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Research Article

ESTIMATION OF KARANJIN IN BARK AND SEED EXTRACT OF *PONGAMIA PINNATA* PLANT AND MARKETED KARANJIN OIL USING THIN-LAYER CHROMATOGRAPHY DENSITOMETRY

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ABSTRACT

Objectives: The present study focuses on the development and validation of a high-performance thin-layer chromatography (HPTLC) method for the quantification of karanjin in ethanolic extracts of bark and seeds of *Pongamia pinnata*, as well as in marketed karanjin oil. *P. pinnata*, a member of the Fabaceae family, is recognized for its traditional medicinal properties, primarily attributed to karanjin, a furanoflavonoid with notable biological activities.

Methods: The HPTLC method was optimized using a silica gel stationary phase and a mobile phase comprising toluene and ethyl acetate in a ratio of 8:2. Parameters such as specificity, linearity, limit of detection (LOD), and limit of quantification (LOQ) were assessed according to ICH Q2 (R1) guidelines.

Results: Karanjin showed good linearity over the range of 100–500 ng/band with a correlation coefficient (r^2>0.99). The LOD and LOQ were 3.15 ng/band and 9.56 ng/band, respectively. Precision studies demonstrated % relative standard deviation values <1% for both intra-day and inter-day analysis. The method showed high accuracy with recovery values ranging from 99.0% to 101.2%. Assay results indicated karanjin content of 3.05±0.02% w/w in bark extract, 30.55±0.30% w/w in seed extract, and 33.40±0.19% w/w in marketed oil.

Conclusion: The developed HPTLC method offers a rapid, sensitive, and reliable analytical approach for the estimation of karanjin, thereby facilitating its application in both pharmaceutical and agricultural contexts.

Keywords: *Pongamia pinnata,* High-performance thin-layer chromatography, Karanjin, Karanjin oil, Flavonoids.

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INTRODUCTION

Pongamia pinnata (Linn.) is a member of the Fabaceae family, which has long been used in traditional medicine to treat a wide range of illnesses in people [1]. One of the main phytoconstituents of *P. pinnata* is karanjin, which belongs to the furanoflavonol group of flavonoids that are mostly derived from the seeds of the karanja tree. Since its chemical structure includes a fused benzene and furan ring, karanjin is classified as benzofuran flavonoids [2]. The phytoconstituents of *P. pinnata* mostly consist of flavonoids and fixed inedible oils. The successful biological activities spectrum is potentially due to the inherent karanjin content in *P. pinnata* [3], which also has agricultural applications, such as in making biodiesel [4,5].

Karanjin (3-Methoxy-2-phenyl-4*H*-furo[2,3-h]chromen-4-one) (Fig. 1) easily crystallizes into a colorless needle-shaped monoclinic crystal at 158°C. Furanoflavonoid' polyphenol backbone enhances its susceptibility to environmental changes, modifying its solubility and spectroscopic characteristics to modulate biological activity [3,6].

The photophysical and photochemical behavior of furanoflavonoid is greatly affected by the addition of a methyl group [3,7]. Similar to this, the structure of karanjin and its functional groups facilitate solubility in a variety of solvents, including benzene, dimethyl sulfoxide, ethanol, methanol and petroleum ether, in addition to imparting hydrophobicity and semi-polarity. Karanjin is characterized by a benzoyl and cinnamoyl backbone with a furan ring and methoxy substituent groups. It is a member of the flavones class of flavonoid compounds [3,8,9]. Two primary absorption bands, associated with cinnamoyl and benzoyl, respectively, at 261 nm (Band I) and 309 nm (Band II), in the ultraviolet (UV)-visible absorption data obtained from karanjin, support the existence of these moieties [3,8]. Furthermore, it has been shown that the molar extinction coefficients (ε) of karanjin are 7380 $M^{-1}cm^{-1}$ [3,10]. In addition, depending on the parameters of the solubilizing medium, Karanjin displays solvatochromic shift features. Karanjin's inherent UV-visible absorption and fluorescence characteristics may therefore be used as its own "signature" to describe the solute-solvent interactions. This would be quite helpful in comprehending and offering preliminary insights into further possible uses of karanjin [3,11].

Several techniques have been described for the analysis of karanjin in herbal products, such as high-performance liquid chromatography combined with UV light (HPLC-UV) [12-17] as well as tandem

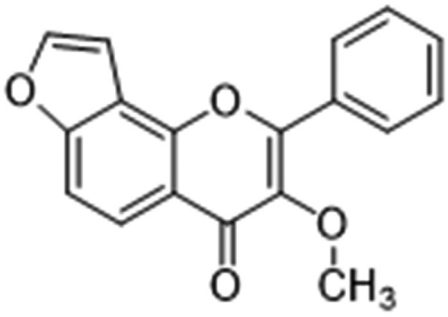

Fig. 1: Chemical structure of karanjin

mass spectrometry–ultra-performance liquid chromatography (UPLC–MS/MS) detection [18]. However, long chromatographic run times, low sensitivity, and poor specificity have been the limitations of earlier HPLC-UV techniques. Fan *et al*. [18] carried out UPLC-MS/MS to characterise and concurrently quantify five flavonoids, including karanjin, in various *Fordia cauliflora* sections; still, this technique is not appropriate for methodological investigations as it necessitates a gradient mobile phase with a somewhat lengthy run time (11 min) [19,20].

The experimental process for isolating and purifying karangin from *P. pinnata* seed oil was presented by Vismaya *et al*. [21]. Following their study, *P. pinnata* seed oil was subjected to liquid-liquid extraction with methanol in the ratio of 1:2 (v/v). Then, the extraction was repeated thrice for a total of 96 h. To produce pure karanjin, the separated methanolic extracts were concentrated and then put through HPLC chromatography with methanol serving as an eluent. Petroleum ether almost renders karanjin insoluble, although it is soluble in benzene, ether, chloroform, acetone, and alcohol [3,5,21].

METHODS

Instruments/equipment/apparatus

High-performance thin-layer chromatography (HPTLC) analysis was performed using a Camag HPTLC system comprising a Linomat V semi-automatic sample applicator (Camag, Muttenz, Switzerland), twin-trough development chamber (Camag, Switzerland), and thin-layer chromatography (TLC) Scanner 3 equipped with WinCATS software version 1.5.3 (Camag, Switzerland). Chromatographic separation was carried out on precoated silica gel 60 F254 aluminium plates (Merck, Darmstadt, Germany). UV spectral analysis was performed using a UV–Visible spectrophotometer (Shimadzu UV-1780, Shimadzu Corporation, Japan). Sample weighing was carried out using an analytical balance (Shimadzu, Japan), and plate activation was done in a hot air oven (Labline Instruments, India).

HPTLC studies were done for the estimation of karanjin in the ethanolic extract of bark and seed, and marketed karanjin oil.

Chemicals and reagents

The standard Karanjin marker was procured from YUCCA enterprises, Pune. Marketed oil was procured from Wellness Medical, Pune. The other chemicals used in the study were of analytical grade.

Chromatographic conditions

Chromatographic separation was performed on 10×10 cm Merck aluminum-backed plates pre-coated with a 250 µm layer of silica gel 60 F254. The TLC plates were prewashed with methanol and dried in an oven at 110°C for 30 min. Samples were spotted on a TLC plate 10 mm from the bottom edge by Linomat V Sample semi-automatic applicator with the following parameters: band width 6 mm, track distance 5 mm; application rate of 0.1 µL/s. The TLC plate was developed in twin trough chamber using toluene: ethyl acetate in the ratio 8:2 as mobile phase at ambient temperature (25°C), chamber saturation time 15 min, and 80 mm migration distance, The TLC plate was scanned and analyzed by TLC Scanner 3 using wincat software version 1.5.3 using having parameters: 5×0.45 slit dimension; 20 mm per second (mm/s) scanning speed; 300 nm detection wavelength; quantity of mobile phase: 10 mL; TLC chamber saturation: 15 min; Migration distance: 80 mm.

Table 1: System suitability parameters

Drug	Concentration (ng/band)	R_f	Area	Asymmetry
Karanjin	200	0.78±0.148	8156.2	1.09

Values are expressed as mean±standard deviation, n=3

Table 2: Linearity data for Karanjin marker

Sr. No.	Concentration (ng/band)	Area 1	Area 2	Area 3	Area 4	Area 5	Average	SD	% RSD
1	100	5344.3	5339.0	5403.0	5349.6	5344.3	5356.044	23.732	0.443
2	200	8067.6	8059.5	8156.2	8148.3	8067.6	8099.849	42.969	0.530
3	300	10721.5	10710.8	10839.3	10732.2	10925.0	10785.760	83.512	0.774
4	400	13314.4	13287.8	13261.2	13234.7	13208.2	13261.249	37.546	0.283
5	500	15132.5	15117.4	15298.8	15268.7	15132.5	15189.964	77.349	0.509

Values are expressed as mean±standard deviation, n=5. RSD: Relative standard deviation, SD: Standard deviation

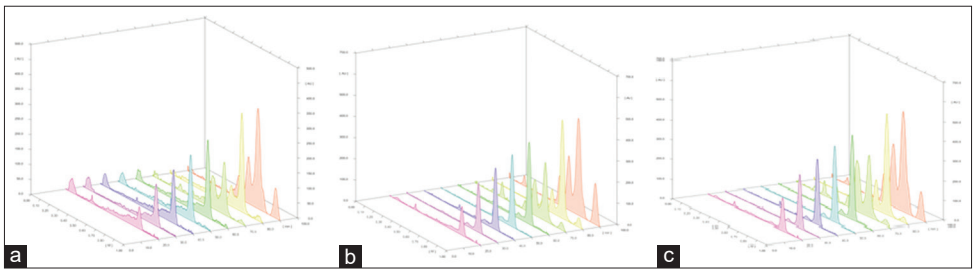

Fig. 2: 3-D Densitogram of high-performance thin-layer chromatography plate at (a) 215 nm, (b) 266 nm and (c) 300 nm

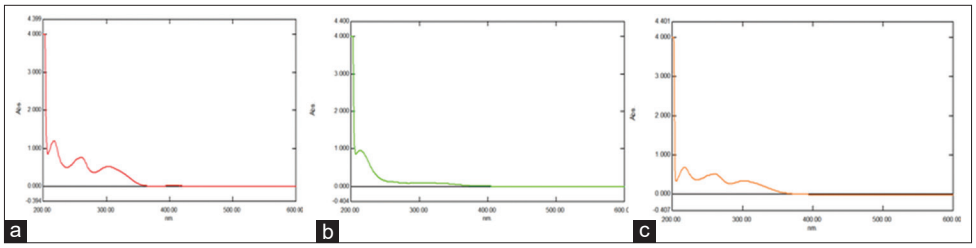

Fig. 3: Ultraviolet spectrum of (a) Karanjin marker, (b) Bark extract and (c) Seed extract respectively

Dilutions for HPTLC studies

1) Standard Karanjin marker was weighed (10 mg) and dissolved in 10 mL of ethanol to make a concentration of 1000 ppm. Then, 1 mL of this solution was diluted to 10 mL of ethanol to obtain a concentration of 100 ppm.
2) Semi-solid bark extract was weighed (10 mg) and dissolved in 10 mL of ethanol.
3) Semi-solid seed extract was weighed (10 mg) and dissolved in 10 mL of ethanol.
4) Marketed oil was weighed (10 mg) and diluted in 10 mL n-hexane.

HPTLC studies were carried out using precoated HPTLC plates with silica gel G60 F_{254}. Nine spots of blank, Karanjin marker, bark extract, seed extract and marketed oil were marked on the plates. Mobile phase used was toluene: Ethyl acetate in the ratio 8:2. Various trials were taken with different volumes of applications, and the final plate was selected with the concentrations and volume of application as mentioned below:

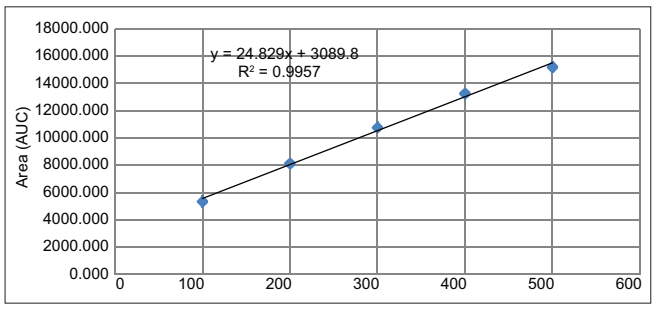

Fig. 4: Linearity curve for karanjin Concentration (ng/band)

Table 3: LOD and LOQ

	SD of Y-intercept	Slope (m)	ng/band
LOD	25.979	24.829	3.453
LOQ	25.979	24.829	10.463
	SD of lowest response	**Slope (m)**	**ng/band**
LOD	23.732	24.829	3.154
LOQ	23.732	24.829	9.558

Sample application

- Track 1: Blank (Ethanol) 2 µL
- Track 2: Karanjin marker 100 ppm 1 µL (100 ng/band)
- Track 3: Karanjin marker 100 ppm 2 µL (200 ng/band)
- Track 4: Karanjin marker 100 ppm 3 µL (300 ng/band)
- Track 5: Karanjin marker 100 ppm 4 µL (400 ng/band)
- Track 6: Karanjin marker 100 ppm 5 µL (500 ng/band)
- Track 7: Bark extract 1000 ppm 10 µL
- Track 8: Seed extract 1000 ppm 2 µL
- Track 9: Marketed oil 1000 ppm 2 µL.

Plates were observed in the UV chamber under short wavelength, long wavelength and visible light.

As shown in Fig. 2, a 3D image of the densitogram of the HPTLC plate was given, which was observed at 215, 266 and 300 nm. In this image, the sample application is done as per the given sample application steps. In this image, on track 1 as a blank solution, no peak was observed. On tracks 2, 3, 4, 5 and 6, the Karanjin marker was applied and shows enhancement in the peak at each wavelength as per the concentration. On tracks 7, 8 and 9, bark, seed and marketed oil samples were applied respectively, and it is clearly seen that the peak of bark extract is lower than that of the seed and marketed oil. The peak of the seed is almost similar to the marketed oil. Hence, the seed contains a higher amount of karanjin than the bark.

System suitability parameters

System suitability was performed by injecting the Karanjin marker, which was prepared by dissolving 10 mg in 10 mL of ethanol. Then, 1 mL of this solution was diluted to 10 mL of ethanol to obtain a concentration of 100 ppm. HPTLC was performed on silica gel plates using toluene: Ethyl acetate (8:2) as the mobile phase. Samples were applied in various volumes of Karanjin marker (100 ppm) in amounts ranging from 1 µL to 5 µL, and analyzing system performance parameters like R_f value, area, and asymmetry (Table 1).

Validation of analytical method

The method was validated as per ICH Q2 (R1) guidelines [22-24].

Statistical analysis

All experimental measurements were performed in replicate (n=3–6), and the results were expressed as mean±standard deviation (SD). Statistical analysis of linearity, precision, accuracy, robustness, and

Table 4: Intra-day and Inter-day precision studies data

Sr. No.	Theoretical concentration (ng/band)	Area (AUC)	Practical concentration (ng/band)	% Recovery	Mean	SD	% RSD
Precision (Intra-day)							
1	200	8132.1	203.081	101.541	101.163	0.276	0.272
2	200	8099.8	201.780	100.890			
3	200	8108.2	202.118	101.059			
4	400	13015.3	399.754	99.939	99.962	0.215	0.215
5	400	12992.7	398.844	99.711			
6	400	13044.8	400.942	100.236			
7	500	15477.5	498.921	99.784	100.119	0.346	0.345
8	500	15578.2	502.976	100.595			
9	500	15501.6	499.891	99.978			
Precision (Inter-day)							
1	200	8091.4	201.443	100.722	100.346	0.274	0.273
2	200	8059.3	200.149	100.075			
3	200	8067.7	200.486	100.243			
4	400	13002.3	399.230	99.808	99.961	0.121	0.121
5	400	13018.7	399.891	99.973			
6	400	13031.8	400.417	100.104			
7	500	15454.3	497.986	99.597	99.703	0.639	0.640
8	500	15570.4	502.663	100.533			
9	500	15377.6	494.897	98.979			

Values are expressed as mean±standard deviation, n=3. AUC: Area under the curve, RSD: Relative standard deviation, SD: Standard deviation

Table 5: Assay (% content analysis)

Sr. No.	Area (Y)	Concentration ng (x)	Volume 1 mL	Volume 100 mg (%)	Avg	SD	% RSD
	Bark extract	10 µL					
1	10647.5	304.390	30438.999	3.044	3.047	0.023	0.761
2	10700.7	306.534	30653.415	3.065			
3	10626.2	303.532	30353.232	3.035			
4	10551.7	300.530	30053.049	3.005			
5	10668.8	305.248	30524.765	3.052			
6	10732.7	307.821	30782.065	3.078			
	Seed Extract	2 µL					
1	18146.2	606.404	303201.881	30.320	30.552	0.299	0.978
2	18418.4	617.366	308683.233	30.868			
3	18109.9	604.942	302471.034	30.247			
4	18509.1	621.021	310510.351	31.051			
5	18182.5	607.865	303932.728	30.393			
6	18200.6	608.596	304298.151	30.430			
	Marketed oil	2 µL					
1	19650	666.970	333485.018	33.349	33.401	0.185	0.554
2	19846.5	674.884	337442.084	33.744			
3	19610.7	665.387	332693.604	33.269			
4	19551.8	663.013	331506.484	33.151			
5	19689.3	668.553	334276.431	33.428			
6	19709.0	669.344	334672.137	33.467			

Values are expressed as mean±standard deviation (SD), n=6. RSD: Relative standard deviation, SD: Standard deviation

Table 6: Accuracy data

Sr. No.	Area (Y)	Slope (m)	Y-intercept (c)	Concentration X (ng)	Sample concentration from extract	Recovered conc.	TC	% Recovery	Avg.	SD	%RSD
Bark extract (Volume applied-10 µL)											
1	14371.1	24.829	3089.801	454.360	304.676	149.684	150	99.790	100.176	0.386	0.386
2	14385.5	24.829	3089.801	454.940	304.676	150.264	150	100.176			
3	14399.9	24.829	3089.801	455.519	304.676	150.843	150	100.562			
4	18125.8	24.829	3089.801	605.581	304.676	300.905	300	100.302	100.512	0.304	0.303
5	18131.2	24.829	3089.801	605.800	304.676	301.124	300	100.375			
6	18167.5	24.829	3089.801	607.260	304.676	302.584	300	100.861			
7	21716.8	24.829	3089.801	750.210	304.676	445.534	450	99.008	99.267	0.297	0.299
8	21738.5	24.829	3089.801	751.085	304.676	446.409	450	99.202			
9	21782.0	24.829	3089.801	752.836	304.676	448.160	450	99.591			
Seed extract (Volume applied-2 µL)											
1	25709.7	24.829	3089.801	911.026	611.032	299.994	300	99.998	100.343	0.346	0.344
2	25735.4	24.829	3089.801	912.062	611.032	301.030	300	100.343			
3	25761.1	24.829	3089.801	913.099	611.032	302.067	300	100.689			
4	33265.2	24.829	3089.801	1215.328	611.032	604.296	600	100.716	100.880	0.265	0.262
5	33268.5	24.829	3089.801	1215.462	611.032	604.430	600	100.738			
6	33335.0	24.829	3089.801	1218.142	611.032	607.110	600	101.185			
7	40496.6	24.829	3089.801	1506.575	611.032	895.543	900	99.505	99.807	0.319	0.319
8	40557.4	24.829	3089.801	1509.026	611.032	897.994	900	99.777			
9	40638.5	24.829	3089.801	1512.293	611.032	901.261	900	100.140			
Marketed oil (volume applied-2 µL)											
1	27067.6	24.829	3089.801	965.718	668.025	297.693	300	99.231	99.534	0.278	0.279
2	27094.7	24.829	3089.801	966.809	668.025	298.784	300	99.595			
3	27108.2	24.829	3089.801	967.355	668.025	299.330	300	99.777			
4	34565.9	24.829	3089.801	1267.717	668.025	599.692	600	99.949	100.119	0.275	0.275
5	34569.4	24.829	3089.801	1267.856	668.025	599.831	600	99.972			
6	34638.5	24.829	3089.801	1270.641	668.025	602.616	600	100.436			
7	42124.3	24.829	3089.801	1572.134	668.025	904.109	900	100.457	100.708	0.238	0.236
8	42187.6	24.829	3089.801	1574.683	668.025	906.658	900	100.740			
9	42229.8	24.829	3089.801	1576.382	668.025	908.357	900	100.929			

Values are expressed as mean±standard deviation, n=3 at each recovery level (50%, 100%, and 150%). RSD: Relative standard deviation, SD: Standard deviation

assay studies was carried out using Microsoft Excel and WinCATS software (version 1.5.3). Linearity was evaluated by least-squares linear regression analysis, and correlation coefficients (r^2) were calculated. Precision was expressed as percentage relative standard deviation (%RSD) for intra-day and inter-day studies. Accuracy was assessed by recovery studies at 50%, 100%, and 150% levels. A %RSD value <2% was considered statistically acceptable, confirming the reliability and repeatability of the method.

Selection of wavelength

As shown in Fig. 3 UV spectra of the marker, ethanolic extract of bark and seed were recorded using a UV spectrophotometer (SHIMADZU 1780). The standard marker, bark extract and seed extract were diluted using ethanol to 10 ppm. The wavelength range selected was 200–600 nm.

The estimation of seed extract, bark extract and marketed oil was done in comparison with the standard karanjin marker using HPTLC. The tracks were scanned at 215 nm, 266 nm, and 300 nm [25].

Table 7: Robustness data

1. Saturation time (15 min±1 min)			
Concentration (ng/band)	**18 min**	**20 min**	**22 min**
200	8023.6	8072.5	8549.3
200	7995.0	8333.3	8650.3
200	8059.0	8073.9	8550.9
Avg	8025.852	8159.918	8583.498
SD	32.039	150.182	57.854
%RSD	0.399	1.840	0.674
2. Time from spotting to development			
Concentration (ng/band)	**immediate**	**30 min**	**1 h**
200	8099.0	7992.9	8150.5
200	8037.2	8114.5	8110.8
200	8051.9	7943.7	8020.7
Avg	8062.719	8017.013	8093.996
SD	32.316	87.908	66.544
%RSD	0.401	1.097	0.822
3. Time from development to scanning			
Concentration (ng/band)	**immediate**	**30 min**	**1 h**
200	8098.8	7889.0	7985.6
200	8235.5	8044.4	8061.8
200	8139.1	8017.4	8035.5
Avg	8157.810	7983.580	8027.62
SD	70.261	83.042	38.73
%RSD	0.861	1.040	0.482
4. Wavelength Change (300±1 nm)			
Concentration (ng/band)	**299 nm**	**300 nm**	**301 nm**
200	7905.8	8136.0	8718.0
200	7944.2	8172.1	8549.4
200	8053.7	8252.3	8582.6
Avg	7967.885	8186.783	8616.674
SD	76.778	59.531	89.276
%RSD	0.964	0.727	1.036

Values are expressed as mean±standard deviation, n=3. RSD: Relative standard deviation, SD: Standard deviation

Specificity

The specificity of the method was ascertained by applying a blank (ethanol) along with the marker on the same plate. No extra peak was observed at R_f value of the marker, showing the specificity of method [26-28].

Linearity and range

From the standard stock solution (1000 µg/mL) of the marker, a dilute solution was prepared containing 100 µg/mL. This solution was further used for a band application. Five replicates per concentration were spotted. The linearity (relationship between peak area and concentration) was determined by analyzing six concentrations over the concentration range of 100–500 ng/band [25,29,30]. The peak area was plotted against the corresponding concentrations to obtain the calibration curve. The linearity data of the marker is shown in Table 2, and the calibration curve is shown in Fig. 4.

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

LOD

The limits of detection (Table 3) of the developed method were calculated from the standard deviation of the intercepts and slope of the calibration curves of karanjin marker using the equation [25].

$$\text{Detection limit} = 3.3 \times \sigma / S$$

σ=Standard deviation of the response (y- intercept or lowest response)
S=Slope of the calibration curve.

LOQ

The limits of quantitation (Table 3) of the developed method were calculated from the standard deviation of the intercepts and slope of the calibration curves of karanjin marker using the equation [25].

$$\text{Quantification limit} = 10 \times \sigma / S$$

σ=Standard deviation of the response (y- intercept or lowest response)
S=Slope of the calibration curve.

Precision [25,26]

The precision of the method was demonstrated by intra-day and inter-day variation studies. In the intra-day studies, 3 replicates of 3 concentrations were analyzed on the same day, and percentage RSD was calculated. For the inter-day variation studies, 1 replicate of 3 concentrations was analyzed on 3 consecutive days and the percentage RSD was calculated. The results obtained are shown in Table 4.

Assay (% content analysis)

Ten mg of semi-solid extract of bark and seed were weighed and dissolved in 10 mL of ethanol. Volume applied was 10 µL and 2 µL, respectively. Similarly 10 mg of Marketed oil was weighed and diluted in 10 mL of n-hexane. Volume applied was 2 µL. The results of % content of analysis are shown in Table 5. Calculations were done using a calibration curve.

Accuracy

The accuracy of the method was evaluated through recovery studies, where the extract solution was spiked with a standard marker at three levels – 50%, 100%, and 150% relative to the estimated sample concentration. The percentage recovery was calculated using the linearity equation, and the results are presented in Table 6 [23].

Robustness

Robustness of the method was determined by carrying out the analysis under conditions during which detection wavelength, saturation time and time were changed from spotting to development and development to scanning. The effect on the area was noted. The method was found to be robust. The results obtained are shown in Table 7.

CONCLUSION

The present work reports the estimation of the percentage of karanjin in the ethanolic extract of bark, seed and marketed oil. Till date, there is no study reported for the estimation of karanjin in the ethanolic extract of bark and seed. The developed HPTLC method was found to be fast, simple, precise, specific and accurate. Statistical analysis proved that the method is repeatable and selective for the determination of Karanjin. Data generated from the current work can be useful for future investigations on karanjin for different therapeutic uses and for justifying the dosage and route of administration of karanjin-containing formulations.

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AUTHOR CONTRIBUTIONS

ASS performed the experimental work and drafted the manuscript. N K assisted in method development and data analysis. G S conceptualized and supervised the study and critically revised the manuscript. RK contributed to analytical support and manuscript editing.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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USE OF AI TOOL

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