RESEARCH PAPER

QUANTIFICATION OF IMPERATORIN ISOLATED FROM AEGLE MARMELOS AND METHOD VALIDATION BY USING HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

Objectives: The study aimed to develop a simple and sensitive High-Performance Thin-Layer Chromatographic method for the quantitative estimation of Imperatorin.

Methods: Chromatography was performed on silica Gel 60 F254 TLC plates using toluene and ethyl acetate as a mobile phase. Imperatorin was detected using a CAMAG TLC scanner 4 with a UV-visible detector over the wavelength range of 200 to 400 nm, exhibiting an Rf value of 0.5 at the wavelength 251 nm and selected for further studies.

Results: The method was validated for linearity, precision, LOD, LOQ, accuracy, and specificity. The HPTLC method exhibited strong retention for Imperatorin with an Rf value of 0.5. The calibration curve demonstrated excellent linearity over a concentration range of 200 – 1200 ng/spot. The slope and Intercept were determined as 0.0019x + 0.6084 (R^2 = 0.9949). The results indicate that the proposed method was accurate, precise and consistent in the determination of Imperatorin. Further AMFE showed comparative anti-oxidants properties.

Conclusion: The method was validated according to ICH guideline Q2 (R1). In that context, the results suggest that this method can be used for routine estimation of Imperatorin.

Keywords: Imperatorin, HPTLC, Method validation, Aegle marmelos.

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INTRODUCTION

Imperatorin is a naturally occurring furanocoumarin in *Agle marmelos (L.) Correa,* family *Rutaceae*. *A. marmelos* is an ethnomedicinally important plant used traditionally for the management of hypertension, diabetes, stroke, diarrhoea, and several other ailments. In addition, various pharmacological properties have been reported for this plant, including antihypertensive, antioxidant, diuretic, blood-brain barrier permeability, neuroprotective, cardioprotective, hepatoprotective, cytoprotective, analgesic, and wound-healing activities [1]. These diverse biological effects make *A. marmelos* a valuable medicinal resource and highlight the need for reliable analytical methods to ensure consistency and quality of its herbal preparations.

The increasing use of herbal medicines has created a growing demand for standardized analytical techniques that can identify and quantify bioactive marker compounds in plant materials and formulations. Quality control of medicinal plants is essential not only for ensuring batch-to-batch consistency but also for confirming the presence and concentration of pharmacologically active constituents. In this context, chromatographic methods play a pivotal role in the authentication, standardization, and evaluation of herbal drugs and natural products [2, 3]. HPTLC is a commonly used method in the quantitative analysis of active compounds in extracts and natural products. HPTLC uses a liquid mobile phase that is adsorbed onto the stationary phase as a thin layer on a chromatography plate. It has a shorter analysis time as the separation of the compound takes place on the thin layer of the chromatography plate [4-6]. Because imperatorin may serve as an important marker compound for *A. marmelos*, the development of a sensitive and validated HPTLC method is necessary for its accurate estimation.

Although imperatorin has been identified in *A. marmelos*, there remains a need for a robust, precise, and validated analytical method for its determination in plant extracts. A validated HPTLC method would be valuable for routine quality assessment, standardization of herbal raw materials, and future pharmacological or phytochemical studies involving *A. marmelos*.

The objective of the present study was to develop and validate a simple, rapid, accurate, and reproducible HPTLC method for the quantitative determination of imperatorin in *Aegle marmelos* and to apply the method for quality control and standardization of the plant extract.

MATERIALS AND METHODS

Materials

Pure Imperatorin (fig. 1) purchased from Yucca Enterprises, Mumbai, Maharashtra. Agle Marmelos fruit was collected from the local area, and its identity was authenticated by Harshad M. Pandit, PhD (Botany), with specimen number (SRJP 02225999).

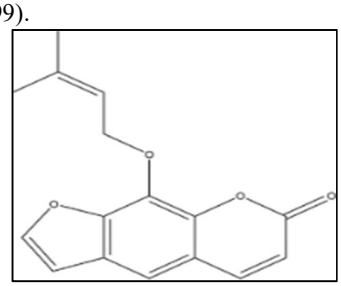

Fig. 1: Structure of Imperatorin

HPTLC analytical conditions and method validation

Preparation of standard solution

A standard stock solution of Imperatorin was prepared by dissolving 10 mg of the standard API in 10 ml of Ethyl acetate to achieve a concentration of 1000 mg/ml. This solution was further diluted to achieve a 100 mg/ml solution of imperatorin as a working standard [7].

Selection of wavelength for detection

The working standard of Imperatorin in Ethyl acetate was scanned by CAMAG TLC SCANNER 4 (S/N: 170422) with UV- visible detector over the wavelength range 200 to 400 nm. Wavelength 251 nm was selected for further studies (Table 1).

Preparation of calibration curve

Different concentrations of working standard solution were applied on the TLC plate, corresponding peak areas were recorded and linear regression was done between the absorbance vs concentration. 200 – 1200 ng/spot range was selected for preparation of the calibration curve, and a linear regression equation was obtained in this range (Table 2).

Chromatographic conditions

Chromatographic conditions are as follows

Table 1: HPTLC chromatographic conditions for Imperatorin

Sr. No.	Parameters	Conditions
1	Stationary phase	Precoated silica gel 60 F 254 HPTLC Aluminum plates (10x10 cm, 0.2 mm thick); Merck
2	Mobile Phase	Toluene: Ethyl Acetate (9:1)
3	Saturation time	15 minutes.
4	Wavelength	251 nm.
5	Lamp	Deuterium
6	Rf value scale	0.1 to 1.00
7	Spectrum speed	20 nm/s.

The HPTLC analysis was performed on CAMAG Twin server LABSERVER, version 3.1.21109.3; Applicator Linomat IV, in an air-conditioned room maintained at 22°C and 55% humidity using precoated silica gel 60 F 254 Aluminium-backed plates (10x10 cm, 0.2 mm layer thickness, 5-6 µm particle size; Merck). On the basis of the trial-and-error method using different solvent systems, the following chromatographic conditions were chosen for analysis. TLC plates were prewashed with methanol and activated at 110°C for 10 min before application. The chromatographic development was carried out using Toluene: Ethyl acetate (9:1 v/v) as mobile phase with chamber saturation time 20 minutes (Fig. 3). The retention factor (Rf) value was calculated as:

$$Rf = \frac{\textbf{Distance travelled by the compund}}{\textbf{Distance travelled by the solvent front}}$$

HPTLC Validation analysis

The objective of validation of an analytical procedure is to demonstrate whether the procedure is suitable for its intended purpose. The proposed method was validated for various parameters such as Linearity and Range, Precision, Limit of detection (LOD) and Limit of Quantification (LOQ), and Accuracy according to ICH Q2 (R1) guidelines [8-11].

Linearity and Range

The linearity was determined by using working standard solutions ranging between 200 and 1200 ng/spot (Table 2). The spectra of these solutions were recorded at a wavelength of 251 *nm*. A Calibration curve of peak area *v/s* concentration was plotted after the suitable calculation, and simple linear regression was performed. Regression equation and correlation coefficient were obtained. The range of solutions has been decided according to the statistical parameters of the generated equation (Table 3).

Precision

Repeatability

The precision of the method was checked by repeatedly injecting (n=6) standard solutions of Imperatorin (200 ng/spot). Absorption of this solution was measured at 251 nm. Data were recorded for every concentration, enabling the calculation of mean area, standard deviation (SD), and Relative standard deviation (% RSD) (Table 4).

Reproducibility

The intra-day precision of the proposed method was determined by taking different concentrations (200, 400, 600, 800, 1000, and 1200 ng/spot) of Imperatorin three times during one day (Table 5). Inter-day precision was checked by analyzing Imperatorin levels of 200, 400, 600, 800, 1000, and 1200 ng/spot on three different days (Table 6). The results have been reported in terms of percentage relative standard deviation (% RSD).

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

Six sets of known concentrations of (200 - 1200 ng/spot) were prepared. Calibration curves were plotted for each set. LOD and LOQ were calculated using the regression equation (Table 2) and the following formulae:

$$\textbf{\textit{Limit of Detection}} = \frac{\mathbf{3.3} * \boldsymbol{SD}}{\boldsymbol{S}}$$

$$\textbf{\textit{Limit of Quantification}} = \frac{\mathbf{10} * \boldsymbol{SD}}{\boldsymbol{S}}$$

Where SD is the standard deviation of the y-intercept of the calibration curve, and S is the mean slope of the calibration curve.

Accuracy

Recovery studies of the compound using the conventional addition method were performed to test the approach's accuracy at 80%, 100%, and 120% of target concentration levels (Table 7). The amount of drug recovered was calculated using the following formula.

$$\%\ \textbf{\textit{Recovery}} = \frac{\textbf{\textit{Amount recovered} - \textbf{\textit{Amount added}}}}{\textbf{\textit{Amount present}}} \boldsymbol{x}\ \mathbf{100}$$

Specificity

The specificity of the method was ascertained by analyzing standard drug and the sample. The spot for the drug in the sample was confirmed by comparing the Rf and spectra of the spot with those of the standard drug spot. The specificity of the method was also ascertained by peak purity profiling studies by analyzing the spectrum at peak start, middle and at peak end.

DPPH radical scavenging Assay

The antioxidant activity of AMFE was evaluated by the DPPH radical scavenging assay. Briefly, the test sample was prepared at the desired concentration and mixed with DPPH solution. The reaction mixture was incubated in the dark at room temperature for a fixed period, after which the absorbance was measured at 517 nm against an appropriate control. Radical scavenging activity was expressed as percentage inhibition using the following formula:

$$\%\ \text{inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100.$$

Statistical Analysis

The statistical analysis was performed using Microsoft Excel LTSC Professional Plus 2021. Data is represented as Mean % Relative Standard Deviation (Mean ± SD).

RESULTS AND DISCUSSION

Ultraviolet-visible spectrophotometric method for Imperatorin

The UV spectrum for the pure Imperatorin as a standard and Agle marmelos ethyl acetate extract is shown in figures respectively. The active compounds show an absorption band at 251 nm (fig. 2).

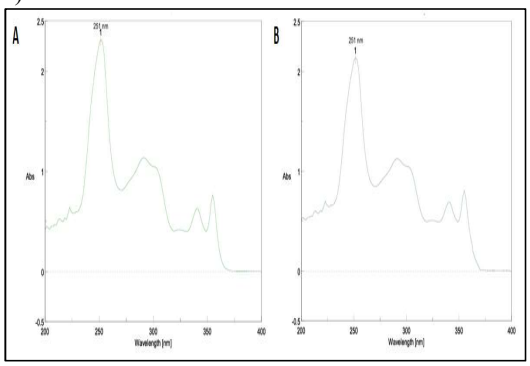

Fig. 2: UV spectrum for (A) pure Imperatorin (100 ppm) and (B) Agle marmelos ethyl acetate extract (100 ppm)

HPTLC analysis and validation

The optimized HPTLC method was validated for Linearity, Precision, Accuracy, Limit of Detection, and Limit of Quantification (fig. 3).

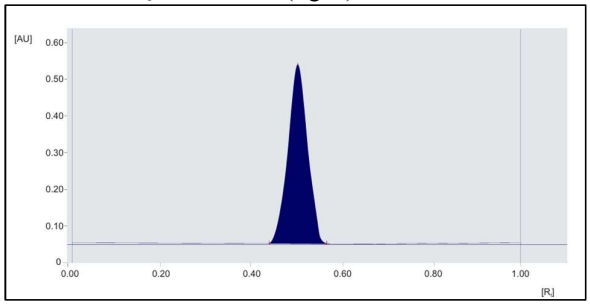

Fig. 3: Densitogram of HPTLC quantitative analysis performed for Imperatorin

Linearity and Range

The results of the linearity test in HPTLC were used to evaluate the concentration linearity with respect to the area under the densitometer response. The HPTLC plate was developed using a selected mobile phase, and the resulting spots were observed under UV light with a wavelength of 251 nm (Table 2 & Table 3).

Table 2: Linearity and Range for Imperatorin

Sr. No.	Drug	Concentration (ng/spot)	Mean peak area	% RSD
1	Imperatorin	200	0.924±0.0007	0.75
2		400	1.405±0.0012	0.85
3		600	1.812±0.0009	0.49
4		800	2.198±0.0006	0.27
5		1000	2.535±0.0011	0.43
6		1200	0.2865±0.0008	0.27

Value expressed as mean ± SD (n=3)

Table 3: System Suitability Parameters

Drug	Linearity Range (ng/spot)	Regression equation	R^2	Slope	Intercept	LOD (ng)	LOQ (ng)
Imperatorin	200-1200	y = 0.0019x + 0.6084	R2 = 0.99949	0.00219	0.60843	1.53	4.644

Precision and Accuracy

The Precision and Accuracy tests were conducted to determine the extent to which the test results approach the value, and to obtain good precision and accuracy values. The results were tabulated in terms of repeatability (Table 4), reproducibility (Tables 5 and 6), and accuracy (Table 7), respectively.

Table 4: Repeatability study for Imperatorin

Sr. No.	Concentration (ng/spot)	Peak area (absorbance)	Mean peak area	SD (n=6)	% RSD
1	200	0.1099	0.1099	0.0006	0.54
2		0.1101			
3		0.1098			
4		0.1103			
5		0.1096			
6		0.1098			

Table 5: Intra-Day precision study for Imperatorin

Sr. No.	Concentration (ng/spot)	Mean peak area (n=3)	SD (n=3)	%RSD
1	200	0.975	0.0009	0.0009
2	400	1.187	0.0011	0.0009
3	600	1.256	0.001	0.0008
4	800	1.296	0.0012	0.0009
5	1000	1.374	0.0009	0.0007
6	1200	1.556	0.001	0.0006

Mean % Relative Standard Deviation = 0.0008, (n=3 per level)

Table 6: Inter-Day precision study for Imperatorin

Sr. No.	Concentration (ng/spot)	Mean peak area (n=3)	SD (n=3)	%RS
1	200	0.984	0.0115	0.011
2	400	1.405	0.0101	0.007
3	600	1.721	0.0092	0.005
4	800	1.998	0.0105	0.005
5	1000	2.335	0.0098	0.004
6	1200	2.565	0.0108	0.004

Mean % Relative Standard Deviation = 0.0063, (n=3 per level)

Table 7: Accuracy study for Imperatorin

Sr. no.	Drug	Level of rec	Amount init	Amount ad	Amount reco	Recovery %	Mean ±

o.		overy (%)	ially present (µg/ml)	ded (µg/ml)	vered (µg/ml)		SD
1	Imperatorin	80	1000	800	178 7.56	99.30	0.94
2		100	1000	1000	195 0.37	97.51	1.12
3		120	1000	1200	219 0.12	99.55	1.09

Mean ±SD (n=3) = average of three determinations.

DPPH Activity

AMFE exhibited marked DPPH radical scavenging activity at 1 µg/mL, with 97% inhibition, compared with 100% inhibition observed for the control. These findings indicate strong antioxidant potential of AMFE (fig. 4).

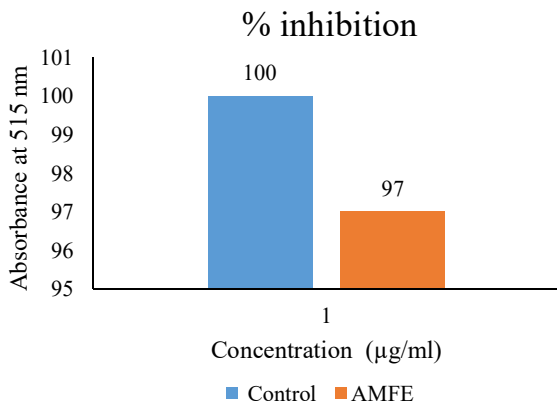

Figure 4: DPPH radical scavenging activity of AMFE. Data represented in Mean±SD (n=3).

DISCUSSION

The determination of LOD and LOQ aims to establish the specified limit for detection and quantification. Based on the linearity test results, standard stock solutions were prepared with the following concentrations: 200 ppm, 400 ppm, 600 ppm, 800 ppm, 1000 ppm, and 1200 ppm. After performing calculations using Microsoft Excel software, the LOD and LOQ were calculated as 1.53 ng/spot and 4.64 ng/spot, respectively.

High-Performance Thin-Layer Chromatography advanced rapidly and gained widespread recognition as a significant analytical instrument for both qualitative and quantitative analysis and became a well-established method for drug detection in mixtures. The application of HPTLC is well-linked and accepted all across the world. It has a wide range of applications in pharmaceutical research, including stability, impurities, synthetic medicine, pharmacokinetics, enantiomeric purity, and drug monitoring in biological fluids.

The calibration curve for Imperatorin was plotted as absorbance versus concentration. The regression equation obtained was $y = 0.0019x + 0.6084$ ($R^2 = 0.9949$). The R^2 value of 0.9949 indicates that the method was linear. The calibration curve was established over the range of 200 - 1200 ng/spot. The proposed method was found to be precise, as the % RSD values for intra-day and inter-day precision were satisfactory. The drug at 80%, 100%, and 120% levels showed recoveries of 99.30%, 97.51%, and 99.55%, respectively, indicating good recovery. Hence, the method was accurate. The LOD and LOQ were calculated as 1.53 ng/spot and 4.64 ng/spot, respectively. Furthermore, DPPH assay showed that AMFE has a strong anti-oxidative potential. The analysis of the pharmaceutical formulation by the developed method was consistent with the label claim, highly reproducible, and reliable. The method can be used for the routine analysis of Imperatorin in the formulation.

CONCLUSIONS

The method presented in this study is accurate, precise and consistent determination of Imperatorin in the formulation. This method was validated as per ICH guideline Q2(R1). This method can be used for routine estimation of Imperatorin in bulk and pharmaceutical dosage forms.

Conflict of interest

Authors declares no conflict of interest

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Author Contributions

Snehal R. Jaiswal: Conceptualization, Investigation, Data Curation, Writing-Original draft and Editing; Jaya Sharma: Conceptualization, Supervision, Data Curation Writing-Review and Editing; Pankaj Sharma: Supervision, Writing-Review and Editing; Suraj M. Sarode: Data Curation, Writing-Review and Editing. All authors have equally contributed and read and agreed to publish the manuscript.

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AI Use Statement

No artificial intelligence (AI) or AI-assisted tools were used in the writing, data analysis, or creation of figures for this manuscript.

Ethics approval
Not Applicable

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