Original Article

DENSITOMETRIC VALIDATION OF LAPACHOL IN *TECOMELLA UNDULATA SEEM* BARK BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

Objective: To develop a validated high-performance thin layer chromatography (HPTLC) method for the quantitative estimation of Lapachol in a Soxhlet extracted bark of *Tecomella undulata seem* (*T. undulata*)**.**

Methods: The bark of *T. undulata* was extracted with chloroform by Soxhlet apparatus. The separation was achieved on a silica-gel 60 F_{254} HPTLC plate using toluene-ethyl acetate–glacial acetic acid (8.5:1.5:0.02 *v/v/v*) as a mobile phase. Densitometric analysis of Lapachol was carried out in absorbance mode at 254 nm.

Results: The proposed method was accurate for the separation and resolution between peaks of the standard and Lapachol (5.04μg) with R_f value 0.77. Calibration curves were found to be linear over the concentration range (10-130μg) for Lapachol and correlation coefficient over (R^2= 0.9973), indicating an excellent correlation between peak areas and concentrations of the marker compound. The experimentally derived LOD and LOQ for Lapachol were determined to be 0.028 μg and 0.086 μg respectively and the developed HPTLC-UV method showed lower %RSD and SEM, value indicating the method to be precise, accurate and robust.

Conclusion: The study concludes that HPTLC-UV validation method can be very efficient and promising technique for the identification and quantitative analysis of Lapachol from *T. undulata* bark. The statistical analysis of data indicates that the developed method is reproducible and specific.

Keywords: *Tecomella undulata,* Lapachol, High-Performance Thin Layer Chromatography (HPTLC)

INTRODUCTION

*Tecomella undulata Seem (*Bigoniaceae*)* is a tree species, locally known as Rohida found in Thar Desert regions of India and Pakistan, which is used as a hepatoprotective agent in various ayurvedic formulations such as Rohitakarishta, Rohitakadyachurna, Rohitakaghrita and Rohitakalauha [1]. *T. undulata* bark consists of various chemical constituents which are primarily used in the treatment of syphilis, painful swellings and cancer. Lapachol (fig. 1) is a naturally occurring 1, 4-naphthoquinone originally isolated from *Tabebuia avellanedae* (Bignoniaceae) [2], used against, *Trypanosome cruzi*, cancer, viruses, bacteria, and Leishmania [3]. This constituent can also be found in other plant families such as, Verbenaceae, Leguminosae, Sapotaceae, Scrophulariaceae, Malvaceae and Proteaceae, however, its occurrence is higher in the Bignoniaceae family, particularly in the gender *Tabebuia*, along with other heterocyclic quinones [3]. Naphthoquinones are natural aromatic compounds that can be found in several plant families, as well as isolated from fungi, algae and bacteria. Traditionally used for their dyeing properties, however, recently a variety of biological activities of these compounds has been reported [3]. They are also categorized in a class of natural phenolic compounds formed on a C6-C4 skeleton. Due to its versatile pharmacological activity, it is isolated and analyzed from natural sources using a simple, efficient and reliable technique. Conventional Soxhlet extraction (CSE) has been used for a long time, especially because of its low cost and easy applicability as compared to the available novel techniques. Extraction of any kind of secondary metabolite by using a wide range of solvents is the added advantage of Soxhlet extraction [17]. Currently, HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost-effectiveness and rapidity [17]. In this investigation, a validated high-performance thin layer chromatography (HPTLC) method was developed for the quantitative estimation of Lapachol in a Soxhlet extracted bark of (*T. undulata*).
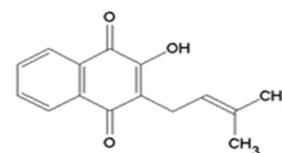

Fig. 1: Chemical structure of Lapachol

MATERIALS AND METHODS

Chemicals and reagents

Standard Lapachol were purchased from Sigma Aldrich fine chemicals, chloroform and methanol (HPLC grade) were used for the preparation of standard and sample and toluene: ethyl acetate-glacial acetic acid (8.5:1.5:0.02*v/v/v*) were used as a mobile phase for HPTLC analysis. All solutions used for analysis were filtered through a 0.1 μm filter. Stem bark of *T. undulata* was collected from Lodhi Garden, New Delhi. The samples were authenticated by the National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India, reference no. NISCAIR/RHMD/Consult/2014/2472-51.

For HPTLC analysis of Lapachol, an HPTLC system from Chemie Erzeugnisse and Adsorptions Technik (CAMAG) AG (Muttenz, Switzerland) was used, equipped with a Linomat IV sample applicator. Lapachol extract was applied to aluminum, baked TLC plates (20X20 cm) precoated with silica gel 60 F_{254} (Merck, Darmstadt, Germany). For developing TLC twin-trough CAMAG (vertical development) chamber was used.

Extraction of plant material

The plant material was collected from Lodhi Garden, New Delhi. Dried fine plant material was subjected to Soxhlet extraction with chloroform for 8 h at 37 °C [11].

Preparation of standard

1 mg compound was accurately weighed in an Eppendorf tube and mixed with methanol (HPLC grade). It was sonicated for 10 min and then filtered through 0.1 µm filter.

Sample preparation and application

Accurately weighed 200 mg of crude extract was dissolved in 50 ml of HPLC grade of chloroform and the solution was sonicated for 10 min, and then made up with 10 ml methanol before filtering through 0.1 µm membrane syringe filter and injecting into the HPTLC system.

Calibration curves for a standard solution

A standard solution was applied in triplicate on silica gel 60 F_{254} plates, using CAMAG linomat-5 Automatic sample spotter. The plates were developed in toluene: ethyl acetate-glacial acetic acid (8.5:1.5: 0.02*v/v/v*) solvent in CAMAG glass twin-trough chamber (20X20 cm) up to a distance of 8 cm, after drying the HPTLC plates were scanned at 254 nm, WINCATS software was used for recording and analyzing the chromatographic data.

The peak areas were recorded. Calibration curves of the Lapachol were obtained by plotting peak areas versus applied crude extract concentration of Lapachol.

Assay validation

The proposed HPTLC-UV method was validated as per the ICH guidelines [22].

Calibration studies

Linearity was evaluated in the range of 10-130 µg/ml for Lapachol. A peak area versus concentration was subjected to least square linear regression analysis and the slope-intercept and correlation coefficient for the calibration was determined.

Limit of detection (LOD) and limit of quantification (LOQ) was evaluated from the following expression:

$$LOD = 3.3 \times \frac{SD}{\alpha} \text{ (slope of curve)}$$

$$LQD = 10 \times \frac{SD}{\alpha} \text{ (slope of curve)}$$

Where SD is the standard deviation and α is the slope of the curve.

Accuracy studies

The accuracy of the method was established by performing recovery experiments using the standard addition method at three different levels, the validity of the accuracy was calculated from the following equation:

$$\left[\frac{\text{spiked concentration} - \text{mean concentration}}{\text{spiked concentration}} \right] \times 100$$

Precision studies

The method was evaluated at inter and intra-day each level of precision was estimated by six sequential replicates of Lapachol at a concentration of 50 and 70µg/spot, the % RSD and SEM of peak areas were calculated.

Robustness

For the determination of the robustness of the method, chromatographic parameters were intentionally altered by a small modification to determine their influence on the retention time and quantitative analysis.

Specificity

The specificity of the method was established by analyzing Lapachol standard and its presence in chloroform extract. The spot for Lapachol in the extract was confirmed by comparing the R_f value, color and peak of the spot in samples with those of a standard.

RESULTS

Method validation

The proposed method was accurate for the separation and resolution between peaks of the standard and Lapachol with R_f value 0.77 (fig. 1, fig. 2). Under described experimental conditions, the linear correlation between peak area and applied concentration was found in the concentration range of 10-130 µg/ml for Lapachol. The correlation coefficient for Lapachol was found to be 0.9973, the regression equation calculated using Y= 15.452X+310.23. The experimentally derived LOD and LOQ for Lapachol were determined to be 0.028 µg and 0.086 µg respectively (table 1).

Precision

Precision data repeatability inter-and intra-day for different concentration level are summarized in (table 2), the precision study showed RSD less than 1%, indicating sufficient precision.

Accuracy

The satisfactory recoveries (table 3) indicate that the HPTLC-UV method is reliable for the quantification of Lapachol in chloroform extracts of *T. undulata* bark.

Robustness

Solvent system having a diverse composition of toluene-ethyl acetate–glacial acetic acid (8.5:1.5:0.02*v/v/v*) were tried at two different concentration levels of 50 and 70 µg/spot and %RSD of peak area were calculated (table 4).
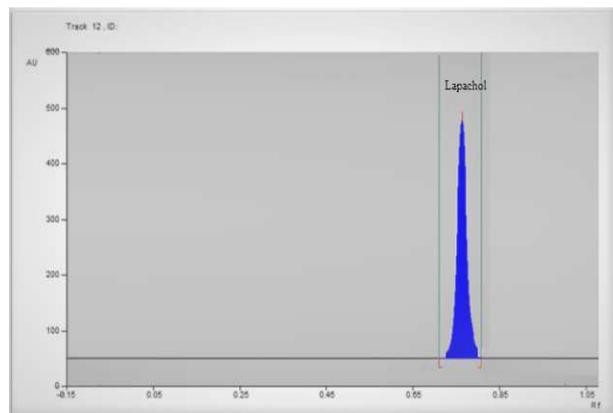

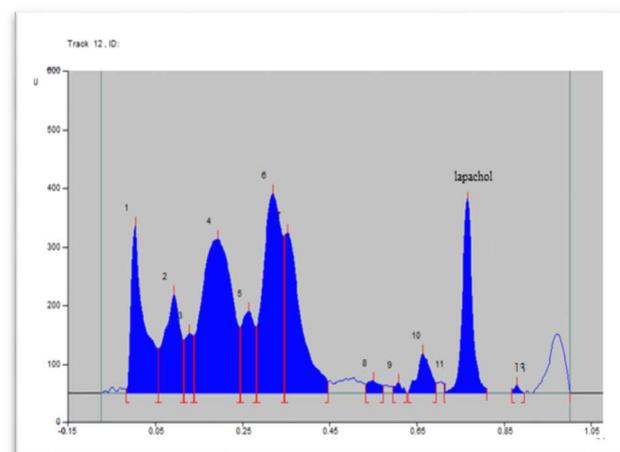**Fig. 2: HPTLC chromatogram of standard Lapachol**

Fig. 3: HPTLC chromatogram of lapachol sample in *T. Undulata* extract

Table 1: Densitometric validation summary of lapachol

Parameter	
Linearity range	10-130µg
Coefficient correlation	0.9973
Regression equation (peak area)	Y= 15.452X+310.23
LOD(µg)	0.028(µg)
LQD(µg)	0.086(µg)
Rf	0.77
Lapachol content (µg)	5.04(µg)
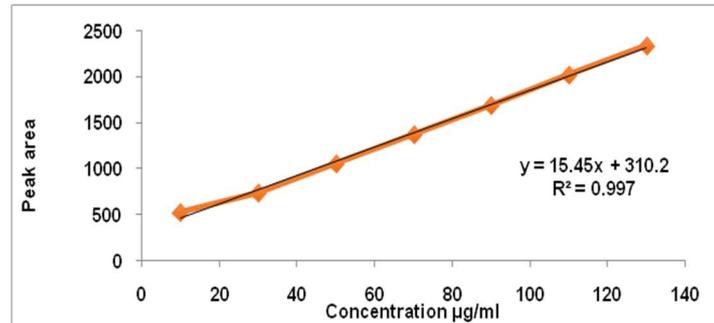
Fig. 4: Calibration curve for Lapachol

Table 2: Intra and inter day

Amount on lapachol spot (µg)	Mean	SD	%RSD	SEM
Intraday				
50	1061	3.74	0.35	1.52
70	1380.5	3.93	0.28	1.60
Inter-day				
50	1051	1.87	0.24	1.05
70	1372.5	1.86	0.13	0.76

Data represent mean±SD and mean±SEM, (n=6)

Table 3: Accuracy analysis

Amount of lapachol in sample (µg)	% of standard lapachol in a sample	% recovery of lapachol (peak area)	% RSD	SEM
5.04	10	527	0.15	0.46
5.04	50	1055	0.14	0.88
5.04	90	1690	0.11	1.16
5.04	130	2341	0.03	0.50

Data represent mean±SEM, (n=3)

Table 4: Robustness

Lapchol (µg)	Toluene–ethyl acetate–glacial acetic acid (8.2:1.8:0.02 v/v/v) %RSD via area	Toluene–ethyl acetate–glacial acetic acid (8.5:1.5:0.02v/v/v) %RSD via area
50	0.95	0.15
70	0.72	0.09

Data represent mean±SD (n=3)

DISCUSSION

Development of new analytical methods for qualitative and quantitative estimation of active compounds in a crude plant extract is essential for pharmacokinetics, biological and toxicological studies and for standardizing them to maintain their quality and batch-to-batch reproducibility [18]. A simple, rapid and accurate method was developed for the first time for estimation of Lapachol from *T. undulata* bark by HPTLC. The standard compound was clearly resolved with sufficient resolution and no interfering peaks of endogenous compounds were observed. Calibration curves were found to be linear over the concentration range 10-130 µg for Lapachol (table 1). The marker compound had a correlation coefficient (R^2) value over 0.9973, indicating that there was an excellent correlation between peak areas and concentrations of the marker compound. The specificity of the method was evidenced in the (*Rf*) results indicated that the developed method was selective and specific to distinguish and quantify Lapachol from a plant extract. Lowest LOD and LOQ values (table 1) of the standard indicated that the developed method was very sensitive and can be used for the detection and quantification of Lapachol over a wide range of concentration. Results of inter-and intra-day precession showed that %RSD value of peak area with high repeatability (table 2). There was not much variation in the inter-and intra-day precision, thus the

method is extremely adaptable because of the good precision and excellent repeatability of the method. The results of inter-and intra-day precisions of the present study were highly precise in comparison with the previously published analytical methods [19]. It was observed that the R_f, peak area and the recovery of the standard compounds were not significantly affected by small changes in the parameters as evident from the low values of %RSD (<2%) indicating the robustness of the method. This rapid and simple HPTLC-UV method was developed for the first time to determine the contents of Lapachol in the chloroform extract of *T. undulata* bark.

CONCLUSION

This study shows that an HPTLC-UV validation method can be very efficient and promising techniques for the identification and quantitative analysis of Lapachol from *T. undulata* bark. In this study, a solvent system was developed for the first time for the HPTLC-UV analysis of Lapachol. The solvent system was found good for resolution of Lapachol peaks. LOD and LOQ were found to be comparatively low which showed the greater sensitivity of the developed method. The statistical analysis of data indicates that the developed method is reproducible and specific.

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AUTHORS CONTRIBUTIONS

Richa Raj, designed, carried out and wrote up the research article, Vidhu Aeri, supervised and helped in designing the work, Nahida Siddiqui guided and proofread the research article.

CONFLICTS OF INTERESTS

The authors declare no conflict of interest

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