Journal of Pharmaceutical Research and Innovation (JPRI)
Volume 6, Issue 2, July 2026

e-ISSN: 2583-0023

HPTLC Method Development and Validation for the Simultaneous Quantification of Pongamol and Karanjin

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Abstract

The objective of the current study is to develop a quantitative method for the simultaneous evaluation of major active components of Pongamia pinnata seed oil – Pongamol and Karanjin - by High-Performance Thin-Layer Chromatography (HPTLC). Chromatographic separations of the active compounds were achieved by silica gel 60 F254 plates utilizing a mobile phase of petroleum benzine:ethyl acetate:formic acid (8:2:0.1, v/v/v), along with densitometric detection at a wavelength of 366 nm. The newly developed method of analysis displayed excellent linearity, and correlation coefficients R^2 = 0.9893 for pongamol and 0.9926 for karanjin, among the concentration ranges of 0.2–1.0 µg/spot and 0.1–2.0 µg/spot, respectively. Less than 2% RSD values for precision with intra- and inter-day proved the reliability of the developed method. Accuracy of the method was confirmed by the recovery rates of 98.5–101.4% for pongamol and 97.9–102.2% for karanjin. The LOD obtained was 0.0238 µg/spot (pongamol) and 0.0279 µg/spot (karanjin), and the LOQ was 0.0721 µg/spot and 0.0848 µg/spot, respectively. This method provides an easy tool for quantification of active compounds in herbal formulations and in the development of cosmeceutical products.

Keywords

HPTLC, Karanjin, Method development, Pongamia pinnata, Pongamol.

INTRODUCTION

Pongamia pinnata (L.) seed oil is known for its various uses in different categories ranging from ayurvedic formulations, feed stuff, lamp oil and as biofuel [1]. It is utilizing for aesthetic and biopesticide purposes, along with the hemostatic characteristics for curing of wounds since ancient times in India [2], [3]. *P. pinnata* seeds have 15–36% oil content with approximately 47–60% oleic acid. The oil is mainly used in leather tanning and soap manufacturing [4], [5], [6]. Due to this high content of oil in the seeds, it is currently gaining interest to use as a source of biofuel [7]. *P. pinnata* seed oil contains different flavonoids, including furano-flavonoids such as pongamol and karanjin which are the major active compound with absorption under ultraviolet region [8], [9]. It also provides enhanced photostabilisation to the formulations [10]. *P. pinnata* seed oil is generally extracted using methods like expelling, solvent extraction, and advanced extraction techniques like supercritical carbon dioxide extraction [11], [12]. As the content of active components is very low, an efficient extraction process will be essential for industrial viability. Accurate evaluation of phytochemicals is critical during the development of extraction process, and simultaneous multi-analyte determination substantially accelerates method optimization.

The measurement of pongamol was standardised previously using an HPLC method [13]. However, the HPLC method involves higher solvent consumption and requires labour-intensive sample preparation procedures, making it less efficient compared to the best alternative technique HPTLC. Typically, HPTLC demand fewer amount of mobile phase, simpler sample handling, and it will be an easy tool for the concurrent measurement of various active components [14]. The *P. pinnata* seeds were standardised by taking karanjin as marker using the same analytical tool [15]. As per the current knowledge, there is no HPTLC method reported in the literature for the concurrent evaluation of pongamol and karanjin in *P. pinnata* seed extract. This study aims to develop a densitometric HPTLC method, and validate as per ICH guidelines, for the simultaneous quantification of pongamol and karanjin in *P. pinnata* seed extracts.

MATERIALS AND METHODS

Raw Materials, Reference Standards and Solvents

The dry seeds of *P. pinnata* were collected from Sangli in Maharashtra, India. The standard of pongamol and karanjin brought from Toronto Research Chemicals Inc., Brisbane, Canada, Hexanes, methanol, petroleum benzine, ethyl acetate, formic acid, and water (HPLC grade), both LR and HPLC grade from Merk were used.

e-ISSN: 2583-0023

HPTLC

Standard Solution of Karanjin and Pongamol

10 mg of pongamol and karanjin were dissolved in methanol and made up to 10 mL in standard flasks. The calibration curves were made based on the guidelines of ICH for accuracy.

Development of Samples

0.10 gm of extract was accurately weighed in a 50 ml in standard flask, dissolved in methanol and made up to the mark with methanol.

Instrumentation

CAMAG HPTLC system containing a CAMAG Automatic TLC Sampler 4 (ATS 4), CAMAG Automatic Developing Chamber 2 (ADC 2), CAMAG TLC Scanner 4, and Vision CATS software.

Extraction of Seeds

The Conventional Extraction (CNE) method was carried out by subjecting dried and flaked *P. pinnata* seeds to four sequential washes with n-hexane. Seed-to-solvent ratio of 1:4 (w/v) was maintained in each wash and allowed to soak for 15 minutes. The miscella were combined and evaporated the solvent under vacuum using a rotary evaporator. For the Microwave Pretreatment and Ultrasound-Assisted Extraction (MUE) method, the seeds first underwent microwave pretreatment and were subsequently extracted with n-hexane under the same solvent ratio. The number of washes were same as used in the CNE method, with each wash exposed to 15 minutes of ultrasonic treatment. The samples from both the methods were taken for HPTLC analysis for the quantification of pongamol and karanjin.

Method Development

- **Optimisation of Mobile Phase:** Different mobile phases were experimented to achieve effective separations, and final optimisation were conducted with different ratios of petroleum benzine: ethyl acetate: formic acid. The best mobile phase ratio was 8:2:0.1 (v/v/v) which showed highest resolution for both pongamol and karanjin.
- **Application of Sample:** Samples were applied as 6 mm bands by CAMAG Automatic TLC Sampler 4 (ATS 4).

Validation

ICH Q2 (R1) guidelines were followed for doing the method validation. Evaluation of reliability and accuracy were assessed by several parameters like, linearity, precision, accuracy, LOD, LOQ and robustness [16], [17], [18].

Linearity

This was assessed by developing bands of reference standard of pongamol and karanjin at different strengths. Triplicates injections were conducted for developing bands of each dilution. Calibration curve and linear regression data were obtained from graph with peak area against strength.

Precision

Intermediate Precision: Intraday and inter-day precision was assessed using mixed standards of pongamol and karanjin with a concentration 0.2 µg/spot for pongamol and 0.1 µg/spot for karanjin. Replicate injections were performed thrice on the same day and on three separate days to assess intraday and inter-day precision.

Instrumental Precision: Instrumental precision was measured with blended standards of pongamol and karanjin at three concentration levels: 0.2-0.1 µg/spot for pongamol and 0.1-0.2 µg/spot for karanjin. Replicate injections were performed thrice in similar day.

Accuracy

It was established through recovery study with different percentages of required concentrations such as 50%, 100%, and 150%. Pongamol and karanjin standards with known dilutions were spiked into samples which was injected previously and measured the % recovery.

LOD and LOQ

Standard deviation of the responses along with calibration curve slope were taken for the evaluation of LOD and LOQ. Effects of the different analytical parameters on the findings were evaluated along with the % RSD values.

Robustness

This was evaluated by intentional variation of key factors, comprising mobile phase components, solvent fronting, wavelength, to ensure consistent performance under slight procedural changes. Robustness was assessed by calculating %RSD of peak areas under varied analytical conditions.

RESULTS AND DISCUSSIONS

HPTLC Method Development

The developed mobile phase consists of petroleum benzine: ethyl acetate: formic acid in the ratio 8:2:0.1(v/v/v), delivered well-separated symmetric bands for karanjin (Rf = 0.38) and pongamol (Rf = 0.62). Figure 1 depicts the bands of both furano-flavonoids at 366 nm. The three-dimensional densitogram of the mixture of reference standard pongamol and karanjin and *P. pinnata* seed extract were presented in Figure 2. It shows clear separations between different active components. Figure 3 shows the HPTLC chromatograms of pongamol and karanjin and Figure 4 depicts the HPTLC chromatogram of *P. pinnata* seed extract. It was observed that both pongamol and karanjin showed definite separations in the plate. These separations confirmed the effectiveness of the developed method for the simultaneous quantification of both active compounds. Compared to a previous work [15] with a mobile phase with toluene: ethyl acetate system, the mobile phase in the newly developed method enhanced the chromatographic resolutions and reduced co-elution. This ensured the advantage of the current method in the simultaneous quantification of karanjin and pongamol. The developed HPTLC method offers a more robust and reliable

JPRI
JOURNAL OF PHARMACEUTICAL RESEARCH AND INNOVATION
e-ISSN: 2583-0023
Journal of Pharmaceutical Research and Innovation (JPRI)
Volume 6, Issue 2, July 2026

approach for the standardization of *P. pinnata* seed extract.

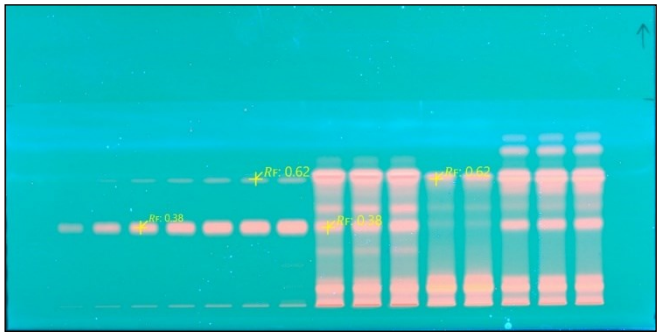

Figure 1. TLC plate of Reference standards Pongamol + Karanjin and *P. pinnata* seed extract

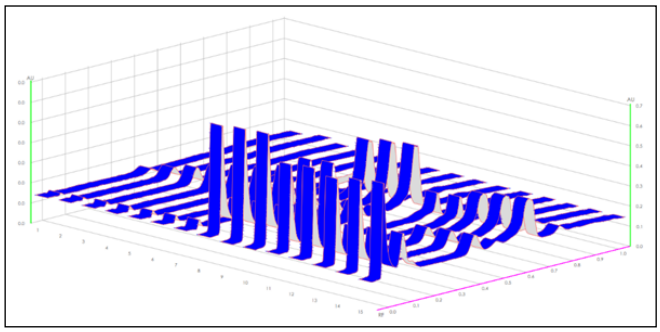

Figure 2. Three-dimensional overlay of HPTLC Densitograms of Reference standards Pongamol + Karanjin and *P. pinnata* extract

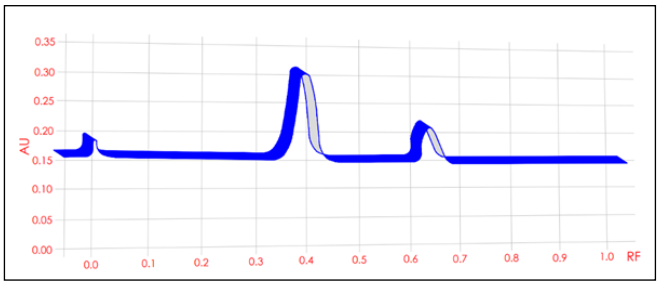

Figure 3. Chromatogram of Reference standards Pongamol + Karanjin

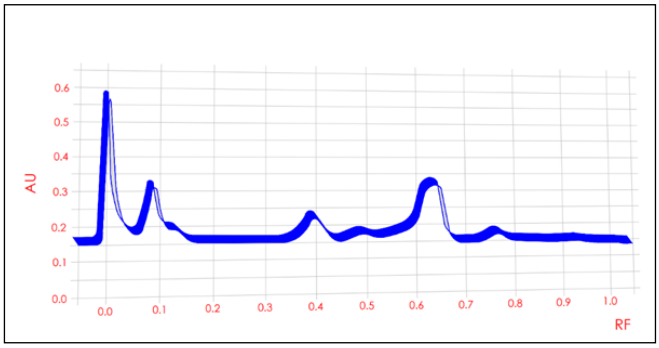

Figure 4. Chromatogram of *P. pinnata* seed extract

Linearity

Accurate measurement of analyte molecules between the studied concentration range essentially requires a linear relationship based on the measured area. The newly developed method provides an excellent linearity for both pongamol and karanjin in the limits of 0.2 – 1.0 µg/spot as exhibited in Table 1.

Table 1. Validation Parameters and Results

Parameter	**Pongamol**	**Karanjin**
Linearity (µg/spot)	0.2 – 1.0	0.2 – 1.0
Regression equation	Y =1224.3x -45.826	Y = 1089.6x + 56.325
Correlation coefficient (R^2)	0.9893	0.9926
Standard deviation of residuals (Sy/x)	8.52 AU	9.43 AU
LOD, µg/spot	0.0238	0.0279
LOQ, µg/spot	0.0721	0.0848
Precision (%RSD)	1.15%	1.38%
Accuracy	98.5 – 101.4 %	97.9 – 102.2 %
Robustness	Method remained unaffected by minor variations in mobile phase (±2 %), detection wavelength (±2 nm), and saturation time (±5 min). %RSD < 2 %	Method remained unaffected by minor variations in mobile phase (±2 %), detection wavelength (±2 nm), and saturation time (±5 min). %RSD < 2 %

The results showed that the areas of the chromatographic peaks increased with respect to the rise in the concentration of pongamol and karanjin within the range and hence exhibited a linear response. The correlation coefficients (R^2) 0.9893 for pongamol and 0.9926 for karanjin show that the new method has outstanding linearity. The linear regression equations for pongamol and karanjin were Y = 1224.3x − 45.826 and Y = 1089.6x + 56.325, respectively, where 'Y' denotes spot area and 'x' the analyte strength. These data verified the validation of newly developed method, as per the specifications documented by previous study [19]. The correlations in the data are shown in Figures 5 and 6.

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Volume 6, Issue 2, July 2026
e-ISSN: 2583-0023

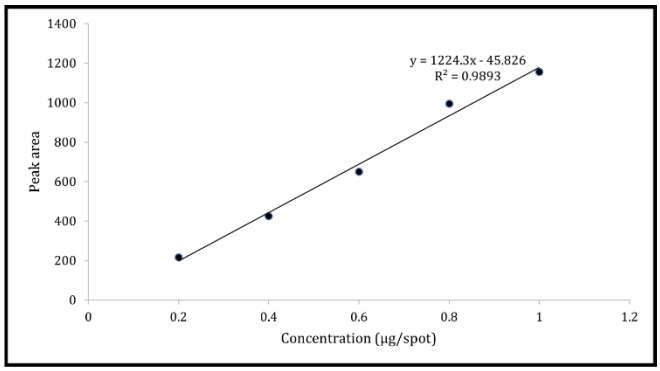

Figure 5. Linear Regression of Pongamol

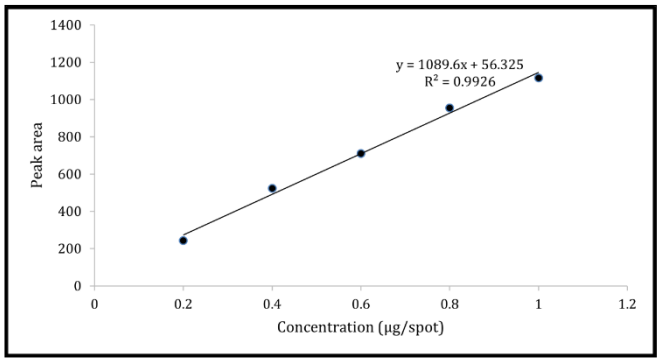

Figure 6. Linear Regression of Karanjin

Precision

Intermediate Precision

RSD values for the peak area obtained during the injection on inter day and intraday are exhibited in Table 1. The values obtained for pongamol were 1.15% and for karanjin 1.38% during the method development. Observed RSD values were less than 2% and it clearly showed the consistency across evaluations and the reliability of newly developed methods under the tested situations. The data verified the precision of current developed method [20].

Instrument Precision

Instrumental precision of the current method was analysed by measuring the area obtained for the peaks of reference standards of pongamol and karanjin in six consecutive injections throughout investigated concentrations as data summarised in Tables 2. The lowest concentration (0.2 µg/spot) showed 1.2% RSD and rest of the higher concentrations showed lesser RSD values than that. The data demonstrated the high precision and reproducibility of instrument, confirming the reliability of the developed method.

Table 2. Linear regression data of Pongamol and Karanjin

Concentration (µg/spot)	Pongamol		Karanjin	
	Area, mean ± SD (n = 6)	RSD	Area, mean ± SD (n = 6)	RSD
0.2	216.93	1.2	243.41	1.4
0.4	425.06	0.65	523.19	0.94
0.6	650.15	0.68	711.24	0.41
0.8	995.58	0.39	955.82	0.27
1	1155.93	0.17	1116.66	0.13

Accuracy

Accuracy of the currently developed method was done by evaluating the percentage recovery during the injections of different known concentration. The known concentrations were achieved by adding known amount of standard of pongamol and karanjin to the previously known concentration of sample solution. One of the previous methods documented that the accuracy for karanjin quantification by HPTLC was 95.05 and 101.05% [4]. It was observed that the percent recovery obtained was 98.5 – 101.4% for pongamol and 97.9 – 102.2% for karanjin, and it clearly indicates a reliable quantification across the tested concentration levels. The reliability of these ranges was confirmed by the low RSD values.

LOQ and LOD

Detailed data shown in Table 1. An LOD 0.0238 µg/spot for pongamol and 0.0279 µg/spot for karanjin and an LOQ of 0.0721 µg/spot for pongamol and 0.0848 µg/spot for karanjin confirmed that the new analytical method has high analytical sensitivity. One of the previous studies reported an LOQ of 0.0739 µg/spot for karanjin but that method showed lesser accuracy than the current method [21].

Robustness

Despite deliberate variations in analytical parameters, peak area reproducibility remained consistent, with %RSD values below 2%, as detailed in Table 1. This proved that the method remained stable and reliable despite minor variations in the evaluated parameters.

Pongamol and Karanjin Contents of the Extracts

CNE yielded seed oil with 0.2895% pongamol and 1.1521% karanjin, while MUE yielded an extract with 0.2918% pongamol content and 1.2961% karanjin. One of the previous studies reported 1.25% karanjin and 0.85% pongamol in *P. pinnata* seed extracts by HPLC method. One another study documented a karanjin content of 3.18% in *P.*

pinnata seeds from West Bengal [22], [23]. Natural molecules show variations in concentrations based on different parameters such as climatic conditions, regions, etc. and hence the deviations observed in this study from previously reported values are in expectance range. It was noticed that microwave pretreatment and ultrasound assisted extraction increased the pongamol content of the extract. Increased pull out other extraneous matter from the seeds reduced the pongamol content in the MUE treatment.

CONCLUSION

Furano flavonoids – pongamol and karanjin – are the major active components in the *P. pinnata* seed extract responsible for its therapeutic usages. Standardization of these active components is essential in the extract to get consistency of activity in the formulations with *p. pinnata* seed extract. Currently developed HPTLC method has outstanding precision, accuracy, and overall analytical robustness in the quantification of pongamol and karanjin simultaneously in *P. pinnata* seed extract. The method offers substantially shorter analysis times, decreases the quantity of sample requirements, and reduces reagent costs, pointing out the practical advantages of this new method.

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