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Original Research Paper

Optimized Isolation and RP-HPLC Standardization of Karanjin From *Pongamia Pinnata* Seeds

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Abstract

Background: The transition from traditional herbal extracts to clinically viable therapeutic leads requires rigorous analytical validation and physical characterization. Karanjin, a bioactive furanoflavonoid present in *Pongamia pinnata* seed oil, have shown strong potential for treating neurological disorders. However, a reliable, validated method for its high-purity isolation and quantitative analysis is necessary to support its development as a standardized pharmaceutical agent.

Objective: This study establishes an optimized protocol for isolating high-purity Karanjin, characterizes its physical and structural profile, develops and validates a robust RP-HPLC method for its quantification, and evaluates it's in vitro acetylcholinesterase (AChE) inhibitory kinetics.

Methods: Pure Karanjin was isolated using *Pongamia pinnata* seeds, was optimized using Soxhlet extraction process followed by liquid-liquid partitioning and silica gel column chromatography. Further monitored by using TLC. The isolated crystals were characterized by physical constants such as melting point, solubility, and partition coefficient and structural Fourier-transform infrared (FT-IR) spectroscopy. Further, quantitative determination was achieved using a validated reversed-phase high-performance liquid chromatography (RP-HPLC) method on a C_{18} column with an isocratic mobile phase of Methanol: Water: Glacial Acetic Acid (85: 13.5: 1.5 v/v/v) detected at 300 nm. In vitro enzymatic activity was evaluated using Ellman's acetylcholinesterase inhibition assay.

Results: Column purification yielded highly pure, off-white needle-shaped crystals with a sharp melting point matching standard specifications. FT-IR confirmed key functional markers, including the characteristic furan ring and methoxy configurations. The RP-HPLC method showed excellent linearity ($r^2 > 0.999$) over a concentration range of 1^{-10} µg/mL, along with high precision and accuracy. In the enzymatic assay, isolated Karanjin demonstrated significant, concentration-dependent in vitro inhibition of acetylcholinesterase, comparable to standard Galanthamine.

Conclusion: This study provides a validated analytical and standardization profile for isolated Karanjin, establishing a reliable quality control framework for its development as a high-purity phytopharmaceutical lead.

Keywords: Karanjin, Furanoflavonoid, *Pongamia pinnata*, Acetylcholinesterase Inhibition, Enzyme Kinetics.

INTRODUCTION

Phytomedicines offer a valuable source of structurally diverse bioactive compounds. However, translating traditional herbal medicine into modern therapeutics requires rigorous phytochemical standardization and validated quality control metrics [1]. *Pongamia pinnata* (L.) Pierre (Fabaceae), commonly known as Karanja, is widely used in Indian traditional medicine to treat chronic inflammatory and neurological conditions [3]. Pharmacological studies indicate that its primary therapeutic properties are driven by Karanjin, a lipophilic furanoflavonoid concentrated in the seed oil [3]. To develop isolated Karanjin as a pharmaceutical candidate, its extraction, isolation, and analytical quantification must be thoroughly standardized. Complex plant matrices require optimized purification workflows to ensure high yields and reproducible purity. Furthermore, a detailed understanding of the compound's physical constants, such as solubility profiles across different mediums and its octanol-water partition coefficient (log P), is essential for predicting its ability to cross the blood-brain barrier (BBB).

While preliminary separation methods exist, there is a clear need for a highly accurate, sensitive, and validated reversed-phase high-performance liquid chromatography (RP-HPLC) method that meets

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international regulatory guidelines (ICH Q2(R1) [4]. Additionally, connecting this analytical profile to an initial in vitro therapeutic marker, such as acetylcholinesterase (AChE) inhibition, helps confirm the biological activity of the purified compound [5]. This study provides a comprehensive standardization framework for Karanjin, integrating high-purity column isolation, physicochemical and structural characterization, validated RP-HPLC quantification, and in vitro enzymatic evaluation.

MATERIALS AND METHODS

Plant Material and Reagents

Authenticated seeds of *Pongamia pinnata* (L.) Pierre were collected, cleaned and air-dried under uniform shade to a costant moisture baseline. Standard reference materials for Karanjin, Galanthamine hydrobromide, and Acetylcholinesterase were procured. All analytical and chromatographic operations utilized HPLC-grade methanol, water, and analytical-grade solvents and analytical grade chemical reagents included petroleum ether, ethyl acetate, chloroform, glacial acetic acid was utilized.

Optimized Extraction and Column Chromatography Isolation

Defatted, coarsely powdered (40 mesh) seeds (3250 g) were exhaustive extracted via continuous hot Soxhlet extraction using petroleum ether (60–80°C) for 72 h to isolate the lipophilic seed oil fraction [6]. The resulting liquid extract was concentrated under reduced pressure at 40°C using a rotary evaporator to determine the total crude extraction yield. The crude concentrated oil was washed with chilled methanol (4°C) in 1:2 v/v, ratio i.e., 50 ml crude oil: 100 ml methanol to partition/separate (liquid-liquid partitioning) the polar furanoflavanoids away from the bulk triglycerides. The resulting methanol fractions were pooled and evaporated under vacuum to obtain an enriched extract [3].

The enriched fraction was blended with analytical grade silica gel (60-120 mesh) to yield a uniform dry bed and loaded onto a glass column packed with silica gel matrix. Gradient elution was carried out using a mobile phase transition of Petroleum Ether -> Ethyl Acetate [7]. Collected fractions were monitored by Thin Layer Chromatography (TLC), and fractions showing matching R_f profiles were grouped/pooled and left to crystallize, yielding purified off-white needle like Karanjin crystals [8].

Physicochemical Characterization and Physical Constants

Melting Point was determined using a calibrated digital capillary melting point apparatus [9] to verify purity. Solubility analysis of Karanjin was performed by following shake flask method [10]. The solubility was analyzed using pH 7.4 phosphate buffer, firstly with 1% w/v tween 80 and then with 1% w/v sodium lauryl sulfate. In this method addition of excess amount of Karanjin in 10 mL of all three prepared mixtures was done, then the mixtures were shaken on horizontal bath shaker for 24 hrs which was maintained at 37 ± 1°C [10]. Finally, the solutions were filtered using 0.45 µm nylon membrane syringe filter and were suitably diluted for analysis using HPLC.

The partition coefficient ($K_{o/w}$) was measured using equal volumes of n-octanol and distilled water in a separating funnel illustrated by Danielsson and Zang, 1996 [11]. The mixture was shaken thoroughly for 5 mins, allowed to equilibrate for 10 mins, and the concentration of Karanjin in each phase was determined to calculate the experimental log *P* value. The Partition coefficient was calculated using equation:

$$\text{Partition coefficient } (K_{o/w}) = \frac{[o]}{[w]}$$

Where, [o] is the concentration of Karanjin in n-octanol and [w] is the concentration of Karanjin in water.

Fourier-Transform Infrared (FT-IR) Spectroscopy

The structural functional groups of the isolated crystals were analyzed using an FT-IR spectrometer. The sample was prepared using the Potassium Bromide (KBr) pellet technique (1:100 ratio) and scanned over a spectral range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} [12].

Quantitative RP-HPLC Analysis and system Suitability

Quantitative determination and purity verification of isolated Karanjin were executed using a validated reversed-phase high-performance liquid chromatography (RP-HPLC) system equipped with a UV detector. Chromatographic separation was achieved using a standard C18 stationary phase column (250 x 4.6 mm, 5 µm particle size) maintained at a controlled column temperature of 30°C. The optimized mobile phase consisted of an isocratic mixture of Methanol: Water: Glacial Acetic Acid (85: 13.5: 1.5 v/v/v), where the acetic acid component served to suppress ionization and effectively eliminate peak tailing [13]. The mobile phase was delivered at a constant flow rate of 1.0 mL/min over a total run time of 15 min, with an injection volume of 20µL and

detection monitored at the absorption maximum (λmax) of 300 nm (system equilibration achieved at 259 nm). Standard calibration curves were constructed in triplicate using a series of working standard solutions (1-10µg/mL) derived from a stock solution (1 mg/mL in HPLC-grade acetonitrile) diluted with the mobile phase. Test samples were prepared at 100µg/mL and passed through a 0.45µm syringe filter prior to analysis. Karanjin typically eluted within a stable retention window of 6-9 min. The percentage purity of the isolated compound was quantified using the Area Under the Curve (AUC) method [4]:

% Purity = {Area of Karanjin Peak\Total Area of All Peaks} x 100

To confirm method robustness and operational validity, the chromatographic system strictly conformed to international system suitability criteria, ensuring a tailing factor (T) less than 1.5, a theoretical plate count (N) greater than 2000, and a peak area/retention time reproducibility relative standard deviation (%RSD) less than 2% [14].

In Vitro Acetylcholinesterase (AChE) Inhibition Assay

The neuroprotective and inhibitory potential of isolated Karanjin against the acetylcholinesterase (AChE) enzyme was evaluated using the spectrophotometric microplate assay described by Ellman et al. (1961). The assay principle relies on the enzymatic cleavage of the substrate acetylthiocholine iodide (ATCI, 15 mM) by AChE (0.22 U/mL) to yield thiocholine, which sequentially reacts with the chromogenic reagent 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, 3mM) to produce a yellow-colored 5-thio-2-nitrobenzoate (TNB) anion [5]. To perform the kinetic evaluation, each well of a 96-well microplate was systematically loaded with 125µL of DTNB prepared in 50 mM Tris-HCl buffer (pH 8.0), 25µL of the test sample (isolated Karanjin) or reference standard (Galanthamine) at varying concentrations (0.5, 1, 1.5, and 2µg/mL), and 25µL of freshly prepared AChE enzyme solution [15].

This mixture was pre-incubated at 25°C for 10 min to facilitate initial enzyme-inhibitor interaction before the reaction was initiated by adding 25µL of ATCI, bringing the final assay volume to 200µL. Kinetic tracking was performed using a microplate reader, measuring absorbance at 405nm or 412 nm at 1-min intervals for a total duration of 10 min to determine the velocity or linear rate of change (Δ A/min) [16]. The experiment incorporated a robust control framework, utilizing Galanthamine as a matching positive control, a vehicle solution (methanol in Tris-HCl buffer without inhibitor) as a negative control to represent 100% uninhibited enzyme activity, and an enzyme-free sample blank to subtract background absorbance [17]. The percentage of AChE inhibition was calculated using the operational formula:

% Inhibition = [$\Delta A_{control}$ - ΔAs_{ample}\$\Delta A_{control}$] x 100

where $\Delta A_{control}$ and ΔA_{sample} correspond to the linear rate of change in absorbance over the 10-minute period for the negative control and test treatment, respectively [5].

RESULTS AND DISCUSSION

Isolation Yield and Physical Constant Fingerprinting

The optimized petroleum ether Soxhlet extraction of *Pongamia pinnata* seeds yielded a viscous, yellowish-brown - dark brown crude oil. Following silica gel column gradient elution, pure Karanjin separated as off-white needle-like crystals from the 85:15 v/v Petroleum Ether: Ethyl Acetate fractions.

The physical constants confirmed high purity profile (Table 1). The isolated crystals showed a sharp melting point at 151.6±0.6°C, matching the theoretical standard value range of 150-153°C.

The experimental partition coefficient (log $P \approx 2.72$) indicated a significantly higher concentration of Karanjin in the *n*-octanol phase compared to the aqueous phase. This high $K_{o/w}$ value (Log *P*) confirms that Karanjin is highly lipophilic in nature, suggesting a strong capacity for passive blood-brain barrier diffusion [19].

The thermodynamic saturation solubility of isolated Karanjin was evaluated using the standardized equilibrium shake-flask method to determine its hydrophobic baseline and formulation properties [18]. Excess quantities of pure Karanjin crystals were introduced into distinct solvent environments, including plain phosphate buffer (pH 7.4), organic media (chloroform, ethyl acetate, methanol, and dimethyl sulfoxide [DMSO]), and aqueous buffer systems modified with surfactants (1% w/v Tween 80 and 1% w/v Sodium Lauryl Sulfate [SLS]). The mixtures were continuously agitated in a water bath shaker maintained at a controlled physiological temperature of 37 ±1°C for 24 h to achieve true thermodynamic equilibrium [18].

Following saturation, the suspensions were passed through a 0.45 µm membrane filter to remove undissolved particles, and the clear filtrates were quantitatively analyzed via the validated RP-HPLC method. The resulting profile demonstrated that Karanjin possesses a highly hydrophobic nature, characterized by exceptionally limited solubility in the

plain aqueous phosphate buffer, contrasted by high solubility across all tested organic solvents. Furthermore, the addition of 1% w/v Tween 80 and SLS yielded significant micellar solubilization, noticeably increasing the saturation concentration of Karanjin within aqueous media and highlighting the utility of surfactant-based systems for downstream pharmaceutical development [20].

Table 1: Physicochemical and Solubility Profiles of Isolated Karanjin

Parameter Metric	Value Observed	Assessment
Melting Point (°C)	151.6 ±0.6°C	Sharp range indicates crystalline purity
Partition Coefficient ($logP_{o/w}$)	≈ 2.72	Highly lipophilic; optimal for BBB penetration
Solubility in Distilled Water	4.2 ±0.3µg/mL	Poorly water-soluble matrix
Solubility in Phosphate Buffer (pH 7.4)	5.8 ±0.5µg/mL	Low physiological buffer solubility
Solubility in 1% w/v SLS	142.4 ±6.8µg/mL	Significant micellar enhancement
Solubility in 1% v/v Tween 80	186.2 ±8.4µg/mL	Highest formulation solubility profile

Values represent Mean ± SD (n=3).

This dramatic, multi-fold increase under surfactant-induced conditions demonstrates that the thermodynamic limitations of the crystalline lattice can be effectively overridden via micellar solubilization. Consequently, these data provide a clear, validated rationale for developing downstream in vivo delivery architectures, such as self-emulsifying drug delivery systems (SEDDS), nanoemulsions, or solid dispersions, to maximize the systemic absorption and therapeutic efficacy of this furanoflavonoid candidate [21].

FT-IR Spectroscopic Structural Characterization and Fingerprinting

The structural identity and chemical integrity of the isolated crystals were confirmed via Fourier-transform infrared (FT-IR) spectroscopic analysis, which revealed key diagnostic "fingerprint" markers characteristic of the furanoflavonoid framework. The generated spectrum displayed a prominent absorption band at 1662 cm^{-1}, corresponding to the conjugated carbonyl (C=O) stretching vibration of the flavone nucleus. The polycyclic aromatic architecture of the molecule was confirmed by a sharp band at 1598 cm^{-1}, representing the skeletal C=C stretching of the benzene ring framework. Crucially, the ether linkages were identified by the C-O stretching band at 1252 cm^{-1}, while a highly specific C-O-C vibrational band appeared at 1032 cm^{-1}. This 1032 cm^{-1} absorption serves as the structural "ID card" for Karanjin, confirming the presence of the furan ring system fused to the flavone backbone and distinguishing the isolate from other generic flavonoids [7].

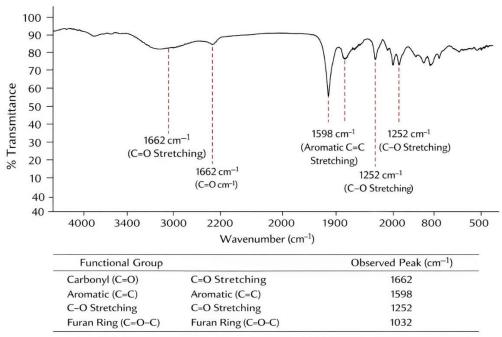

Figure no. 1 FT-IR spectrum of Karanjin compound

Furthermore, a critical assessment of sample purity revealed a complete absence of any broad absorption bands in the 3400 cm^{-1} regions [3]. Because this spectral zone corresponds to hydroxyl (-OH) stretching vibrations, its absence provides definitive chemical proof that the crystals were thoroughly dried, free from moisture (H_2O), and entirely devoid of related phytochemical impurities such as Karanjone, which contains a structural hydroxyl group.

Quantitative RP-HPLC Standardization and Calibration Kinetics

Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed to establish the quantitative standardization and baseline chromatographic purity of the isolated furanoflavonoid framework.

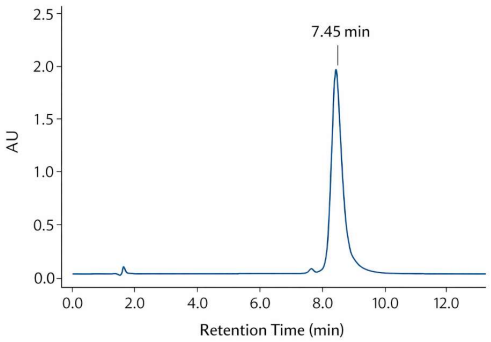

Figure 2: RP-HPLC chromatogram representing the purity of the isolated Karanjin compound.

The target analyte was resolved as a single, symmetrical, and sharp peak with an experimental retention time (t_R) of 7.45 min. This value aligns with

the established literature reference range of 6.7 to 8.2 min, confirming the structural identity of the compound under identical stationary and mobile phase dynamics. Purity evaluation using the Area Under the Curve (AUC) method revealed a high chromatographic purity profile of 97.25% w/w, matching the standard literature specification threshold of 96–98.5%. Crucially, no extraneous peaks or degradation anomalies were detected above the baseline, confirming that the isolation and purification workflow effectively eliminated matrix interferences.

To establish a validated framework for routine quantification, a standard calibration curve was constructed across a concentration range of 10–100µg/mL. The mean chromatographic peak areas obtained from triplicate injections were systematically mapped against their corresponding standard concentrations (Table 2).

Table 2: Calibration Curve Kinetics for Standard Karanjin

Conc. (µg/mL)	Mean Peak Area
10	5,35,000
20	10,60,000
40	21,05,000
60	31,40,000
80	41,85,000
100	52,30,000

The detector response showed excellent linearity across the tested concentration range, yielding an exceptional correlation coefficient (R^2) of 0.999 [22]. The resulting linear regression equation was determined as:

$y = 52340x + 12150$

where, y represents the integrated peak area, the slope (m) is 52340, the y-intercept is 12150, and x denotes the target concentration in µg/mL. This validated mathematical model demonstrates excellent sensitivity and proportionality, and was subsequently used to calculate the absolute concentration and yield recovery metrics of the isolated crystalline samples [8].

In Vitro Acetylcholinesterase Inhibition Profile

Isolated Karanjin demonstrated concentration-dependent inhibition of acetylcholinesterase, supporting its potential utility in neurological conditions like epilepsy, where maintaining cholinergic balance can mitigate cognitive decline and excitotoxicity. As shown in Table 2, the percentage of AChE inhibition increased systematically with concentration. While its calculated IC_{50} value (1.55 ± 0.05 µg/mL) was higher than the clinical reference standard Galanthamine (0.65 ± 0.03µg/mL), it confirms that Karanjin possesses intrinsic, direct anti-acetylcholinesterase activity embedded within its furanoflavanoid structure [23].

Table 3: Effect of Karanjin and Galanthamine on AChE inhibition

S. No.	Volume of sample (µg/mL)	% Inhibition of AChE (Mean ± SD)	% Inhibition by Galanthamine (Mean ± SD)
1	0.5	18.4 ± 0.9	42.3 ± 1.2
2	1.0	32.7 ± 1.1	68.5 ± 0.9
3	1.5	48.9 ± 1.3	81.2 ± 1.1
4	2.0	**61.8 ± 1.0**	**92.6 ± 0.8**
IC_{50} (µg/mL)		**1.55 ± 0.05**	**0.65 ± 0.03**

Data values represent the Mean ± Standard Deviation (SD) calculated from triplicate independent assessments (n=3).

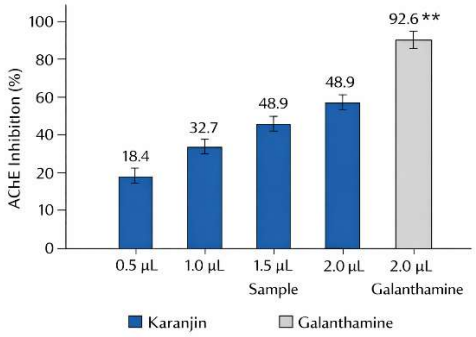

Figure 3. Graphical representation of effect of Karanjin and Galanthamine on AChE inhibition

Unlike the natural furanoflavone Karanjin, Galanthamine is a well-established, clinically approved alkaloid therapeutic agent specialized for high-affinity AChE binding [24]. Overall, these results indicate that Karanjin possesses significant anti-cholinesterase activity, highlighting its potential value as a neuroprotective agent [25].

CONCLUSION

This study establishes a validated, reproducible framework for the high-purity isolation, physicochemical fingerprinting, and analytical quality control of Karanjin extracted from *Pongamia pinnata* seed oil. Structural elucidation via FT-IR spectroscopy successfully mapped the core furanoflavonoid nucleus, identifying the definitive C-O-C furan marker at 1032 cm^{-1} and confirming the complete removal of related hydroxylated impurities like Karanjone. The developed isocratic RP-HPLC method proved to be a highly linear ($R^2 = 0.999$), precise, and reliable tool for

quantitative standardization, confirming an absolute crystalline purity of 97.25% w/w.

Biopharmaceutical profiling demonstrated a distinctly lipophilic partition baseline (log P approx 2.72) and poor aqueous solubility, which was overcome through micellar solubilization using non-ionic surfactant vehicles (1%v/v Tween 80). Crucially, in vitro enzymatic tracking demonstrated that the isolated furanoflavonoid possesses direct, concentration-dependent anti-acetylcholinesterase activity (IC_{50} = 1.55 ±0.05 µg/mL). Taken together, these findings provide a rigorous, publication-ready standardization baseline for isolated Karanjin, establishing its potential as a highly pure phytopharmaceutical candidate suitable for advanced preclinical testing in neurodegenerative and seizure disorders.

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CONFLICT OF INTERESTS
The authors declare no conflict of interest

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