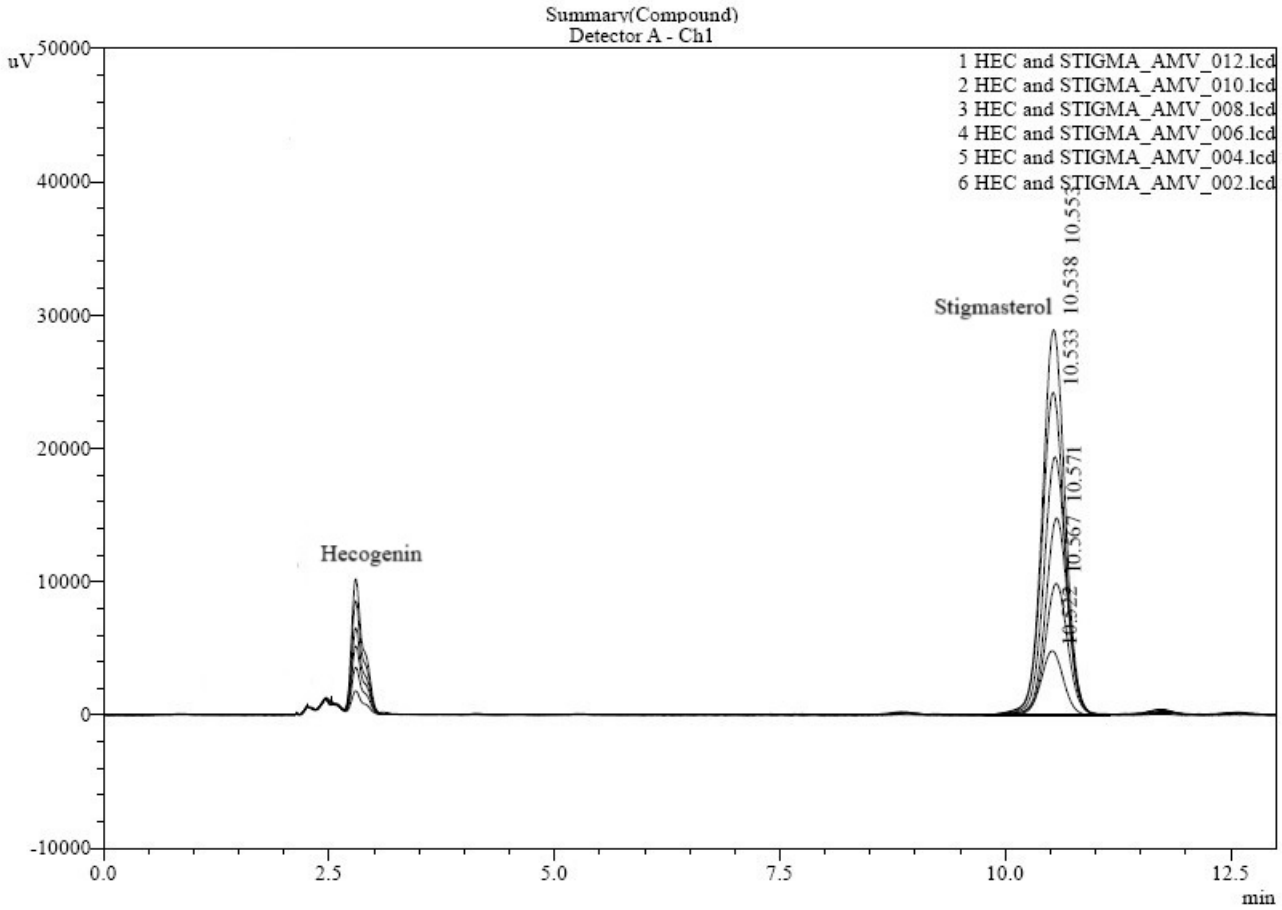


Figure 3. Chromatogram showing linearity of standard Hecogenin and Stigmasterol.

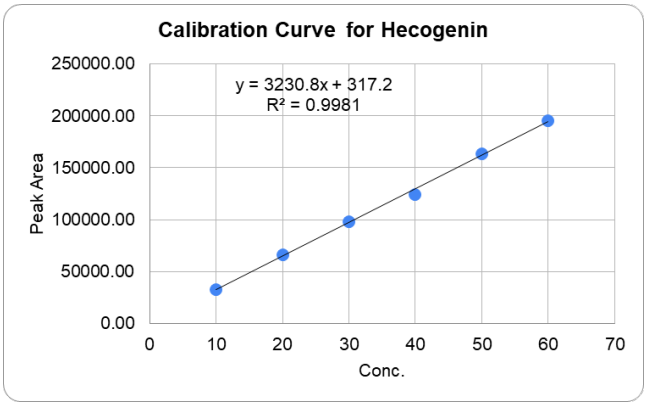


Figure 4. Calibration curve for linearity of Hecogenin.

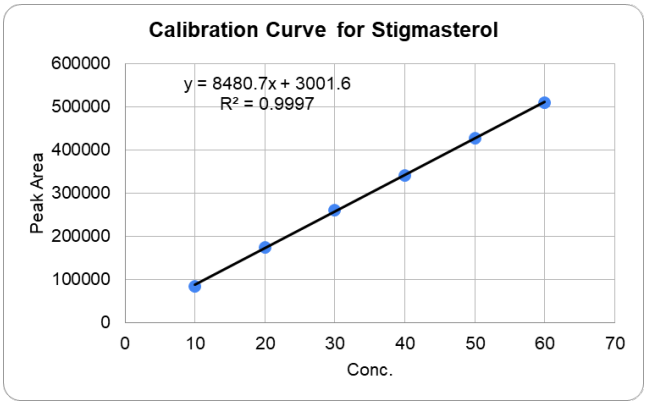


Figure 5. Calibration curve for linearity of Stigmasterol.

Table 2. Outcomes of accuracy (% recovery) for Hecogenin and Stigmasterol

Drug	Level (%)	Amount of sample (ppm)	Amount of standard spike (ppm)	Total Amount (ppm)	% Recovery (avg.)
Hecogenin	50	10	15	25	101.73
	100		30	40	99.24
	150		45	55	99.13
Stigmasterol	50	10	15	25	100.87
	100		30	40	98.66
	150		45	55	100.66

Table 3. Outcomes of intra-day precision study for Hecogenin and Stigmasterol

Analyte	Conc. (ppm)	Mean Area	SD	%RSD (Intraday)
Hecogenin	15	57772.8333	147.4058	0.2551
	30	98433.6667	194.4836	0.1976
	45	155684.0000	169.7194	0.1090
Stigmasterol	15	131583.0000	255.3011	0.1940
	30	262338.8333	304.7203	0.1162
	45	401128.6667	449.9502	0.1122

Table 4. Outcomes of inter-day precision study for Hecogenin and Stigmasterol

Analyte	Conc. (ppm)	Mean Area	SD	% RSD (Intraday)
Hecogenin	**15**	57702.0000	263.8585	0.4573
	30	98416.8333	216.9965	0.2205
	45	155284.0000	270.6043	0.1743
Stigmasterol	**15**	131807.3333	194.2976	0.1474
	30	262741.8333	420.8188	0.1602
	45	400896.1667	603.8563	0.1506

method's accuracy. The results of the precision study have been depicted in Table 2.

3.5 Precision

Accuracy was performed at 15, 30 and 45 µg/mL (50, 100 and 150 %) of both Hecogenin and Stigmasterol solutions. The precision for intra-day and for inter-day studies, yielded % RSD values of less than 2%, indicating the method's reliability and reproducibility. The results of the precision study have been depicted in Tables 3 and 4.

3.6 Robustness

The robustness of the technique was also evaluated by slight variations in chromatographic conditions, such as mobile phase composition, flow rate and detection

Table 5. Results of robustness study for Hecogenin and Stigmasterol

Parameter	Variation	Hecogenin			Stigmasterol		
		Mean Area	SD	%RSD	Mean Area	SD	%RSD
Flow rate (mL/min)	1 ±0.1	98503.8889	388.3669	0.3943	262579.5000	496.5144	0.1891
Detection Wavelength(nm)	210±2, 240±2	97910.0000	597.3390	0.6101	262308.8889	334.6864	0.1276
Mobile phase composition	Water: ACN (10:90, %v/v) ± 1% of ACN	98520.5556	185.4847	0.1883	262253.1667	436.8500	0.1666

Table 6. The result of assay in the plant extract

Analyte	Amount in Extract (mg/g)	% w/w
Hecogenin	88.3	8.83%
Stigmasterol	29.4	2.94%

wavelength, and the method showed consistent performance. The results of the precision study have been depicted in Table 5.

3.7 Estimation of Markers from Plant Extract

The assay of the plant extract containing Hecogenin and Stigmasterol was performed using the established HPLC technique. The concentration of Hecogenin and Stigmasterol in the extract was estimated by comparison of the peak areas of the sample with those of the standard solutions. The analysis indicated that the extract contained 8.83% w/w of Hecogenin and 2.94% w/w of Stigmasterol (Table 6).

4. Discussion

The developed HPLC method demonstrated excellent linearity for both analytes, Hecogenin and Stigmasterol, across the concentration range of 10–60 µg/mL, with coefficients of correlation (R^2) of 0.9981 and 0.9997, respectively, confirming the method's reliability for quantitative analysis. The method exhibited high sensitivity with LOD values of 0.1373 µg/mL for Hecogenin and 0.3521 µg/mL for Stigmasterol, and LOQ values of 0.4161 µg/mL and 1.0670 µg/mL, respectively. Recovery rates between 98.66% and 101.73% highlight the method's accuracy, while % RSD values below 2% for both intra-day and inter-day precision underscore its reproducibility. The quantification of Hecogenin (8.83% w/w) and Stigmasterol (2.94% w/w) in the *C. borivilianum* root extracts further validates the method's applicability for phytochemical analysis, offering a robust and reliable tool for the estimation of these bioactive compounds in complex matrices.

5. Conclusion

A novel, simple and reliable HPLC technique was established and validated for the simultaneous estimation of Hecogenin and Stigmasterol. The technique adheres to ICH Q2(R1) guidelines, demonstrating specificity, precision, accuracy and robustness. This validated technique can be effectively applied for routine quality control analysis of Hecogenin and Stigmasterol in pharmaceutical formulations.

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7. Author Contribution

Lata Mansukhbhai Manani: Conducted experimental work, data analysis, and initial manuscript drafting. Manan Raval: Supervised the research, provided conceptual guidance, and critically reviewed the manuscript. Samir Patel: Contributed to methodology development and validation processes. Parixit Prajapati: Assisted in data interpretation and final manuscript

preparation. Arun Soni: Contributed to methodology development.

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