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RESEARCH ARTICLE

Development and Validation of Novel High-Performance Liquid Chromatography Method for Simultaneous Estimation of *p*-Cymene and Aloe-emodin

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Abstract

The objective of the present investigation was to develop a novel, accurate, precise, and linear High-Performance Liquid Chromatographic (HPLC) method for the simultaneous estimation of *p*-Cymene and aloe-emodin in the novel topical herbal formulation and validated as per ICH guidelines. In the current study, good chromatographic separation was achieved in isocratic mode using an HPLC C18 column (250mm × 4.6), 5 µm, and a mobile phase consisting of acetonitrile:water in the ratio of 80:20, at a flow rate of 1.0 mL/min and column temperature maintained at 25°C. The response obtained was monitored at 225 nm wavelength with a UV-Visible detector. The retention times of Aloe-emodin and *p*-Cymene were found to be 4.3 min and 9.0 min respectively. Linearity was established for both *p*-Cymene and aloe-emodin in the range of 10-90 µg/mL, respectively. For the method, % Recovery was found in the range of 99.67-100.51 % for *p*-Cymene and 98.68-100.4 % for aloe-emodin respectively. The LOD and LOQ were found to be 0.01 and 0.04 for *p*-Cymene and 0.12 and 0.36 for aloe-emodin respectively. This method can be successfully employed for simultaneous quantitative analysis of *p*-Cymene and Aloe-emodin in the novel topical herbal formulation.

Keywords: Aloe-emodin, HPLC, *p*-Cymene, Simultaneous Method, Validation

1. Introduction

Ayurvedic medicines are polyherbal formulations that contain a wide variety of chemical constituents in each herb. Plants, which are regarded as a traditional source, are the source of a significant number of phytochemicals. Ayurvedic medicines are polyherbal formulations with a variety of chemical constituents in each herb. The effectiveness of herbal remedies must be evaluated to support their inclusion in the current medical system. Manufacturing and primary processing of herbal substances have an impact on the quality of the active medicinal constituent[1]. It is challenging to quantify markers in any polyherbal composition and to standardise polyherbal formulations. Herbal products can be standardised using advanced techniques like UV, visible, infrared, thin-layer chromatography, High-Performance Liquid Chromatography (HPLC), high-performance thin-layer chromatography, gas chromatography with mass spectrometry, liquid chromatography with a mass spectrometer, atomic absorption spectrometry, spectrofluorimetric, and other techniques. Various methods for estimating *p*-Cymene and aloe-emodin alone or in conjunction with other markers have been developed, but no HPLC analysis method for aloe-emodin and *p*-Cymene has been measured simultaneously.

More than 100 different plant species contain the monoterpene *p*-Cymene, which is used in both medicine and food. Its chemical name is 1-methyl-4-(1-methyl ethyl)-benzene. It has antibacterial, anticancer, calming, painkilling, and anti-inflammatory properties.

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Monoterpenes are part of a wider family of chemical compounds known as "terpenes," which are the most common constituents of essential oils. Figure 1 depicts the chemical structure of *p*-Cymene as a benzene ring with substitutions for methyl and isopropyl[2]. The gel, sap, or leaves of aloe vera contain the anthraquinone and isomer of emodin known as aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)anthraquinone). Aloe-emodin does not cause cancer when applied to the skin, however, it may make certain rays more carcinogenic[3]. The chemical structures of *p*-Cymene and aloe-emodin are shown in Figures 1 and 2 respectively.

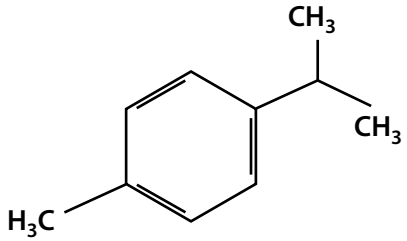

Figure 1. Chemical structure of *p*-Cymene.

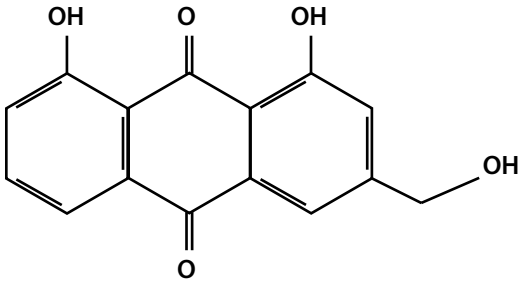

Figure 2. Chemical structure of Aloe emodin.

2. Materials and Methods

UV detector is part of the RP-HPLC Shimadzu LC-20A type instrument. Lab Solution was the program employed in HPLC. The maximum wavelength (max) of the relevant substances was determined using a UV-visible spectrophotometer.

2.1 Chromatographic Conditions

In this procedure, HPLC was used together with Lab Solution software. Column: C18 (250mm × 4.6), 5 µm was employed in the analysis. With a flow rate of 1.0 mL/min and an injection volume of 20 µl, acetonitrile, and water were used as the mobile phase. The temperature in the column was fixed at 28°C. Using a UV detector, *p*-Cymene, and aloe-emodin were found at 225 nm.

2.2 Selection of Wavelength

p-Cymene and aloe-emodin standard solutions were produced and examined using a UV spectrophotometer. The resulting overlay spectra of *p*-Cymene and aloe-emodin, which were determined using a detection range of 200 to 400 nm, are presented in Figure 1. For the study of *p*-Cymene and aloe-emodin, 225 nm was used as the detection wavelength because both markers displayed noticeable absorption at this wavelength.

2.3 Preparation of Solutions

2.3.1 Preparation of Stock Solution for p-Cymene

p-Cymene (10 mg) was carefully weighed out and put into a 10mL volumetric flask, which gives (1000) µg/mL and sonicated for 5 min.

2.3.2 Preparation of Stock Solution for Aloe-emodin

p-Cymene (10 mg) was carefully weighed out and put into a 10mL volumetric flask., which gives (1000) µg/mL and was sonicated for 5 min.

2.3.3 Preparation of a Working Standard Solution for p-Cymene

From a standard stock solution of *p*-Cymene (1000 g/mL), (0.1, 0.3, 0.5, 0.7 and 0.9) were taken, transferred to a 10 mL volumetric flask, and the remaining volume was filled with a diluent to obtain the following concentrations: (10, 30, 50, 70 and 90 g/mL). The above-prepared solutions' additional absorbances were measured.

2.3.4 Preparation of Working Standard Solution for Aloe-emodin

Aloe-emodin (0.1, 0.3, 0.5, 0.7 and 0.9) were obtained from the standard stock solution (100 g/mL) and put into a 10 mL volumetric flask, where they were diluted to give the final concentrations of (10, 30, 50, 70 and 90 g/mL). The produced solutions mentioned above underwent further absorbance measurements.

2.4 Method Development

Based on improved peak resolution and peak symmetry after a number of runs, acetonitrile:water (80:20) was chosen as the mobile phase. Analysis was conducted

using a Prontosil C18 column (250 x 4.6 mm, 5µ), with an injection volume of 20µl. The runtime was 10 minutes, with a flow rate of 1.0 mL/min. The detection was done at 225 nm with the column temperature set to 28°C. Aloe-emodin and *p*-Cymene both had Retention Times (RT) of 4.32 and 9.05 minutes, respectively. Standard and sample chromatograms of aloe-emodin and *p*-Cymene.

2.5 Method Validation

For system suitability, linearity, the limit of detection, the limit of quantitation, precision, accuracy, specificity, and robustness, the enhanced chromatographic process was validated using the International Conference on Harmonization (ICH) (2005) Q2R(1) recommendations.

2.6 System Suitability

A system suitability test was run to ensure that the analytical system is operating properly and producing precise and accurate data. The chromatograms were recorded after six injections of a standard solution (10µg/mL) for both *p*-Cymene and aloe-emodin. There should be a 2.0 tailing factor and a 2.0% RSD for the area response from six replicate injections of the standard solution. The resolution of drug peaks should be greater than 2.0 and theoretical plates should be greater than 2000.

2.7 Linearity

Analyzing solutions with concentrations between 10 and 90µg/mL for aloe-emodin and *p*-Cymene from the same solution allowed for the determination of the linearity peak area response. The developed technique was used to measure the peak area of each solution. The peak area vs. concentration calibration curve was plotted. Regression line equations and correlation coefficients were calculated for *p*-Cymene and aloe-emodin.

2.8 Precision

2.8.1 Repeatability

Based on six measurements of the same concentration of aloe-emodin and *p*-Cymene (10µg/mL) and chromatograms were recorded and RSD was calculated.

Intraday Precision

Aloe-emodin (30, 50 and 70), *p*-Cymene (30, 50 and 70), and standard solutions containing 3 replicates of 3 concentrations of a standard solution were evaluated in a single day. %RSD was computed.

Interday Precision

On three different days, standard solutions containing *p*-Cymene and aloe-emodin (30, 50, and 70 g/ml) were examined. Each sample's chromatogram was noted. There were calculated SD and RSD.

2.9 Accuracy

Aloe-emodin and *p*-Cymene were spiked in triplicates at concentrations of 50%, 100% and 150% of the working level to conduct % Recovery tests.

Each spiked solution's chromatogram was obtained, and the amount of drug present overall and the percentage of recovery were determined.

2.10 Robustness

Three replicates of three concentrations (30µg/mL, 50µg/mL, and 70µg/mL) were analyzed at the three different flow rates (± 0.1 mL/min) and three different wavelengths (±1nm). %RSD was calculated with the measured peak area.

2.11 Quantification Limits

The following equation was used to determine the Limit of Detection (LOD): The Limit of Quantitation (LOQ) was calculated using the equation: DL = 3.3/S QL = 10/S, where S is the calibration curve's slope and DL is the responses' standard deviation.

3. Result and Discussion

3.1 Method Development and Optimization

The largest absorption peak (max), which is displayed in Figure 3, was found at 225 nm during the acquisition of the UV absorption spectrum to start the research. A C18 column was used to begin the technique development tests. Considering the properties of the pharmaceuticals being studied, acetonitrile: water (80:20) was chosen as the mobile phase due to its improved peak resolution and peak symmetry. A 20µl

injection volume was maintained. The runtime was 10 minutes, with a flow rate of 1.0 mL/min. The detection was done at 225 nm with the column temperature set at 28°C. *p*-Cymene and aloe-emodin both had Retention Times (RT) of 9.05 ± 0.20 min and 4.35 ±0.20 min, respectively.

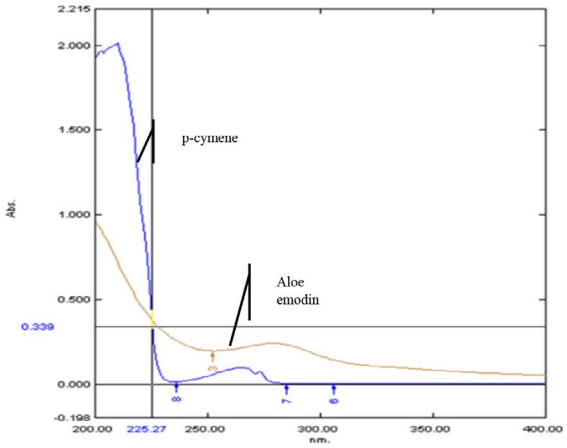

Figure 3. UV overlay spectra of *p*-Cymene and aloe-emodin.

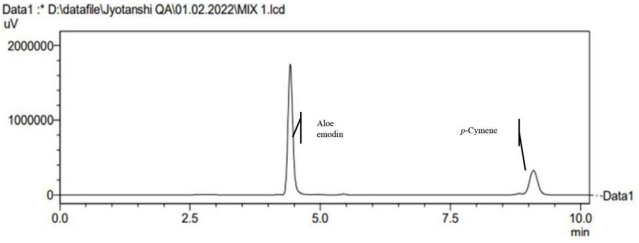

Figure 4. Chromatogram of a *p*-Cymene and aloe-emodin mixture produced under ideal circumstances.

UV- overlay spectra of *p*-Cymene and aloe-emodin are presented in Figure 3 for wavelength selection for HPLC.

Figure 4 displays the HPLC chromatograms of *p*-Cymene and aloe-emodin. Table 1 lists the optimal chromatographic settings.

Table 1. Conditions for *p*-Cymene and aloe-emodin chromatography that are optimal

Parameters	**Optimal Circumstances**
Column	Prontosil C18, (250×4.6 mm, 5 µ)
Mobile phase	Acetonitrile:water(80:20)
Detector	UV detector
Wavelength for Detection	225 nm
Column temperature	28 degrees Celsius
Injection volume	20 µl
Flowrate	1.0 mL/min
Runtime	10 min
Retention time	9.05min and 4.35 min

3.2 Validation of Method

3.2.1 System Suitability

Data for System suitability parameters of *p*-Cymene and aloe-emodin in the mixture are shown in Table 2. System suitability parameters were in acceptance criteria where theoretical plates were more than 2000, a resolution was more than 2, tailing factor was less than 2.

Table 2. Data for system suitability parameters of *p*-Cymene and aloe-emodin in the mixture

Sr. No.	**Retention Period (min)**		**Tailing Factor**		**Theoretical Plates**		**Resolution**
	Aloe-emodin	*p*-Cymene	Aloe- emodin	*p*-Cymene	Aloe-emodin	*p*-Cymene	
1.	4.320	9.055	1.237	1.093	9552.155	16018.460	19.928
2.	4.320	9.059	1.226	1.089	9552.597	16101.320	19.965
3.	4.321	9.056	1.224	1.092	9563.904	16071.110	19.989
4.	4.323	9.042	1.233	1.068	9542.017	16099.119	19.955
5.	4.326	9.056	1.235	1.094	9529.507	16126.718	19.945
6.	4.328	9.054	1.234	1.099	9568.539	16046.121	19.905
Mean	4.323	9.053666667					
S.D	0.00334664	0.00595539					
% RSD	0.077414761	0.065778761					
Limit	< 2		< 2		>2000		>2

3.2.2 Linearity

Both *p*-Cymene and aloe-emodin showed a linear relationship between peak area and drug concentration in the range of 10-90µg/mL, respectively. Plotting the graph of concentration v/s peak area allowed for the creation of the calibration curves for *p*-Cymene and aloe-emodin. The linearity of *p*-Cymene and aloe-emodin were found to be 0.995 and 0.996 respectively, as shown in Table 3.

Table 3. Linearity of *p*-Cymene and aloe-emodin

Linearity parameters	p-Cymene	Aloe-emodin
Range(µg/mL)	10-90	10-90
Slop	118490.59	58328.93
Intercept	1520987.15	2012770.30
Coefficient of determination (r^2)	1.0	0.999

3.2.3 Precision

The % RSD for repeatability was found to be 0.792 for *p*-Cymene and 0.400 for aloe-emodin. Measured in terms of % RSD, intra-day and inter-day precision were <2% within the selected range of (30µg/mL, 50µg/mL, and 70µg/mL) for both *p*-Cymene and aloe-emodin. The results are presented in Table 4.

Table 4. Study of precision

Drug	Drug Concentration (µg/mL)						
	Repeatability	Intra-day			Inter-day		
p-Cymene	10	30	50	70	30	50	70
% RSD	0.109	0.123	0.029	0.656	0.191	0.029	1.411
Aloe-emodin	10	2	4	6	2	4	6
%RSD	0.031	0.038	0.120	0.58	0.207	1.761	0.120

3.2.4 Accuracy

In the accuracy study % recovery was found to be in the range (of 99.6-100.5 %) for *p*-Cymene and aloe-emodin (98.6-100.4 %). A known amount of standard spike (80%, 100%, and 120%) to a pre-quantified sample solution. The results are presented in Table 5.

Table 5. Study of accuracy

Drug	Level	The volume of the sample (µg/mL)	Standard Spiked Quantity (µg/mL)	Total amount (µg/mL)	Amount Recovery (µg/mL)	% Recovery
Aloe-emodin	80%	30	15	45	45.11	100%
	100%	30	30	60	59.21	98.68%
	120%	30	45	75	75.32	100.4%
p-Cymene	80%	30	15	45	45.23	100.51%
	100%	30	30	60	61.99	100.50%
	120%	30	45	75	74.75	99.67%

3.2.5 Robustness

The change was done in wavelength of detection (±1nm) and flow rate (±0.1mL/min). The % RSD of robustness for both changes was found to be <2%. The results for robustness are shown in Tables 6 and 7.

Table 6. Study of robustness - change in wavelength

Drug	Conc. (µg/mL)	Peak area 225nm	Peak area 224nm	Peak area 227nm	Mean	SD	%RSD
Aloe-emodin	30	4773579	4776015	4758187	4769260.333	9666.827	0.202
	50	7081420	7016108	6903591	7000373	8995.2659	1.284
	70	9864375	9885979	9866604	9872319.333	1188.2002	0.120
p-Cymene	30	3771672	3779923	3781672	37777.55.667	534.0.692	0.141
	50	4917595	4943851	5017142	49595.29.333	5159.2.236	1.040
	70	6022279	5922279	6023491	59893.49.667	5808.8.062	0.969

n = 3 concentration/3 replicates

Table 7. Study of robustness - change in flow rate

Drug	Conc. (µg/mL)	0.5 ml/min	1.0 ml/min	1.1 ml/min	Mean	SD	%RSD
Aloe-emodin	30	4672897	4773379	4775853	47407.09.667	5874.0.518	1.239
	50	6986143	7081420	6837012	69681.91.667	12318.8.901	1.767
	70	9885979	9866604	9881604	98780.62.333	1016.1.457	0.102
p-Cymene	30	3653954	3779923	3712765	37155.47.333	6303.0.574	1.696
	50	4976234	4917595	4871309	49217.12.667	5258.3.555	1.068
	70	6024587	6022279	6187938	6078.268	9498.4.016	1.562

n = 3 concentration/3 replicates

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

Results from five calibration curves and the average standard deviation of the intercept were calculated, and LOD and LOQ were calculated from the equation. The LOD and LOQ for *p*-Cymene were found to be 0.1 µg/mL and 0.04 µg/mL respectively, and the LOD and LOQ for aloe-emodin were found to be 0.12 µg/mL and 0.36 µg/mL respectively. The results are shown in Table 8.

Table 8. Study of LOD and LOQ

Parameter	Aloe-emodin	p-Cymene
S.D. of the 5 calibration curves' Y-intercepts	4583.587	5106.04
The five calibration curves' average slope	1220744	1430659
LOD = (SD/Slope) × 3.3 (µg/mL)	0.12	0.01
LOQ = (SD/Slope)× 10 (µg/mL)	0.36	0.04

4. Conclusion

To estimate *p*-Cymene and aloe-emodin simultaneously, a novel HPLC method was created and validated. According to the ICH Q2 (R1) requirements, this method was verified in terms of linearity, precision, LOD, LOQ, accuracy, and robustness. For the determination of *p*-Cymene and aloe-emodin, the established approach is simple, linear, robust, precise, and accurate. The peaks of both markers were sharp and well-resolved. As a result, the proposed method can be used to regularly conduct qualitative and quantitative analyses of *p*-Cymene and aloe-emodin in a novel topical herbal formulation that contains these phytoconstituents.

5. Acknowledgement

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6. References

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