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Isolation and chromatographic resolution of calophyllolide, an anti microbial and cytotoxic coumarin from *Calophyllum inophyllum*

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ABSTRACT

Calophyllolide, a 4-phenyl pyranocoumarin is the major component in the seed of *Calophyllum inophyllum*. As the seed oil was reported to be showing anti HIV activity, isolation of calophyllolide was attempted using a simple and efficient soxhlet extraction method. A new reverse phase HPLC method was developed to check the extraction efficiency and purity of the isolated compound. The method was validated as per ICH guidelines. Forced degradation studies were performed to ensure the specificity of the method. The LOD and LOQ of calophyllolide was found to be 0.011% (w/w) and 0.034% (w/w) respectively in this method.

KEYWORDS

Calophyllolide;
Calophyllum inophyllum;
Cytotoxic coumarin;
Soxhlet extraction;
Method validation.

INTRODUCTION

Calophyllum inophyllum (also known as Tamanu tree) is the most abundant species of the genus Calophyllum belonging to the Clusiaceae family[1]. The seeds of *Calophyllum inophyllum* contain several coumarins and xanthones with antimicrobial properties[2]. Calophyllolide is the major component present in the seeds along with several inophyllum such as inophyllum A, B, C, D, E and P of which inophyllum B and P were found to be inhibitors of HIV-1 reverse transcriptase[3,4]. Calophyllolide was found to exhibit significant inhibitory activity against Gram (+) Staphylococcus aureus. Also calophyllolide has been proved to be showing significant cytotoxic activity against nasopharynx cell (KB)[2,3,5] To further study the anti HIV property of calophyllolide by various synthetic structural modifications[6,7], a research study was undertaken initially to isolate and characterize the major component, calophyllolide present in the oil (Figure 1). A normal phase methodology with three silica columns in series was found reported recently for the screening of anti HIV inophyllums by HPLC-DAD[8,9]. A simple reverse phase HPLC method was developed to facilitate the isolation process. To our knowledge as there is no reverse phase HPLC method reported so far for calophyllolide.

EXPERIMENTAL

General

Agilent 1100 HPLC with Photo Diode Array (PDA) detector was used for developing the method and for analyzing the samples. UV spectra of the components were extracted from the PDA detector. NMR spectra were recorded using $CDCl_3$ as solvent on a Varian NMR system spectrophotometer equipped with a 5mm multinuclear probe operating at 500 and 125 MHz for ^1H and ^{13}C NMR respectively. TMS was used

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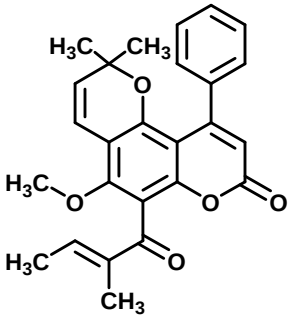

Figure 1 : Structure of Calophyllolide

as internal standard and chemical shifts were reported in δ values in ppm. Mass spectra were recorded using API 4000 QTRAP mass spectrometer equipped with an ESI interface in positive ionization mode.

Material

Calophyllum inophyllum fruits were collected from coastal region of south Tamil Nadu in India. The harvested fruits were dried and the nuts were extracted. The nuts were then dried in shade in thin layers over a period of about two months. Then the seeds were crushed to get a coarse powder.

Extraction and isolation

Soxhlet extraction was carried out for about 5 hours using 50g of the seed powder each with 250ml of solvent. The solvent extract was further purified by column chromatography packed with silica, n-hexane and ethyl acetate (8:2) were used as eluents for the purification. The collected fractions were pooled together and concentrated in Buchi rota evaporator under vacuum at room temperature. The collected fractions were analysed by HPLC.

HPLC method development

The HPLC method was developed by using an YMC Pack ODS-A column of 100mm length and 4.6mm ID with 3µ particle size. A mobile phase composition of water and acetonitrile in the gradient mode was used. The initial mobile phase composition was kept as 90% water and 10% acetonitrile and linearly increased to 90% of acetonitrile over a period of 50 minutes. A UV detector at 254nm was used for monitoring the peaks. Calophyllolide was found to elute at about 36 minutes as sharp peak in this method (Fig 2A). The run time was fixed as 70 minutes for monitoring the possible non polar impurities during the extraction. Later on it was reduced to 50 minutes. A 7:3 mixture of acetonitrile and water was used as diluent for dissolving the samples.

Method validation

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Stress studies were performed for calophyllolide to provide an indication of the stability-indicating property and specificity of the proposed method. Intentional degradation was attempted with a stress condition of UV light (254nm), heat (60°C), acid (1N HCl), base (0.1N NaOH) and oxidation (5.0% H_2O_2) to evaluate the ability of the proposed method to separate calophyllolide from its degradation product. For heat and light studies, study period was 10 days whereas for hydrolytic, acid, base and oxidation, it was 24 h. Peak purity test was carried out for the calophyllolide peak by using PDA detector in stress samples.

Precision

The precision of the related substances method verified by repeatability and by intermediate precision. Repeatability was checked by injecting six individual preparations of calophyllolide. %RSD of area for calophyllolide and each impurity was calculated. The intermediate precision of the method was also evaluated using different analyst and performing the analysis on different days.

Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ for impurities were determined by measuring signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations of calophyllolide[10]. Precision study was also carried out at LOQ level by injecting six individual preparations of calophyllolide and calculating the % RSD of the area.

Linearity

Linearity test solutions were prepared by diluting stock solution of calophyllolide to the required concentrations. The solutions were prepared at six concentration levels from LOQ to 150% of the sample concentration.

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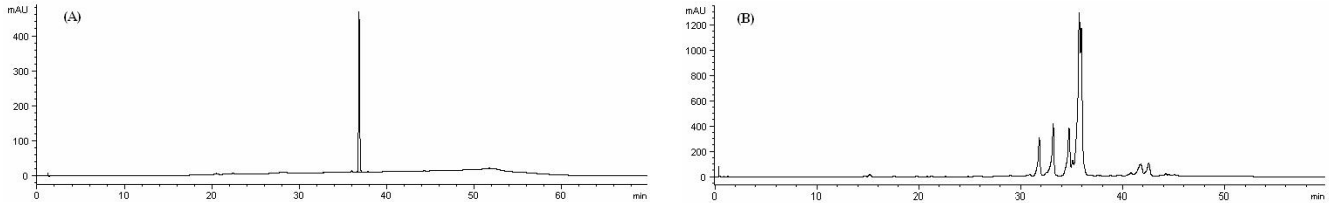

Figure 2 : HPLC chromatograms of calophyllolide purified (A) and seed powder (B)

TABLE 1 : Summary of forced degradation studies

Stress condition	% Degradation
Oxidative degradation	4.3%
Acid degradation	4.2%
Base degradation	47.4%
Thermal degradation	ND
Photolytic degradation	ND

ND-Not detected

TABLE 2 : Regression and precision data

Parameter	Result
LOD (w/w)	0.011%
LOQ (w/w)	0.034%
Regression equation (y)	
Slope (b)	49127
Intercept (a)	282858
Correlation coefficient	0.999
Precision (% RSD)	1.57%
Intermediate precision (% RSD)	0.30%

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between calophyllolide and its impurities and tailing factor for calophyllolide were recorded. The actual flow rate of the mobile phase was 1.0mL/min. To study the effect of flow rate on the resolution, flow was changed by 0.1 units from 0.9 to 1.1mL/min.

Solution stability

Solution stability of calophyllolide and its impurities was carried out by leaving the sample solutions in tightly capped volumetric flasks at room temperature for 48 h. Content of impurities were determined for every 12 h interval up to the study period.

RESULTS AND DISCUSSION

Soxhlet extraction

The seed powder was initially analysed by HPLC and was found to contain about 20% of calophyllolide by area normalization method (Figure 2(B)). Soxhlet extraction of calophyllolide from the seed powder was carried out using various solvents such as n-hexane, ethyl acetate, acetone and ethanol. The solvents were chosen initially considering the non polar nature of calophyllolide. n-Hexane and ethyl acetate were found to be the best solvent for selectively extracting calophyllolide followed by acetone and ethanol. About 125mg of calophyllolide was isolated by this procedure from about 50g of seed (2.5mg/g) using n-hexane and ethyl acetate as solvent by soxhlet extraction, whereas about 122 mg (2.4mg/g) and 52mg (1.1mg/g) respectively from acetone and ethanol solvents. The optimum duration of soxhlet extraction was found to be about 5 h. Further extension of extraction time beyond 5 h has not resulted in any substantial increase in the content of calophyllolide. The purity of the isolated compound was found to be about 99% by HPLC.

Structure confirmation

The mass of the isolated calophyllolide was found to be m/z 416. The structure was confirmed by ^{1}H, ^{13}C and 2D NMR techniques (^{1}H-^{1}H COSY, HSQC and HMBC). The observations were matching with the reported literature values[3].

HPLC method development

Initially a mobile phase composition of phosphate buffer with various pH range from 2.0 to 7.0 and acetonitrile were attempted. As calophyllolide was essentially non polar, there was no much impact of pH of the mobile phase on the separation of impurities. So it was decided to use water as the aqueous component in the mobile phase. Methanol was checked for its suitability for organic component. But due to the higher back pressure generated by the mixing of water and methanol, acetonitrile was fixed as organic component. The developed HPLC method was able to separate various other components present in the solvent extract from

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calophyllolide.

Results of forced degradation studies

Calophyllolide was found to degrade significantly in base hydrolysis. LC/MS analysis of this major degradant in the base hydrolysis showed a mass of m/z 434 with an addition of 18 mass units. Mild degradation was observed in acid and peroxide stress conditions. Calophyllolide was found to be stable under photolytic degradation and hydrolytic conditions. PDA detector was employed to check and ensure the homogeneity and purity of calophyllolide peak in all the stressed sample solutions[11]. The results are tabulated in TABLE 1.

Validation of the method

Precision

The % RSD of calophyllolide and impurities in the precision study and the intermediate precision study was within 2.0 % confirming good precision of the method. The % RSD values are presented in TABLE 2.

Limits of detection and quantification

The limit of detection and limit of quantification for calophyllolide are tabulated in TABLE 2.

Linearity

Linear calibration plot was obtained over the calibration ranges tested, i.e. 25% to 150%. The correlation coefficient obtained for calophyllolide was 0.999. The above result shows that an excellent correlation existed between the peak area and the concentration of calophyllolide proving the correctness of area normalization methodology for assessing the impurity content.

Accuracy

The accuracy of the method was confirmed during method development by changing the sample concentration and ensuring a similar impurity content. Further evaluation of accuracy by physical spiking could not be done due to non availability of isolated impurities.

Robustness

In the deliberate varied chromatographic condition of flow rate, the resolution between all pairs of compounds was greater than 2.0 and tailing factor for calophyllolide was less than 1.2.

Solution stability

No significant changes were observed in the content of impurities during solution stability experiments. The solution stability experiment data confirms that the sample solutions used during analysis was stable for 48 h.

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