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Validated stability indicating method for *Karanjin* using HPTLC and HPLC

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Abstract

Karanjin is an active marker present in Karanja seed. Seed powder is valued for Anti-Plasmodia activity, Anti-Inflammatory activity, Anti-ulcer Activity, Hypoglycemic and Hypolipidemic Activity, Antiviral Activity, Antidiabetic activity, renal protective activity. The present study was aimed to develop and validate stability indicating method for karanjin by HPLC and HPTLC. The development was done using TLC plates precoated with silica gel 60F₂₅₄. Toluene-Ethyl acetate (8:2 v/v) were used as mobile phase and scanning was done by using TLC Scanner III. The developed HPLC method involved use of RP-C18 (150 × 4.6 mm) column. The detection done at 260 nm using PDA detector having mobile phase Methanol: ACN: Water (70:15:15 v/v/v). The flow rate was kept as 1 mL/min. The linearity range 80-400 ng/band was set for HPTLC and for HPLC 10-50 µg/ml. Stress degradation studies were carried out for karanjin as per the ICH guidelines. The characterisation of stress sample were done using LCMS.

Keywords: *Pongamia pinnata*, HPTLC, HPLC, LCMS, forced degradation studies, validation

Introduction

Karanja consists of dried root bark, leaf, root, seeds and stem bark of *Pongamia pinnata* (Linn.) Merr. Syn. *P. glabra* Vent. (Fam. Fabaceae), a glabrous tree [1]. A semi evergreen glabrous tree with up to 18 m or more in height. Different parts of Karanja plant Bark, Leaves, Flowers, Fruits, and Seeds are used. Pulp of seed has an application in leprosy. Commonly used in Bronchitis and whooping cough. Other Uses are keloid tumors, hypertension, skin ailments and rheumatic arthritis. Seed powder valued for Anti-Plasmodia activity, Anti-Inflammatory activity, Anti-ulcer Activity, Hypoglycemic and Hypolipidemic Activity, Antiviral Activity, Antidiabetic activity, renal protective activity. *Pongamia pinnata* also used as Bio fuel [2, 3]. Since there are number of pharmacological activities reported of karanjin and one HPTLC method are reported according to literature survey, karanjin is selected for further study [4, 5].

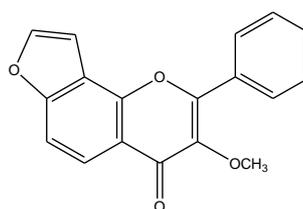


Fig 1: Structure of karanjin

In the field of pharmaceutical industry HPLC and HPTLC is playing important role for the resolution of drugs. The development of the method for the quantitative analysis of compound. A rapid simple reproducible stability-indicating HPTLC method was developed and validated. So far to our knowledge there was no stability indicating method has been reported using HPTLC OR HPLC for karanjin.

Materials and Methods [6, 7]

Collection of Plant material

The whole plant *Pongamia pinnata* was collected from Medicinal garden, AISSMS College of pharmacy pune and authenticated from Botanical survey of India, Pune. The authentication no. BSI/WRC/IDEN.CER./2016. Plant seeds were collected and powdered using a mixer grinder and stored in air tight container. Marker karanjin was procured from M/S Yucca Enterprises.

Chemicals

The reagents used for present study are as follows Methanol, Acetonitrile, Toluene, Ethyl

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acetate, Distilled water, HCl, NaOH, Hydrogen peroxide (30% w/v) were of analytical grade from Loba Chemie Pvt. Ltd., Mumbai, India.

Preparation of extracts^[8]

Extract was obtained using methanol as solvent. Methanolic extract was prepared by using the maceration method 24 hours at room temperature. The Extract was concentrated on a water bath at 45°C. Extract were collected and quantified.

Wavelength selection

The λ_{max} of active marker karanjin was determined from spectrum using UV Spectrophotometer (V-730 model) make JASCO.

Mobile phase optimization

The mobile phase optimized for karanjin marker such that the Rf should be in the range of 0.2-0.8 and the band should be compact.

Preparation of standard

10 mg of marker karanjin was dissolved in methanol and further diluted to achieve final concentration of 20 μ g/ml.

Calibration curve

The calibration range for karanjin was set as 80 to 400ng/band. The different volumes of stock solution 4, 8, 12, 16 and 20 μ l were spotted on the HPTLC plate to attain the concentration of 80, 160, 240, 320 and 400ng/ band.

Forced degradation studies

According to ICH guidelines, the stress testing aims at the identification of the probable degradation products which may further assist in determination of the intrinsic stability of the molecule and validate the stability indicating procedures adopted. The conditions are optimized in such a way that degradation achieved in 10 to 30%.

Alkali hydrolytic condition

For alkali degradation study, 10 ml of (0.1mg/ml) stock solutions of marker karanjin was taken into 100ml round bottom flask and 10 ml of 2N sodium hydroxide was added and refluxed for 2hr. cooled at room temperature. Then solution was neutralized with 2N hydrochloric acid and diluted with methanol to make up the volume 50ml (20 μ g/ml).

Acid hydrolytic condition

For acid degradation study, 10 ml of 2N hydrochloric acid was added and this procedure was same as alkali hydrolytic condition. Then solution was neutralized with 2N sodium hydroxide before making up the concentration.

Neutral hydrolytic condition

For neutral degradation study, 10 ml of distilled water was added and further procedure same as alkali hydrolytic condition.

Oxidative stress degradation

For oxidative stress degradation, 2 ml of (0.1mg/ml) stock solutions of standard karanjin was taken into 10 ml volumetric flasks and 2 ml of 30% V/V hydrogen peroxide (H₂O₂) was added and made up the volume with methanol. The mixtures were kept overnight at room temperature.

Dry heat degradation

Marker karanjin was kept in oven at 60°C for 4 hours. Then the sample was allowed to cool. 10 mg each of standard karanjin were accurately weighed and dissolved in few ml of methanol in volumetric flasks (10 ml) and makeup the volume with the methanol. Dilute appropriately to prepare the solution with final concentration of 20 μ g/ml.

Photolytic degradation

The standard karanjin were exposed to UV lamp (200 watt hours/square meter) and cool white fluorescent lamp (1.2 million lux hours). 10 mg was accurately weighed and dissolved in few ml of methanol into volumetric flasks (10 ml) and made up the volume with methanol. Further diluted with methanol to attain the working standards of 20 μ g/ml concentration.

All the sample solutions were applied on TLC plate and densitograms were recorded using HPTLC. Chromatography was performed on pre-coated TLC plates with Silica gel 60 F254. Each of the degraded samples of standard karanjin were separately applied to the plate with an automatic TLC applicator Linomat-5 with N₂ flow. Densitometric scanning was performed at 260 nm on CAMAG TLC scanner 3. Development of densitogram was done in twin glass chamber previously saturated with the mobile phase Toluene: Ethyl acetate (8: 2v/v) for 15min.

Same sample was parallelly tested on HPLC using PDA detector and Methanol: ACN: Water (70:15:15 v/v/v) were used as mobile phase.

Method validation

Specificity

The specificity of the method was confirmed by analyzing marker karanjin and peak of karanjin in extract. The peak of karanjin detected in the extract was confirmed by comparing the Rf and spectra of the peak with that of marker karanjin at same Rf. The peak purity of karanjin was examined by comparing the spectra at peak start, peak middle and peak end positions of the bands.

Precision

The precision of the system was ascertained by verifying the instrumental precision, intra-day precision and inter-day precision. Instrumental precision was verified by replicate (n=6) applications of same karanjin solution. Intraday precision and inter- day precision was assessed by replicate (n = 6) applications of freshly prepared standard solution of same concentration (80ng/spot), on the same day and on 3 different days respectively.

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection and limit of quantification is the lowest concentration of analyte in a sample which can be detected and quantified with acceptable accuracy and precision. LOD and LOQ were estimated on the basis of signal to noise ratio. 80-400ng/spot concentrations of the standard karanjin were applied along with methanol as blank.

Linearity

The linearity range for karanjin was set as 80 to 400ng/ band. A stock solution (20 μ g/ml) of standard karanjin was prepared in methanol. The different volumes of stock solution 4, 8, 12, 16 and 20 μ l were spotted on the HPTLC plate to attain the concentration of 80, 160, 240, 320 and 400ng/ band,

respectively and for HPLC linearity range set to be 10-50 $\mu\text{g/ml}$.

Accuracy

Accuracy of the method were carried out to check recovery studies. The different amount of karanjin was added at different level i.e. 80, 100 and 120% and analysed from

Methanolic extract and result was analysed.

Results and Discussion

Extractive value

The Extractive value of Methanolic extract was found to be 3.2 % w/w.

UV spectrum for marker karanjin

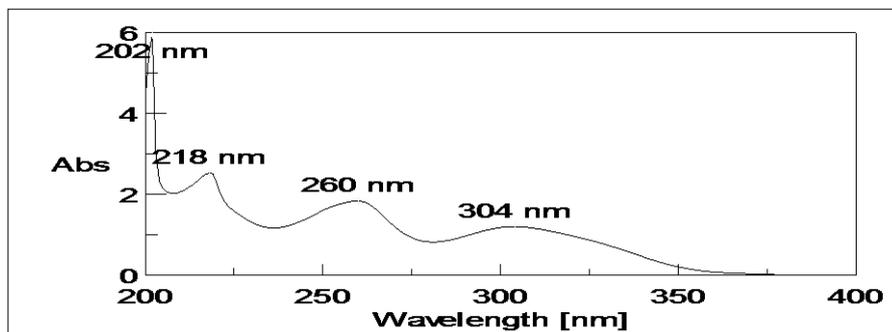


Fig 2: UV spectrum for marker karanjin (10ppm)

Development of the optimum mobile phase

Various mobile phases were tried for analysis of marker karanjin. HPTLC method was developed by various trial methods using different solvent systems according to their polarity. The solvent system that exhibits good resolution and

gives good chromatogram with sharp and symmetric peak was selected. The solvent system Toluene: Ethyl acetate (8:2 v/v) was found optimum for the studies and the Rf value of karanjin was found to be 0.67.

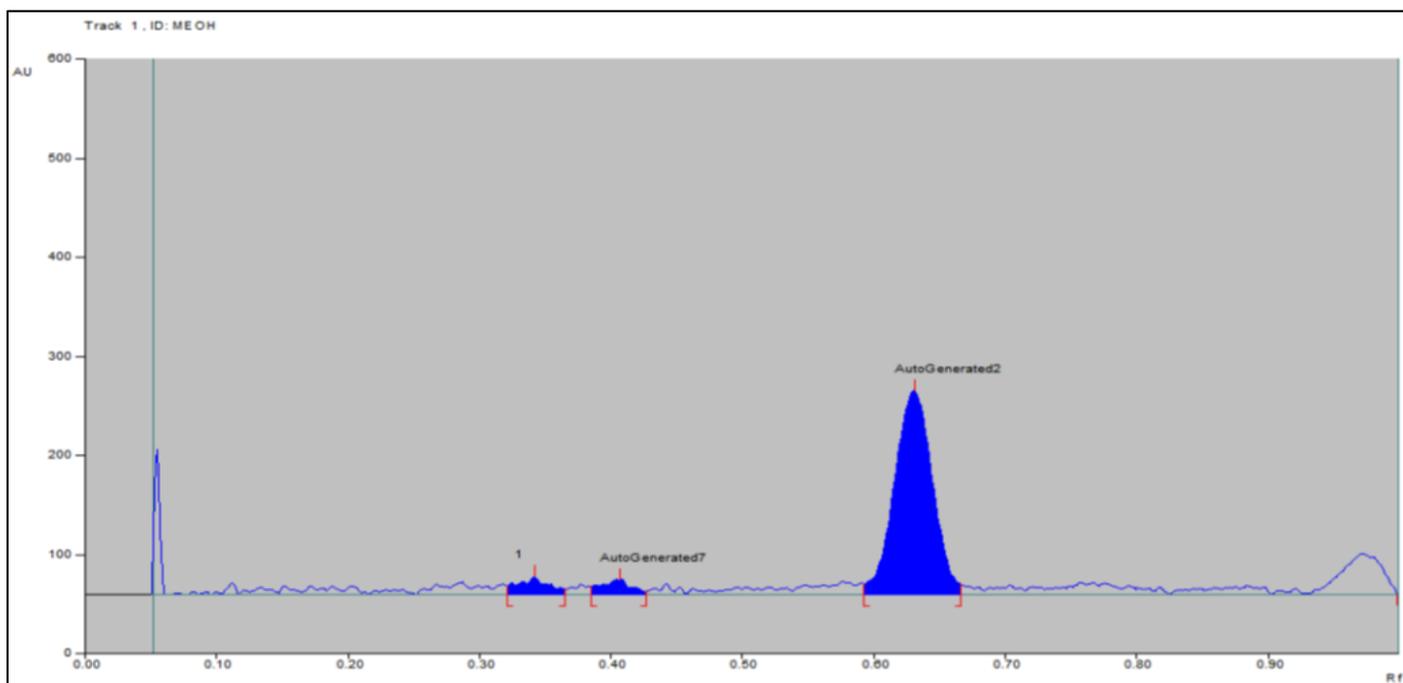


Fig 3: Densitogram of marker karanjin

HPTLC study of standard karanjin with extract of *Pongamia pinnata*

HPTLC study was carried out an extracts of *Pongamia pinnata*. Standard was quantified accurately using precoated HPTLC plates with silica gel F254 and the mobile phase used

was Toluene: ethyl acetate (8:2 v/v). The Rf value was found to be 0.67. The chromatograms of karanjin and Methanolic extract of *Pongamia pinnata* are shown in Figure 4. The Rf value of karanjin was compared with the Rf value of extract i.e 0.67. Percent karanjin obtained in extract 4.41% w/w.

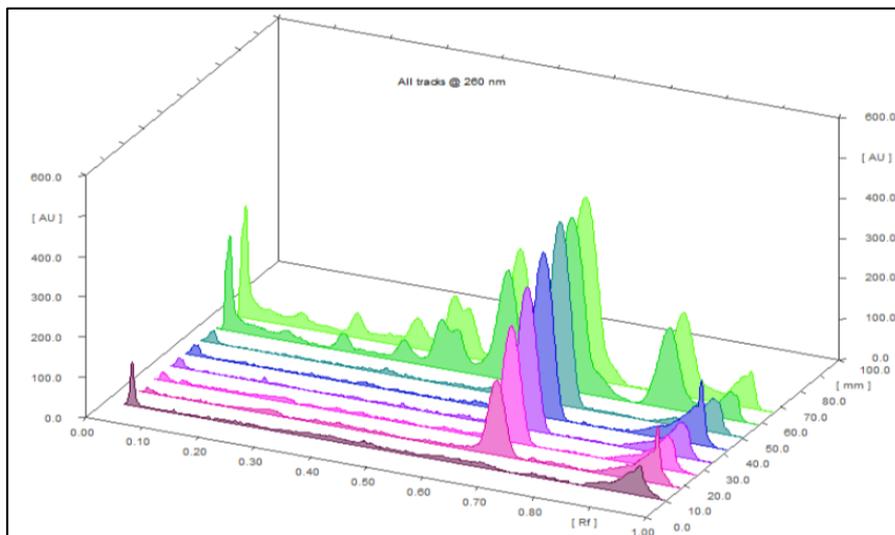


Fig 4: Densitogram of marker karanjin and Methanolic extract (Track 1: Methanol, Track 2: Marker 2-6, Track 3: Methanolic extract 7-8)

Forced degradation studies [9, 10]

Degradation were seen in HPLC and HPTLC for Acidic and Alkaline condition. No degradation seen in Neutral, Thermal and photolytic conditions for pure karanjin in HPLC as well as in HPTLC. Also there is no separate degradation peak were seen in any condition.

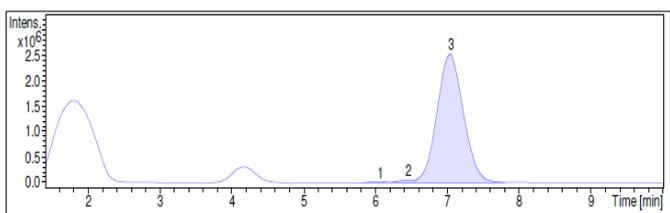
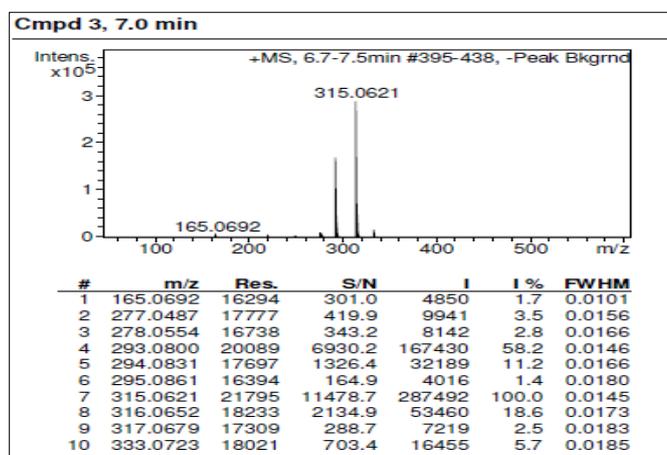


Fig 5: Chromatogram for standard karanjin

Acid hydrolytic condition

Degradation seen in terms of decrease in area, no additional peak is seen in densitogram in HPTLC and chromatogram in HPLC.

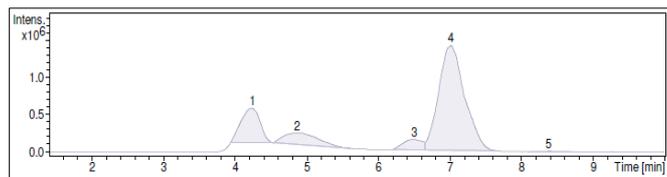
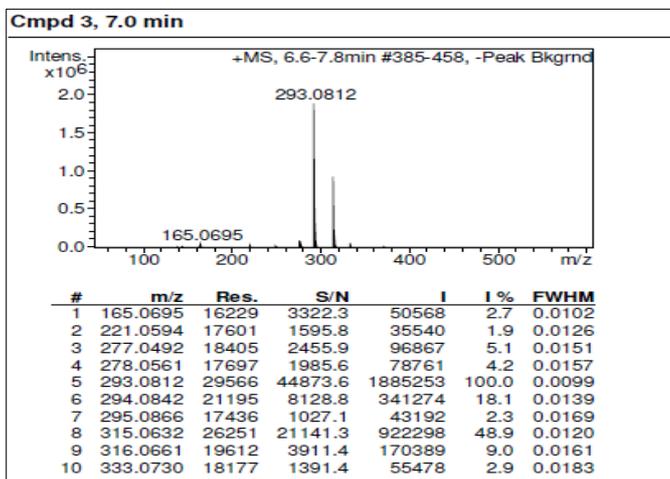
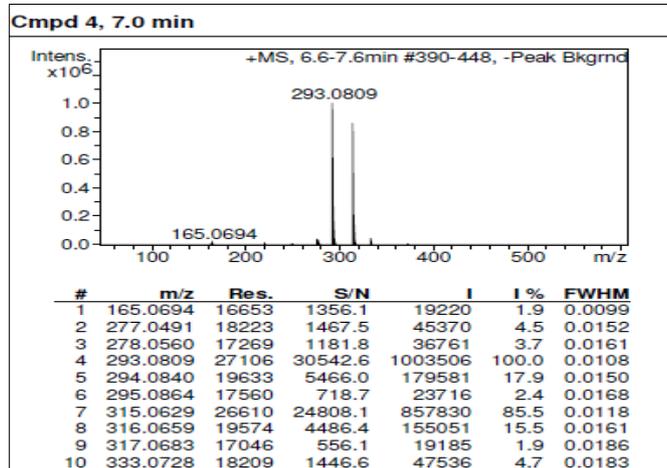


Fig 7: Chromatogram for acid hydrolysis



Alkali hydrolytic condition

Degradation seen in terms of decrease in area, no additional peak is seen in densitogram in HPTLC and chromatogram in HPLC.

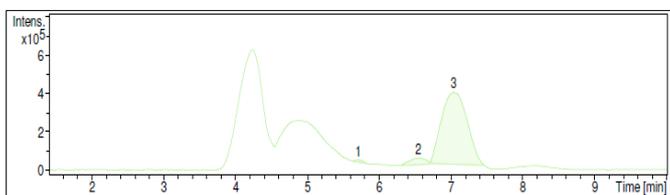


Fig 6: Chromatogram for alkali hydrolysis

Neutral hydrolytic condition

No Degradation is seen in chromatogram of HPLC and densitogram of HPTLC.

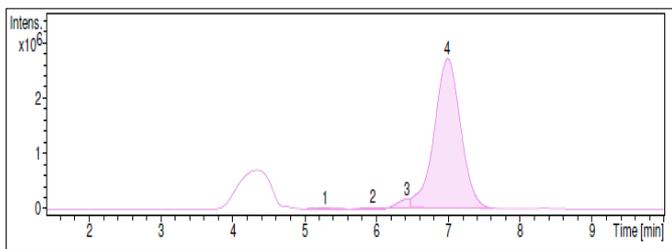
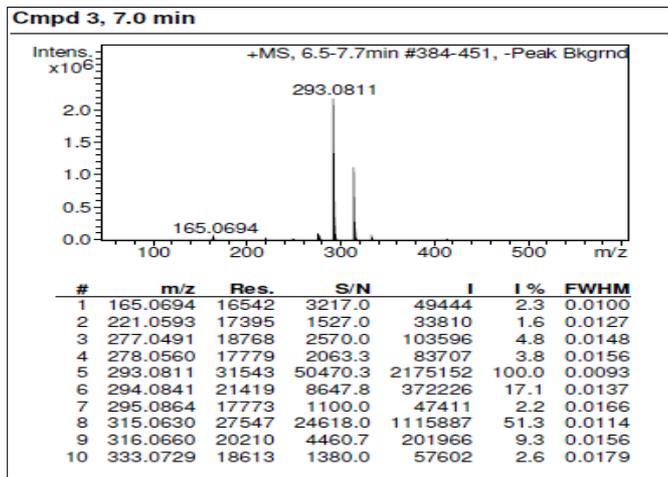


Fig 8: Chromatogram for Neutral hydrolysis



Thermal degradation

Densitogram of dry heat samples exhibited the no degradation products peaks for standard karanjin.

Table 1: Results of the degradation studies for standard karanjin.

Sr. no.	Parameters	Condition	Time	HPTLC
				Recovery
1	Alkali hydrolysis	2N NaOH at 60°C reflux	2hrs	85.72
2	Acid hydrolysis	2 N HCL at 60°C reflux	2hrs	97
3	Neutral hydrolysis	Distilled water at 60°C reflux	2hrs	103
3	Oxidative stress Degradation	30% H ₂ O ₂	overnight	101
4	Photolytic degradation	UV light cool white fluoro light	200 watt hours/ square meter 1.2 million lux hours	102
5	Dry heat degradation	Dry heat, hot air oven at 60°C	4hrs	103

Oxidative stress degradation

No degradation is seen in oxidation. No additional peak in densitogram of HPTLC and chromatogram of HPLC.

Photolytic degradation

No Degradation seen in photolytic condition. No additional peak in densitogram in HPTLC and chromatogram in HPLC.

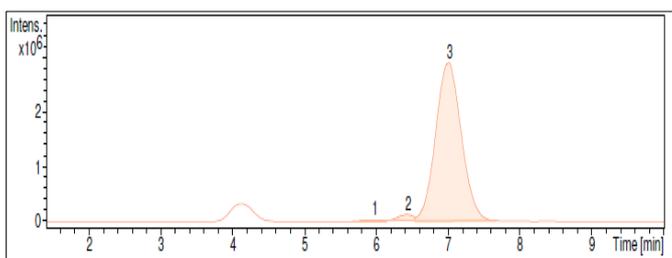


Fig 9: Chromatogram for Photolytic degradation

Validation of the method

Specificity

The specificity of the method was confirmed by analyzing marker karanjin and extracts of *Pongamia pinnata*. The spot for karanjin in the extract was validated by comparing the R_f and spectra of the spot with that of marker karanjin. The peak purity of karanjin was verified by comparing the spectra at peak start, peak middle and peak end position of the spot/bands.

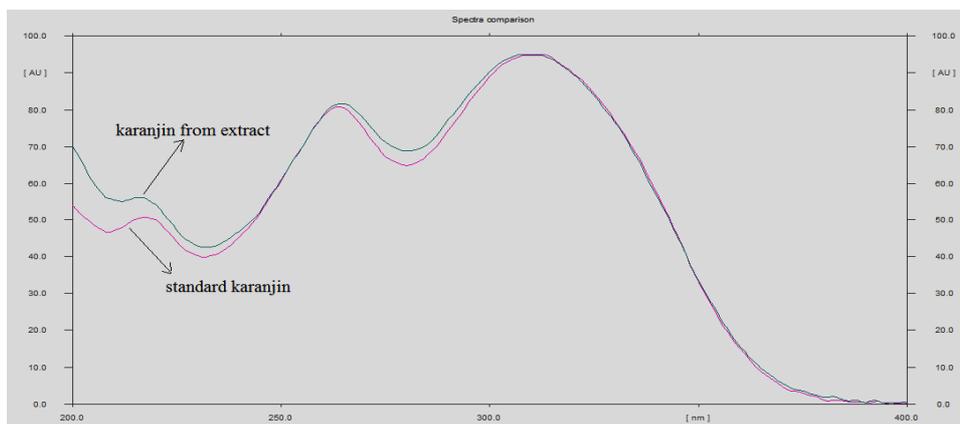


Fig 10: Overlay spectrum of standard karanjin over extract.

Linearity

The linear regression data for the calibration curves (n= 5) as shown in Table 1. Results showed a good linear relationship

over the concentration range 80-400ng/band with respect to peak height and peak area, slope 0.9975, $Y = 11.461x + 424.8$ (Figure 4).

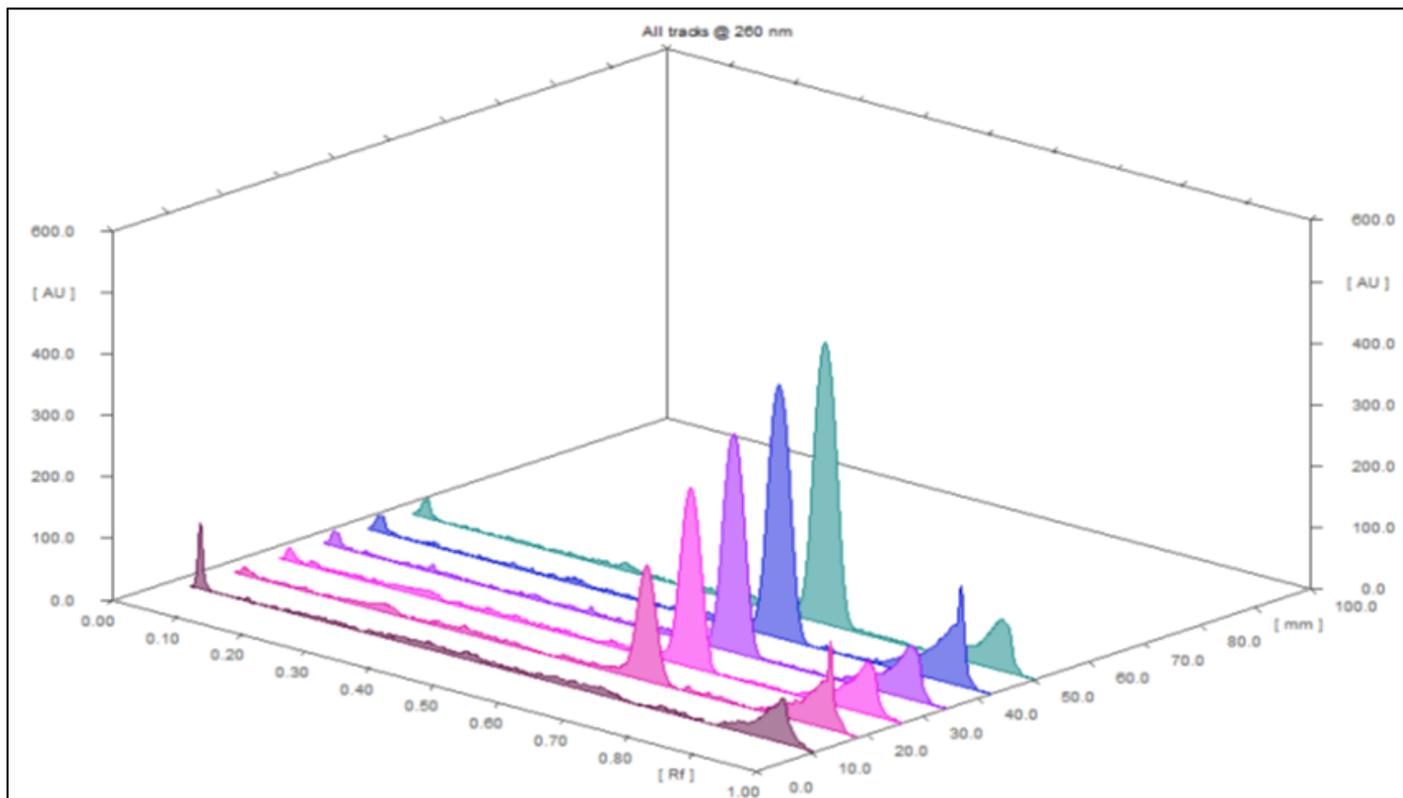


Fig 11: Chromatogram of standard karanjin

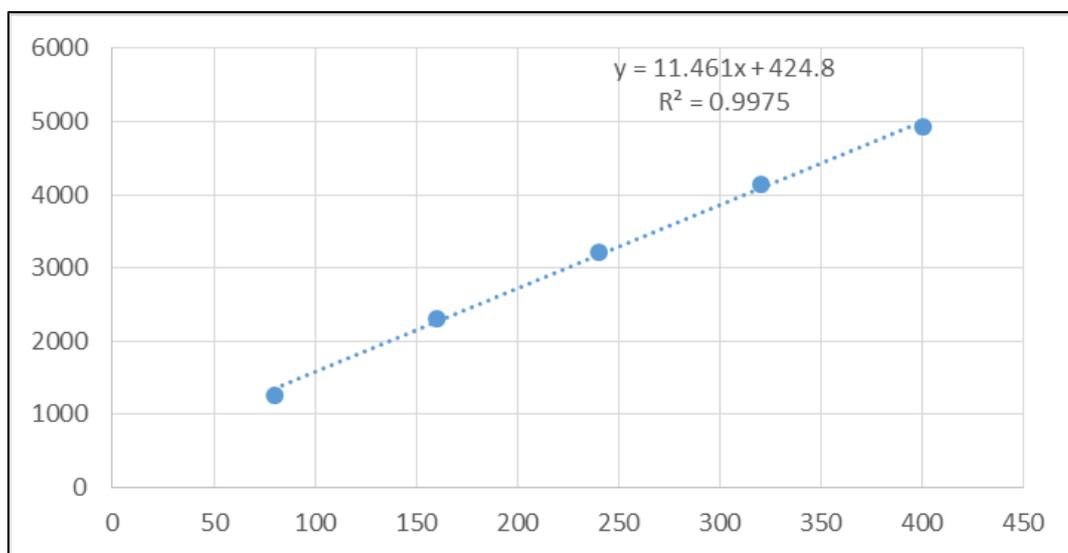


Fig 12: Calibration curve

Precision

The inter and intra- day precision of HPTLC method (n= 6) is shown in Table 2 results expressed in terms of %RSD,

which describes intra- and inter-day variation of karanjin at different concentration levels of 80ng/band (n= 6).

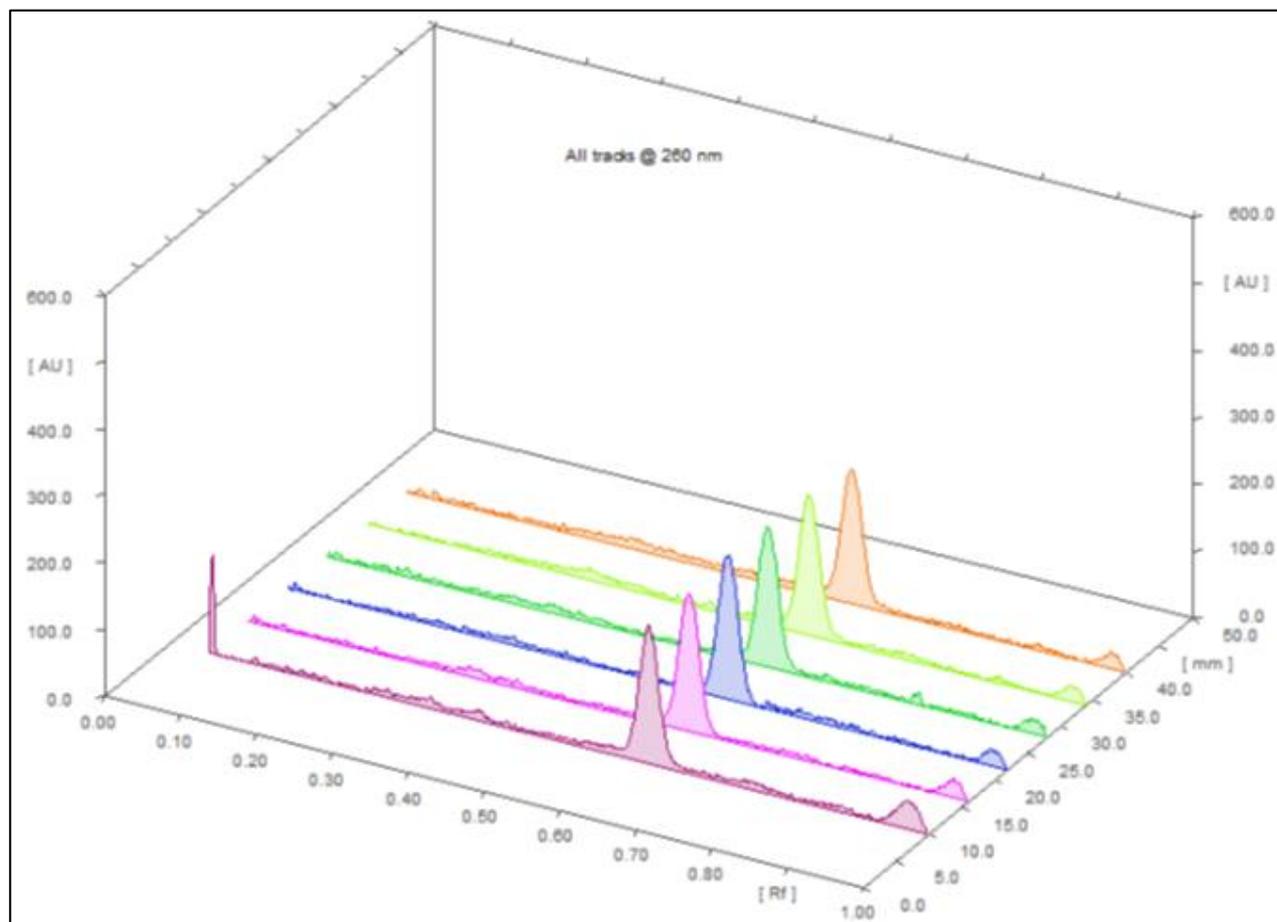


Fig 13: Precision (n=6)

Table 2: Inter and intra- day precision of HPTLC method (n=6).

Sr. No.	Amount ng/spot	Inter-day precision	SD	% RSD	Intra- day precision	SD	% RSD
1	80	5199.7	108.912	2.09	4458.24	79.33	1.73
2	80	5190.7			4553.45		
3	80	5398.6			4427.51		
4	80	5221.2			4524.38		
5	80	5169.2			4644.6		
6	80	5063.3			4575.85		

Limit of Detection (LOD) and limit of quantification (LOQ)

Detection limit and quantification limit were calculated using the formula $LOD = 3.3 \times \sigma/S$, $LOQ = 10 \times \sigma/S$ Where, σ = standard deviation of the response, S = slope of the calibration curve. LOD and LOQ were found to be 23.78 ng/band and 78.48 ng/band respectively, which shows the sufficient sensitivity of the method.

Table 3: Method performance parameters for validation of HPTLC protocol.

Parameters	Method
Selectivity	Selective
Specificity	Specific
Linear range (ng/band)	80-400
Correlation coefficient (r)	0.9975
Linear regression equation ($Y = mx + c$)	$y = 11.461x + 424.8$
LOD (ng/band)	23.78 ng/band
LOQ (ng/band)	78.48 ng/band
Interday (n=6)	2.09
Intraday (n=6)	1.73

Conclusion

The present study of stability indicating method for karanjin using HPLC and HPTLC method confirms that the developed methods were precise, specific, and accurate. HPTLC consumes less amount of solvents and therefore can be regarded as more economic and environment friendly. This work illustrates a simple, sensitive and robust HPTLC method for estimation of Karanjin. The proposed method met the ICH validation criteria such as linearity, ranges, precision and accuracy, specificity. Stability testing performs a significant role in the testing program for drug substances as the instability of the product affects the prime essential requisites, i.e. quality, efficacy and safety of any drug. The study results indicate that karanjin is prone to hydrolysis. Hence during formulation of Pongamia pinnata extract, pH may be maintained at neutral value to maintain marker stability.

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