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Bioavailability of karanjin from *Pongamia pinnata* L. in Sprague dawley rats using validated RP-HPLC method

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ABSTRACT

Pongamia pinnata L. (fabaceae) is a small evergreen tree reported in traditional literature as 'Karanj'. Seeds of this plant have been used in the management of skin diseases, ulcer, piles, bronchitis; leprosy etc. Seeds are reported to possess phytoconstituents like pongapin, pongamol, pongaglabrone, kanjone, karanjin etc. The therapeutic activities of seeds are mainly attributed to its major furanoflavone karanjin. Thus, in the present research work the pharmacokinetics of karanjin was determined in Sprague dawley female rats using a validated RP-HPLC method from the ethanolic extract of *P. pinnata* (*p.o.*). The pharmacokinetic parameters were calculated using non compartmental model of analysis. Findings of the present work would be applicable for the future investigation on *P. pinnata* and karanjin for their different therapeutic uses and would be useful for justifying their dosage and route of administration from their allied formulations.

INTRODUCTION

Pongamia pinnata L. Pierre (fabaceae; synonym *Pongamia glabra* Vent.), is a commonly used plant in traditional Indian medicinal systems. All parts of *P. pinnata* are used therapeutically for treating tumors, piles, skin diseases, wounds and ulcers (Tanaka et al., 1992). Leaves are reported to be efficacious in treating inflammation (Shrinivasan et al., 2001). Seeds are reported in Ayurveda and Siddha systems of medicine for treating bronchitis, chronic fever and rheumatism (Muthu et al., 2006). Extracts of seeds have been reported to possess anti-inflammatory (Singh and Pandey, 1996), anti-oxidative, analgesic, hypoglycaemic and anti-ulcerogenic properties (Dahanukar et al., 2000). Seeds are reported as anthelmintic, and good in treatment of leprosy, piles, ulcers, chronic fever and in liver pain (Warrier et al., 1995). They are useful in rheumatism arthritis and scabies (Prasad and Reshmi, 2003) and to treat dermatitis of domestic animals (Sridhar, 2009). Karanjin, a major bioactive furanoflavone of *P. pinnata* seeds is reported as antioxidant, H+, K+ - ATPase inhibitor (Vismaya et.al., 2010) and showed anti-inflammatory activity in rats by inhibiting Lipoxygenase- 1 and 5- Lox (Sapna et al., 2007). The present research work was aimed to develop and validate a RP – HPLC method for determination of karanjin in rat plasma from ethanolic extracts of *P. pinnata* seeds (*p.o.*).

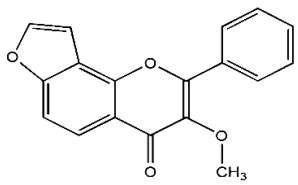

Fig. 1: Structure of karanjin.

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade acetonitrile was procured from Merck (Mumbai, India) while karanjin (Figure 1) and galangin from Sigma chemicals (Mumbai, India). *Raughan-e-Karanj*; an oil based formulation of *P. pinnata* seeds was purchased from Dawabazar (Mumbai, India). All the other chemicals used were of analytical grade.

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Plant materials and extract preparation

P. pinnata seeds (dried and mature) were collected from Lakhangaon (Pune, India). The plant material was authenticated by Botanical Survey of India, Pune (Authentication no. BB789502). The sample was oven dried for three days, powdered, sieved through BSS sieve (80 mesh) and stored in air tight container. Ethanolic extract of *P. pinnata* seeds was prepared by mixing 5 g of the accurately weighed powdered drug in 50 mL ethanol and kept on shaker overnight followed by filtration using Whatman filter paper (No. 1). The filtrate was evaporated under reduced pressure (yield 22.21 %).

Instruments and chromatography conditions

Jasco's HPLC system with pneumatic (PU-980) pump, auto sampler (AS 2057), and fixed wavelength UV detector were used for the HPLC analysis. The chromatographic separation was achieved on Cosmosil® C_{18} column (150 x 4.6 mm, 5 µm) using acetonitrile: 10 mM KH_2PO_4 (pH-4, adjusted with dilute glacial acetic acid), 50: 50 v/v delivered at flow rate of 1.0 mL/min. Sample injection volume was 30 µL, detection wavelength was 219 nm and run length was 18 min. Data was acquired and processed by Borwin PDA and Borwin chromatography software.

Animal procurement and maintenance

Female Sprague dawley rats, aged 10-12 weeks, weighing 200-250 g were procured from Haffkine biopharmaceuticals (Mumbai, India). All animals were housed at Animal Testing Centre (CPCSEA/315) with standard conditions of temperature (22 ± 5ºC) and humidity (65 ± 10%) and 12 h light dark cycles. Animals were given free access to standard laboratory food (Amrut Feeds, Chakan) and potable water. All animals were acclimatized for at least one week prior to experimentation.

Preparation of standard and calibration samples

Stock solution of karanjin (970 µg/mL) and galanjin (1000 µg/mL, internal standard i.e. I.S.) were prepared in methanol. Working karanjin solutions of 0.02, 0.05, 0.15, 0.49, 0.97, 1.95 and 2.92 µg/mL were prepared by appropriately diluting the stock solutions. Galanjin working solution of 10 µg/ mL was prepared by 1: 100 dilution of galangin stock solution.

Determination of karanjin from *P. pinnata* seeds and *Raughan-e-Karanj*

Prior to the pharmacokinetic evaluation of karanjin from ethanolic extract of *P. pinnata* seeds; its karanjin content was determined using the regression equation obtained from the seven point calibration curve of standard karanjin. As an application of the method *Raughan-e-Karanj* was also subjected to determination of its karanjin content.

Method validation

Selectivity, LOD and LOQ

To evaluate selectivity, five independent rat plasma were analyzed by comparing with the plasma spiked-analytes for excluding the endogenous material interference. Quantification was based on I.S. method of plotting peak areas to ratios of analyte/I.S. versus the concentration of the sample with a weighting factor 1, the calibration curves were reduplicated five times. The LOD was considered as the final concentration that produced a signal to noise (S/N) ratio 3 and LOQ as the final concentration that produced signal to noise (S/N) ratio 10 (CDER, 1994).

Linearity and quantification

Calibration samples for plasma analysis of karanjin were prepared by spiking, 80 µL blank plasma with 10 µL galanjin working solution and 10 µL karanjin working solutions, to produce final concentration of 0.02, 0.05, 0.15, 0.49, 0.97, 1.95 and 2.92 µg/mL. Resulting galanjin concentration was 1.0 µg/mL. All the working standard solutions were stored at 4º C and brought at room temperature before use. The calibration curve for the plasma assay was constructed by plotting the ratio of peak areas of karanjin to peak areas of I.S. The lower limit of quantification (LLOQ) was determined as the lowest concentration on the calibration curve at which precision was within ± 20% and S/N ≥ 5.

Precision and accuracy

The precision and accuracy of method were assessed by performing replicate (n=7) analysis of calibration curve samples. The precision was determined from five inter- day and intra- day using five determinations of calibration curve samples and expressed as relative standard deviation (% R.S.D.). Coefficient of variance (% C.V.) was calculated for precision and ≤ ± 15 % considered as the limit of acceptance except LLOQ (≤ ± 20 %).

Pharmacokinetic evaluation

Study design, dose administration and sample collection

Karanjin from ethanolic extract of *P. pinnata* seeds was evaluated for pharmacokinetics in rats using non compartmental model of analysis. Accurately weighed sample of ethanolic extract of *P. pinnata* seeds (equivalent to karanjin 10 mg/kg) was administered to each animal by steel feeding gavage no. 16. Twelve blood collection points were decided at 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h post administration. Sparse sampling method was used to distribute the sampling points in three sets (3 female rats per set). Four sampling points were assigned to each set. First set (0.0, 3.0, 4.0, 24.0 h), second set (0.5, 1.5, 2.5, 8.0 h) and third set (1.0, 2.0, 6.0, 12.0 h.) Blood collection volume and no. of sampling points (400 µL, 4 each rat) were decided so that the blood loss of each animal within 24 h has not been more than 1.6 mL. After collecting 400 µL blood from retro orbital plexus of every rat in heparinised tube, blood was centrifuged at 4000 x g for 10 min and separated plasma was stored immediately at -20 ºC until analysis. Aliquots of 90 µL plasma samples were processed and analysed for karanjin concentration. Pharmacokinetic parameters were determined by using the plasma concentration –time data (Gibaldi, 2005).

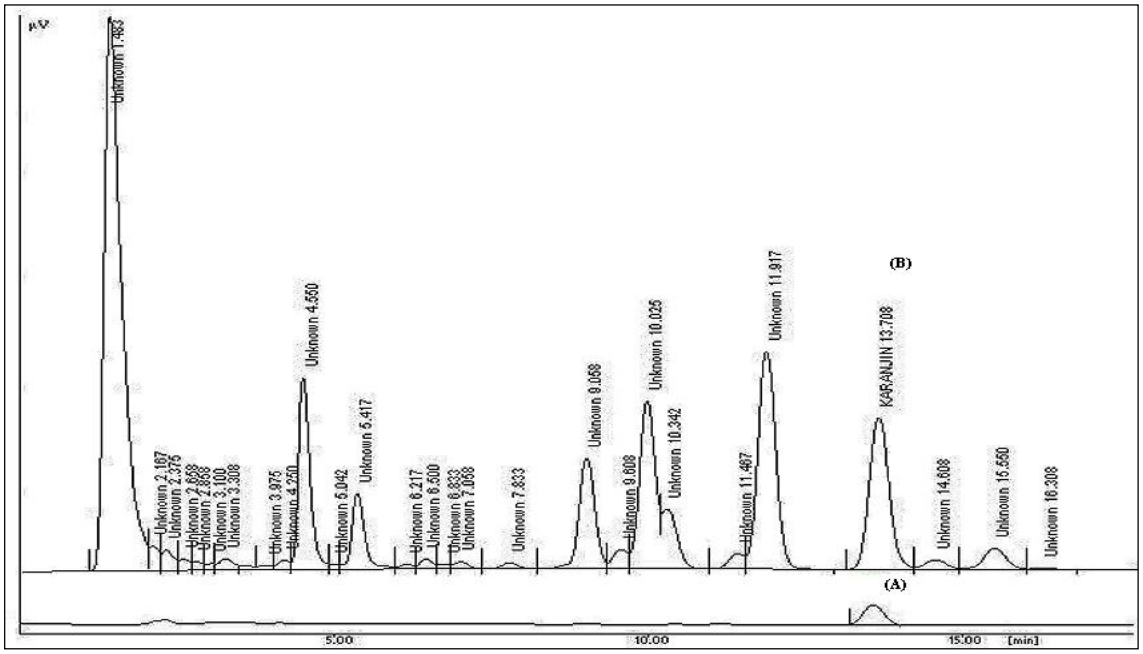

Fig. 2: Chromatograms of (A) karanjin standard (1.03 µg/mL) and (B) fingerprint of ethanolic extract of *P. pinnata* seeds.

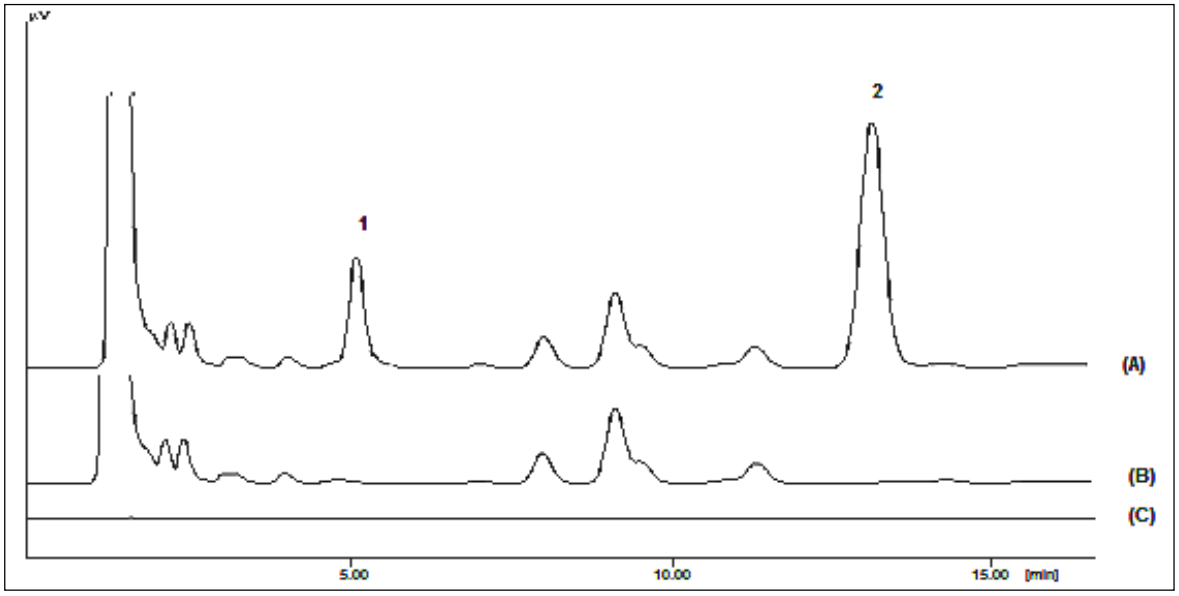

Fig. 3: Chromatograms of (A) plasma spiked with internal standard galanjin (1) and karanjin (2); (B) blank rat plasma and (C) mobile phase.

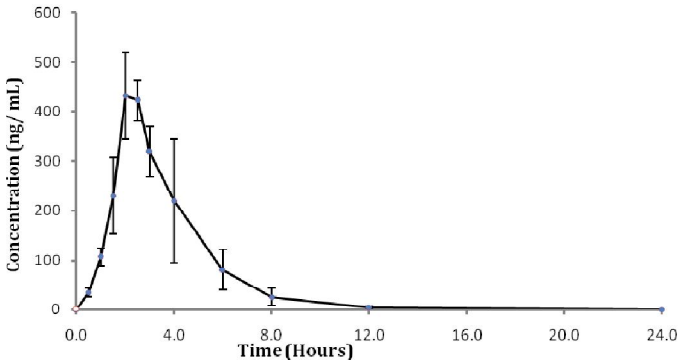

Fig. 4: Plasma concentration - time curve during pharmacokinetics of karanjin from ethanolic extract of *P. pinnata* seeds in rats.

The concentration of karanjin at different time intervals was evaluated by means of linear regression analysis. The relevant pharmacokinetic parameters were calculated by using WinNonlin statistical program.

Sample preparation for HPLC analysis

Solvent protein precipitation method was used (Nair et al., 2012). To 90 µL of plasma, 10 µL of internal standard galanjin (10 µg/ mL), and 1.0 mL acetonitrile was added followed by vortex mixing for 1 min and centrifugation at 4000 x g for 10 min. One millilitre of the supernatant was collected and evaporated to dryness under nitrogen stream at 60 ºC for 10 min. The residue was reconstituted with 100 µL mobile phase and 30 µL of it was injected in to the HPLC system.

Statistical analysis

Parameters for method validation were expressed as mean ± S.D. Pharmacokinetic parameters were evaluated using WinNonlin using non compartmental model of analysis (NCA 200 module) with sparse sampling.

RESULTS AND DISCUSSION

Earlier published chromatographic methods have reported quantitation of karanjin from plant matrices (Ravikumar et al., 2011). Dermal absorption of karanjin from an oil based Ayurvedic drug *Jatyadi Taila* has been reported by our group (Shailajan et al., 2011). There is scanty literature available on oral bioavailability of karanjin from the ethanolic extract of *P. pinnata* seeds and RP-HPLC method along with Sparse sampling which has been used to design this pharmacokinetic study. Karanjin content in the ethanolic extract of *P. pinnata* seeds and *Raughan-e-Karanj* was determined to be 69.12 and 11.64 mg/g respectively (Figure 2). Chromatographic characterization of *Raughan-e-Karanj* in terms of its karanjin content has been carried out for the first time. During pharmacokinetic study, sampling points were assigned to reduce the blood loss per animal (≤ 15% of blood volume in systemic circulation) within 24 h ensures no change in pharmacokinetics of drug in later sampling hour (Rani and Padh, 2001).

Bioanalytical method validation and pharmacokinetics

The developed analytical method did not show any matrix interference with the peaks of I.S. and karanjin. Figure 3 shows typical chromatogram of mobile phase, blank plasma and blank plasma spiked with I.S. (1.0 µg/mL) and karanjin (1.01 µg/mL). LOD and LLOQ were observed to be 0.01 and 0.02 µg/mL. Good linear relationship was observed for karanjin from 0.02 – 2.92 µg/ mL in plasma ($y = 1.982\ x+0.0121$, $r^2 = 0.998$).

Table 1 reports intra-day and inter-day precision and accuracy analysis of karanjin in blank rat plasma, coefficient of variations were observed below 15 % and accuracy (R.S.D.) was observed below 7 %. Recovery of karanjin from plasma was observed to be 77.12 %. Figure 4 shows mean plasma karanjin concentration (µg/mL) – time (h) profile after oral administration of Ethanolic extract of *P. pinnata* seeds showing karanjin. Absorption and elimination pattern shows that karanjin was found to be eliminated from systemic circulation at 24 h. Other pharmacokinetic parameters are summarized in Table 2.

Table. 1: Inter-day and Intra-day accuracy and precision of karanjin in plasma.

Linearity Concentrations (µg/mL)	Intra-day (n=5)		Inter-day (n=5)	
	Precision Mean ± S.D. (%C.V.) [a]	Accuracy (%R.S.D.)	Precision Mean ± S.D. (%C.V.) [a]	Accuracy (%R.S.D.)
0.019	0.020 ± 0.00 (13)	10.83	0.020 ± 0.00 (13)	9.95
0.049	0.050 ± 0.00 (6)	3.76	0.052 ± 0.00 (13)	5.70
0.146	0.148 ± 0.01 (9)	6.09	0.143 ± 0.01 (5)	6.85
0.486	0.468 ± 0.01 (2)	1.39	0.482 ± 0.02 (3)	3.40
0.973	0.996 ± 0.07 (12)	6.99	0.963 ± 0.05 (5)	5.24
1.945	1.949 ± 0.06 (5)	3.08	1.971 ± 0.06 (5)	3.04
2.918	2.956 ± 0.09 (5)	3.04	2.954 ± 0.09 (5)	2.99

[a]Coefficient of variance is expressed as ≥ ± (100-n)%, where n is the largest value in observations.

Table. 2: Pharmacokinetic parameters of karanjin.

PK parameters	Results (Mean ± S.E., n=3)
C_{max} (µg/mL)	0.498 ± 0.01
t_{max} (h)	2.307 ± 0.11
AUC_{0-t} (µg.h/mL)	1.430 ± 0.01
$AUC_{0-\infty}$ (µg.h/mL)	1.492 ± 0.01
$t_{1/2}$ (h)	3.78 ± 0.30
K_{el} (h^{-1})	0.49 ± 0.01

CONCLUSION

Till date there is no data reported for oral pharmacokinetics of karanjin in any biological system. The present work reports oral bioavailability of karanjin in rat plasma and a validated RP-HPLC-UV method for determination of karanjin. Data generated from current work would be applicable for the future investigation on karanjin for different therapeutic uses and would be useful for justifying the dosage and route of administration of karanjin containing formulations.

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