

Sample Preparation for Green Isolation of Bioactive Furanoflavonoid and Disaccharide from *Pongamia pinnata* seeds

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Abstract

Pongamia pinnata popularly known as Karanj, is a legume. The traditional and pharmacological properties of Karanj are well known. Karanjin a bioactive furanoflavonoid is the major constituent of karanj seeds. The present research work aimed at sample preparation for green isolation of furanoflavonoid (karanjin) and disaccharide (sucrose) with an emphasis on achieving a higher yield of extracts, fractions and pure compound using different methods. The samples for isolation of compounds were prepared by the process of fractionation. The methanol extract of *P. pinnata* seeds was fractionated with four different solvents (hexane, chloroform, butanol and water). The chloroform fraction and methanol extract were used for green isolation of karanjin and sucrose by crystallization. The identification of karanjin was done by melting point, HPLC analysis and NMR spectroscopy and sucrose by melting point and NMR as reported in literature. The antimicrobial activity of karanjin against *Mycobacterium kansasii* was reported for the first time with MIC of 0.05 µg/ml. Thus a successful attempt for isolation of compounds without use of column chromatography and large amount of solvents was made in this research work. The isolation of sucrose from water fraction in large amount has economic value whereas isolation of karanjin in higher yield from chloroform fraction has medicinal value.

1. INTRODUCTION

Pongamia pinnata (Linn.) Pierre syn. *P. glabra*, belongs to family Fabaceae, and popularly known as 'Karanj' or 'Karanja'. This tree is indigenous to the Indian subcontinent and south-east Asia. It is medium-sized forest tree with 0.11 million tons of seeds collected every year in India [1]. In addition to being a well-known natural fuel source, the Karanja plant and seeds are also recognised for its therapeutic qualities. Karanja seed oil is non edible but has been identified as good source of biodiesel preparation in India [2]. It has been utilized in Indian folk and traditional medicine, including Ayurveda and Siddha [3]. Diarrhea, ulcers, skin diseases, rheumatic arthritis are treated using different parts of the plant [4]. It has been reported that the primary phenolic compounds from different parts of the plant include flavones, isoflavones, chalcones, furanoflavonoids, and pyranoflavonoids [5]. Among the 5–6% flavonoids in the seed oil, karanjin, a furanoflavonoid, is the principal component. Karanjin has insecticidal and pesticidal properties, as well as potential anti-inflammatory, anti-hyperglycemic, and anti-ulcer properties [6]. Sucrose, a natural disaccharide is also reported in the seeds of this plant [7], this fermentable sugar is used for bioethanol production [8]. Other applications of sucrose involve polyurethane foams production using sucrose as a complex diol in condensations with diisocyanates [9], sucrose esters are widely used as natural surfactants in drug delivery systems [10]

This research work aims a new search for sample preparation and green isolation of furanoflavonoid and disaccharide from *Pongamia pinnata* seed extract. Sample preparation is a crucial stage in many analytical procedures as it enables the development of more sensitive and selective methods. However, because of the necessary resources (energy, solvents, materials), it is seen as a stage with a significant

potential impact on the environment [11]. For this research study samples have been prepared for isolation of compounds using minimum amount of solvents.

2. MATERIAL AND METHODS

2.1 Plant material and chemicals

The *P.pinnata* seeds collected from local areas of Lucknow near Kukrail Reserve Forest, Lucknow (UP), India, were identified as *P.pinnata* species of leguminosae family from Plant Diversity, Systematics and Herbarium Division (LWG) of CSIR- National Botanical Research Institute, by Dr. K.M. Prabhukumar. A voucher specimen (reference no. 118409) of *P.pinnata* was deposited at institutional herbarium at CSIR-NBRI, Lucknow. This plant species collected is wild growing in above mentioned area however it is now widely cultivated due to its medicinal values. The molecules were isolated using analytical grade chemicals and Reference standards obtained from sigma Aldrich. Pre-coated TLC plates (silica gel 60F₂₅₄) were procured from Merck.

2.2 Plant material extraction and sample preparation for isolation

Seeds of *P.pinnata* were collected in the month of May 2023. The seeds were shade dried, separated from the pods and were grinded to a fine powder. The powdered seeds were extracted with methanol by two different methods of extraction i.e. maceration and ultrasound assisted extraction (UAE). Both the extracts were separated from the solid (powdered seeds residue) by filtration with Whatman No. 1 filter paper. The remaining solid was extracted twice with the same solvent in both methods. The extracts were concentrated under reduced pressure at 55°C, on rotary evaporator (IKA RV 10 Digital). Concentrated extracts were stored in a refrigerator at 4°C for further analysis. The extractive yield was calculated as follows:

$$\text{Extract yield (\%)} = (W_1/W_2) \times 100$$

Where, W_1 is the weight of extracted plant residues in grams, and W_2 is the weight of seeds in grams.

The methanol extracts obtained by maceration (E_1) and UAE (E_2) were then subjected to fractionation by two different method i.e. method I and II for comparative study of their percentage yield in different solvents.

Method I- E_1 was dissolved in distilled water in a separating funnel, fractionated and extracted first with hexane then with chloroform and butanol successively to produce fractions of different polarities. Fractions obtained were concentrated under reduced pressure on a rotary evaporator, followed by air drying at room temperature [12, 13]. The water fraction was freeze-dried. About 10 gm of methanolic extract was kept unfractionated for further analysis. The fractionation protocol is presented in Fig. 1.

Method II- The extract 'E₂' was fractionated with slight modification in the conventional method, each step of fractionation was ultrasound assisted. The water extract of 'E₂' was subjected to sonication in hexane for 40 minutes, thereafter the layers of both the solvents were separately collected with the help of separating funnel and concentrated on rotary evaporator. Same was repeated with other solvents like chloroform and butanol. The fractionation in step 1,2,3 and 4 was ultrasound assisted as described in Fig. 1.

The fractions of hexane, chloroform, butanol, water were used as sample A, B, C, D respectively and the methanolic extract as sample 'E' for further analysis through Thin Layer Chromatography. The TLC of sample 'A' was developed in 100% chloroform, sample 'B' and 'E' in 98:2 (chloroform: methanol) and sample 'C' in 90:10 (chloroform: methanol). The TLC were observed under UV fluorescence analysis cabinet at 366 nm (Camag® UV Lamp 4) .

2.3 Isolation of Karanjin: A furanoflavonoid

The isolation of karanjin was carried out with the chloroform fraction (sample B) of *P. pinnata* seeds (18.2 gm). The fraction was dissolved in ethanol and kept for few hours, for settling down of precipitate in a beaker. After precipitation the beaker was decant for separation of precipitate from rest of the extract. The precipitate was further dissolved in chloroform to form saturated solution and kept for few hours for crystallization. The crude crystals (4.4gm) obtained were washed with methanol and recrystallized 2–3 times with chloroform for further purification. The TLC for colourless needle- shaped crystals was developed in 99:1, chloroform: methanol and observed in UV Lamp at 366 nm.

2.4 Isolation of Sucrose: A disaccharide

The unfractionated methanolic extract (10 gm), sample 'E' was used for isolation of sucrose from *P.pinnata* seeds. The dry methanolic extract was again dissolved in methanol and kept for few hours, in a beaker. After few hours the crystals of sucrose settled down at the bottom of the beaker. The crystal separated by filtration and washed with chloroform. These crystals were insoluble in chloroform and methanol, therefore they were recrystallized 2–3 times with distilled water.

2.5 Identification of Karanjin and sucrose

Isolated furanoflavonoid was identified as Karanjin (3-methoxy-2-phenylfuro-(2,3-h-chrome-4-ol)) ; C₁₈H₁₂O₄ by direct comparison of co-TLC (99:1, chloroform: methanol), retention time in HPLC analysis and melting point of 158°C Singh et al. [14] with that of Reference standard obtained from Sigma-Aldrich. The melting point was recorded on 'Biochem melting point apparatus'.

The structure was also confirmed by the ¹H NMR and ¹³C NMR spectra acquired on 'Bruker Avance 600 MHz' and compared to 'δ values' as reported in the literature.

The identification of sucrose was done by comparison of melting point 186 °C Beckett et al. [15] and ¹H NMR and ¹³C NMR as reported in the literature.

2.6 HPLC Analysis of sample 'E' and isolated compound-Karanjin

HPLC analysis of methanolic extract and isolated compound (karanjin) was performed on Shimadzu prominence system equipped with an auto sampler (SIL-20 AC), LC Solution 1.0 software and DAD detector. The seed methanolic extract and compound were analyzed in triplicate at the concentration of 1mg/ml as described by Prabhu et al. [16] under the following conditions: stationary phase: C₁₈ column (dimensions 250mm x 4.6mm and particle size of 5 µm); mobile phase: methanol and water (80:20, considering the elution isocratic). The injection volume and the flow rate were set at 10 µl and 1ml/min respectively at the constant temperature of 25°C. Before, analysis the solutions were degassed and filtered through 0.22 µm filter membrane. The wavelength was set at 254 nm for analysis. All the reagents used for chromatographic analysis were of HPLC grade.

2.7 Anti –microbial activity assay

0.5 Mc Farland Standard dilution of microbes were used for study. 100 µl diluted log cultures of bacteria *Mycobacterium kansasii* was added to the micro centrifuge tube and added with 5 µl of prepared treatment dilutions (karanjin) of different concentrations (0, 0.1, 01, 10, 100, 500, 1000 µg/ml) to the defined tubes and incubated for 24 hours. After incubation all content was transferred to the 96 well plate and turbidity reading was taken by ELISA Plate Reader (iMark Biorad) at 630 nm. Ciprofloxacin (10 µg) was used as positive control.

3. RESULTS AND DISCUSSIONS

3.1 Extraction and fractionation

The seeds of *P. pinnata* were extracted with methanol by two different methods of extraction – maceration and UAE to produce the crude methanolic extract. It was observed that, UAE method showed higher extractive yield with less time and solvent consumption as compared to maceration as given in Table 1. The methanolic extracts were later subjected to fractionation and the percentage yield of different fractions (hexane, chloroform, butanol and water) of E₁ and E₂ with respect to raw material is given in the Table 1. It was observed that fractions of E₂ (method II) showed higher yield as compared to fractions of E₁ (method I). Fractionation results in separation of different components of plant extract. The hexane fraction contains non polar components oils and fatty acid, chloroform fraction are found rich in terpenoids, flavonoids and alkaloids, butanol fraction contains steroids and sugars and water fraction consists of sugars of high molecular weight.

Table 1
Percentage yield of extract and fractions with respect to
raw material (seeds)

S.No.	Extract / Fractions	Percentage yield	
		Method I	Method II
1	Methanolic extract	12	14
2	Hexane fraction	1.4	2.2
3	Chloroform fraction	1.1	2.0
4	Butanol fraction	1.2	2.1
5	Water fraction	8	7.6

The fractions were used as samples (A, B, C, D, E) for TLC analysis as presented in Fig. 2. The TLC of different samples showed that all the samples had different class of compounds present in them.

3.2 Isolation and identification

The crude crystals of karanjin and sucrose were obtained by the method of crystallization from samples 'B' and 'E' respectively. The two compounds were later purified by recrystallization (2–3 times). The compounds were isolated using minimum amount of solvent without use of column chromatography. The melting point of compounds were observed as 157.5 °C and 185° C respectively. The TLC of karanjin with reference standard showed R_f of 0.56 under UV Lamp at 366 nm as presented in Fig. 3. The percentage yield of karanjin and sucrose in *P. pinnata* seeds was found to be 0.52% and 0.33% respectively.

3.3 Karanjin characterization using HPLC- DAD analysis

The analysis, of the sample 'E' and isolated karanjin using high-performance liquid chromatography coupled to a DAD detector (HPLC–DAD), enabled the identification of karanjin, the analytical marker previously reported in this plant species (16). The retention time (R_t) of the furanoflavonoid, was 10.6 and 10.7 min in the sample 'E' and isolated karanjin respectively. The analysis of the peak area percentage quantified the content of karanjin to be 41.5% and 99.1% in the sample 'E' and isolated compound,. Figure 4 presents the chromatographic profile of the sample 'E', isolated and reference standard of karanjin (R_t 11.0).

3.4 Structure confirmation with ¹H and ¹³C NMR spectra

The structures of the isolated compounds were confirmed by NMR spectroscopy. ¹H and ¹³C spectra of karanjin was compared to δ values values reported by Vismaya et al. [1, 6] as shown in table 2 and Fig. 5

Table 2. NMR spectral data of karanjin

Reported δ values in ppm (Vismaya et. al)			Isolated karanjin δ values in ppm	
	$^1\text{H}[\delta, \text{mult.}]$	^{13}C	$^1\text{H}[\delta, \text{mult.}]$	^{13}C
A	7.70 d	145.4	7.78 d	145.7
B	7.20 d	103.9	7.20 d	104.2
O-CH ₃	3.85 s	59.9	3.95 s	60.24
2		154.5		154.8
3		141.5		141.8
4		174.7		175.1
5	8.22 d	121.6	8.23 d	121.9
6	7.57 d	109.7	7.54 d	109.9
7		157.8		158.1
8		116.7		117.0
9		149.6		149.9
10		130.7		131.0
1'		119.4		119.7
2'	8.17 m	128.0	8.18m	128.3
3'	7.60 m	128.3	7.59 m	128.6
4'	7.60 m	130.3	7.58 m	130.6
5'	7.60 m	128.3	7.56 m	130.6
6'	8.17 m	128.0	8.16m	128.0

^1H (600 MHz) and ^{13}C (150 MHz) spectral data (δ in ppm, CDCl) for karanjin

^1H and ^{13}C spectra of sucrose was compared to δ values reported in literature [17, 18] as presented in Table 3 and Fig. 6

Table 3. NMR spectral data of sucrose

	Reported δ values in ppm		Isolated sucrose δ values in ppm	
	$^1\text{H}[\delta, \text{mult.}], \text{no. of protons}$ (Hernández-García et al.)	^{13}C (Sun et al.)	$^1\text{H}[\delta, \text{mult}]$	^{13}C
1	5.38 d, 1H	92.05	5.19 d	92.21
2	3.52 t, 1H	71.08	3.59 t	72.09
3	3.72 t, 1H	74.56	3.56 t	74.78
4	3.43 t, 1H	70.11	3.48 t	70.33
5	3.83 m, 1H	73.20	3.89 m	73.23
6	3.78 d, 2H	60.81	3.66 d	60.94
1'	3.63 s, 2H	61.50	3.63 s	62.49
2'	-	104.26		104.49
3'	4.18 d, 1H	77.37	4.80 d	77.53
4'	4.01 t, 1H	74.56	4.50 t	74.73
5'	3.86 m, 1H	82.72	3.87 m	83.02
6'	3.79 d, 2H	62.40	3.77 d	62.60

^1H (600 MHz) and ^{13}C (150 MHz) spectral data (δ in ppm, DMSO- d_6) for sucrose

3.5 Anti –microbial activity of karanjin

Based on the study, it was observed that when test organism *M. kansasii* was exposed with different concentrations of the karanjin, the organism growth was inhibited and Minimum Inhibitory concentration (MIC) was estimated for Approx 0.05 $\mu\text{g/ml}$ against *M. kansasii*. Concentration inhibiting at least 20% of bacterial growth is considered MIC. The anti microbial activity of karanjin against *M. kansasii* was reported for the first time. Figure 7 describes the anti- microbial activity of karanjin.

LIMITATION

The present study provides rapid and easy method of isolation of compounds like sucrose and karanjin yet the study has some limitations such as there is more research work needed to be done for finding methods for isolation of more compounds using different fractions such as hexane and butanol. Further more biological activity of extract and fractions can be studied.

CONCLUSION

In this research study, sample preparation and green isolation of compounds was performed, both these processes used on present study required small amount of solvent as compared to column chromatography. The extraction and fractionation through UAE (method II) resulted higher percentage yield with less solvent and time consumption. The isolation of both the compounds through crystallization was fast and easy. The different samples prepared through fractionation can be used for further analysis and isolation of other compounds as well. The compounds isolated were identified by comparing melting point, HPLC analysis and NMR data to those reported in literature. The antimicrobial activity of karanjin against *M. kansasii* was reported for the first time. Thus successful attempt for green isolation was made in this research which also contributes to green chemistry.

Declarations

Acknowledgement

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Authors' contributions

Meenu Verma, Vaishali Mishra and Kajal Srivastava conceptualized the study, conducted the experiments and collected the data. Meenu Verma wrote the initial draft of the manuscript. Manjoosha Srivastava, Mahesh Pal and Meenakshi Singh guided, reviewed and edited the final manuscript. All authors read and approved the final version of the manuscript.

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Data availability

Data available on request from the corresponding author.

Ethics approval

All sampling of the *P.pinnata* seeds was conducted meticulously, adhering to both local and international regulations. Necessary authorizations were secured from the relevant Forest authorities before commencing collection. No endangered species was involved in this study, in compliance with IUCN guidelines and relevant national legislation. The *P.pinnata* seeds collected from local areas of Lucknow near Kukrail Reserve Forest, Lucknow (UP), India, were identified as *P.pinnata* species from Plant Diversity, Systematics and Herbarium Division (LWG) of CSIR- National Botanical Research Institute. A

voucher specimen (reference no. 118409) of *P.pinnata* was deposited at institutional herbarium at CSIR-NBRI, Lucknow.

Consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Eipeson WS, Manjunatha JR, Srinivas P, Kanya TS. Extraction and recovery of karanjin: A value addition to karanja (*Pongamia pinnata*) seed oil. *Ind Crops Prod*. 2010;32(2):118–22. <https://doi.org/10.1016/j.indcrop.2010.03.011>.
2. Susarla RS, Murthy MM, Rao BV, Chakrabarti PP, Prasad RB, Kanjilal S. A method for isolation of karanjin from the expelled cake of *Pongamia glabra*. *Eur J Lipid Sci Technol*. 2012;114(9):1097–101. <https://doi.org/10.1002/ejlt.201200016>.
3. Rekha MJ, Bettadaiah BK, Kanya TS, Govindaraju K. A feasible method for isolation of pongamol from karanja (*Pongamia pinnata*) seed and its anti-inflammatory activity. *Ind Crops Prod*. 2020;154:112720. <https://doi.org/10.1016/j.indcrop.2020.112720>.
4. Shoba FG, Thomas M. Study of antidiarrhoeal activity of four medicinal plants in castor-oil induced diarrhoea. *J Ethnopharmacol*. 2001;76(1):73–6. [https://doi.org/10.1016/S0378-8741\(00\)00379-2](https://doi.org/10.1016/S0378-8741(00)00379-2).
5. Sajid ZI, Anwar F, Shabir G, Rasul G, Alkharfy KM, Gilani AH. Antioxidant, antimicrobial properties and phenolics of different solvent extracts from bark, leaves and seeds of *Pongamia pinnata* (L.) Pierre. *Molecules*. 2012;17(4):3917–32. <https://doi.org/10.3390/molecules17043917>.
6. Katekhaie SD, Kale MS, Laddha KS. A simple and improved method for isolation of karanjin from *Pongamia pinnata* Linn. seed oil.3(1) (2012), 131–134.
7. Shameel S, Usmanhane K, Ali MS, Ahmad VU. Chemical constituents from the seeds of *Pongamia pinnata* (L.) Pierre. *Pak J Pharm Sci*. 1996;9(1):11–20.
8. Zabeed H, Faruq G, Sahu JN, Azirun MS, Hashim R. Nasrulhaq Boyce, A. Bioethanol production from fermentable sugar juice. *Sci world J*. 2014. <https://doi.org/10.1155/2014/957102>.
9. Queneau Y, Jarosz S, Lewandowski B, Fitremann J. Sucrose chemistry and applications of sucrochemicals. *Adv carbohydr Chem Biochem*. 2007;61:217–92. [https://doi.org/10.1016/S0065-2318\(07\)61005-1](https://doi.org/10.1016/S0065-2318(07)61005-1).

10. Szűts A, Szabó-Révész P. Sucrose esters as natural surfactants in drug delivery systems—A mini-review. *Int J Pharm.* 2012;433(1–2):1–9. <https://doi.org/10.1016/j.ijpharm.2012.04.076>.
11. Lucena R. The best sample preparation is green sample preparation. *Adv Sample Preparation.* 2022;4. 100016.<https://doi.org/10.1016/j.sampre.2022.100016>.
12. Mtunzi FM, Ejidike IP, Ledwaba I, Ahmed A, Pakade VE, Klink MJ, Modise SJ. Solvent–solvent fractionations of *Combretum erythrophyllum* (Burch.) leave extract: Studies of their antibacterial, antifungal, antioxidant and cytotoxicity potentials. *Asian Pac J Trop Med.* 2017;10(7):670–9. <https://doi.org/10.1016/j.apjtm.2017.07.007>.
13. Abubakar AR, Haque M. Preparation of medicinal plants: Basic extraction and fractionation procedures for experimental purposes. *J Pharm bioallied Sci.* 2020;12(1):1. https://doi.org/10.4103%2Fjpbs.JPBS_175_19.
14. Singh A, Bhatt G, Gujre N, Mitra S, Swaminathan R, Limaye AM, Rangan L. Karanjin Phytochemistry. 2021;183. 112641.<https://doi.org/10.1016/j.phytochem.2020.112641>.
15. Beckett ST, Francesconi MG, Geary PM, Mackenzie G, Maulny. A. P. DSC study of sucrose melting. *Carbohydr Res.* 2006;341(15):2591–9. <https://doi.org/10.1016/j.carres.2006.07.004>.
16. Prabhu TM, Devakumar C, Sastry VRB, Agrawal DK. Quantification of karanjin using high performance liquid chromatography in raw and detoxified karanj (*Pongamia glabra vent*) seed cake. *Asian-Australasian J Anim Sci.* 2002;15(3):416–20. <https://doi.org/10.5713/ajas.2002.416>.
17. Sun S, Zhao Z, Umemura K. Further exploration of sucrose-citric acid adhesive: Synthesis and application on plywood. *Polymers.* 2019;11(11). 1875.<https://doi.org/10.3390/polym11111875>.
18. Hernández-García E, García A, Avalos-Alanís FG, Rivas-Galindo VM, Delgadillo-Puga C. & del Rayo Camacho-Corona, M. Nuclear magnetic resonance spectroscopy data of isolated compounds from *Acacia farnesiana* (L) Willd fruits and two esterified derivatives. *Data brief.* 2019;22:255–68. <https://doi.org/10.1016/j.dib.2018.12.008>.

Figures

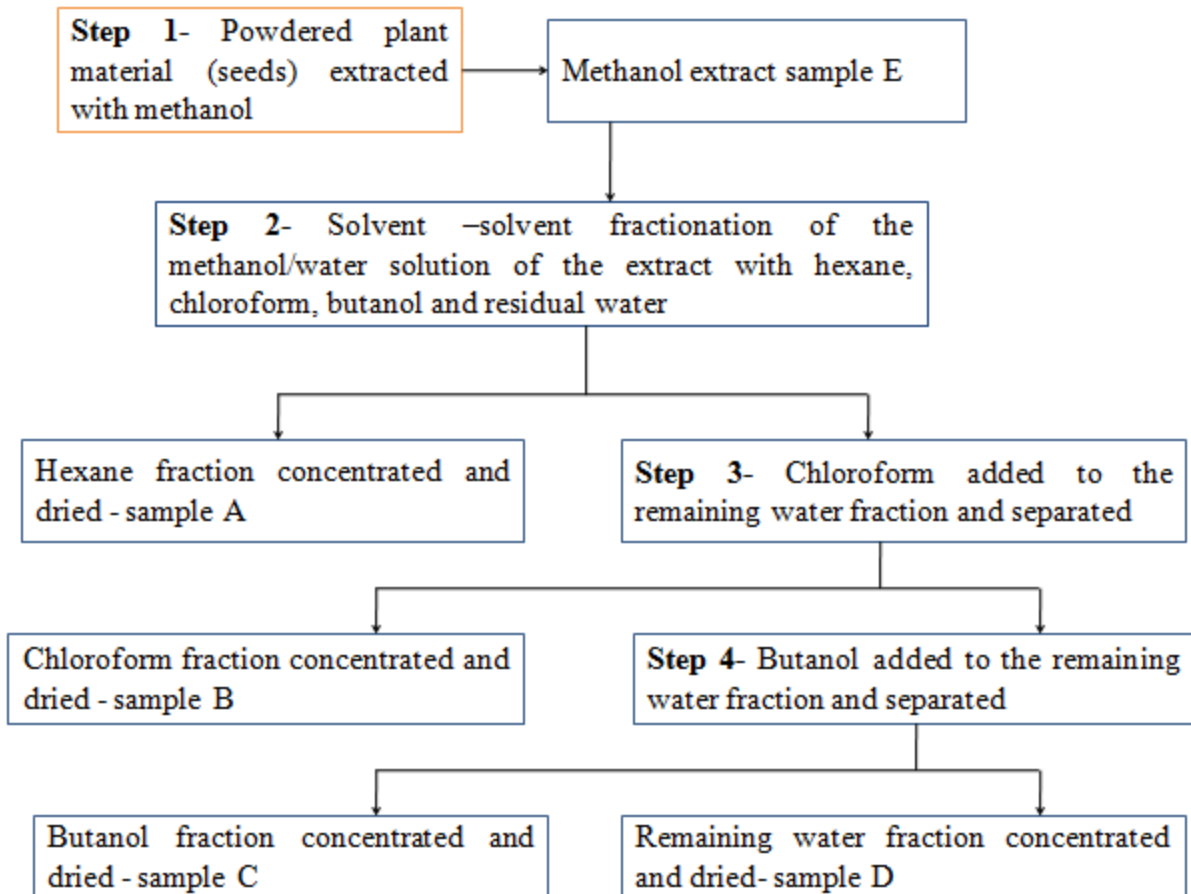


Figure 1

Protocol for fractionation

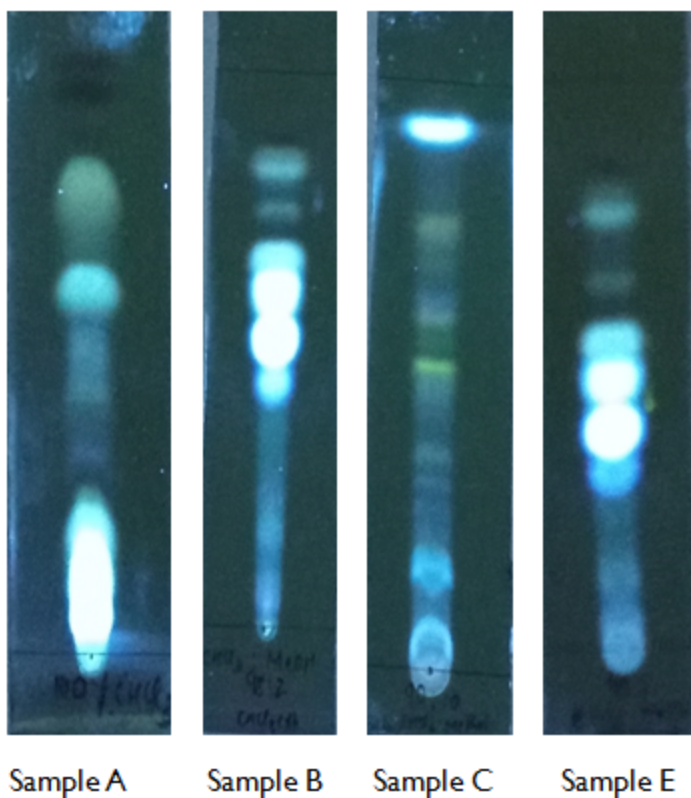


Figure 2

TLC of samples under UV Lamp at 366 nm

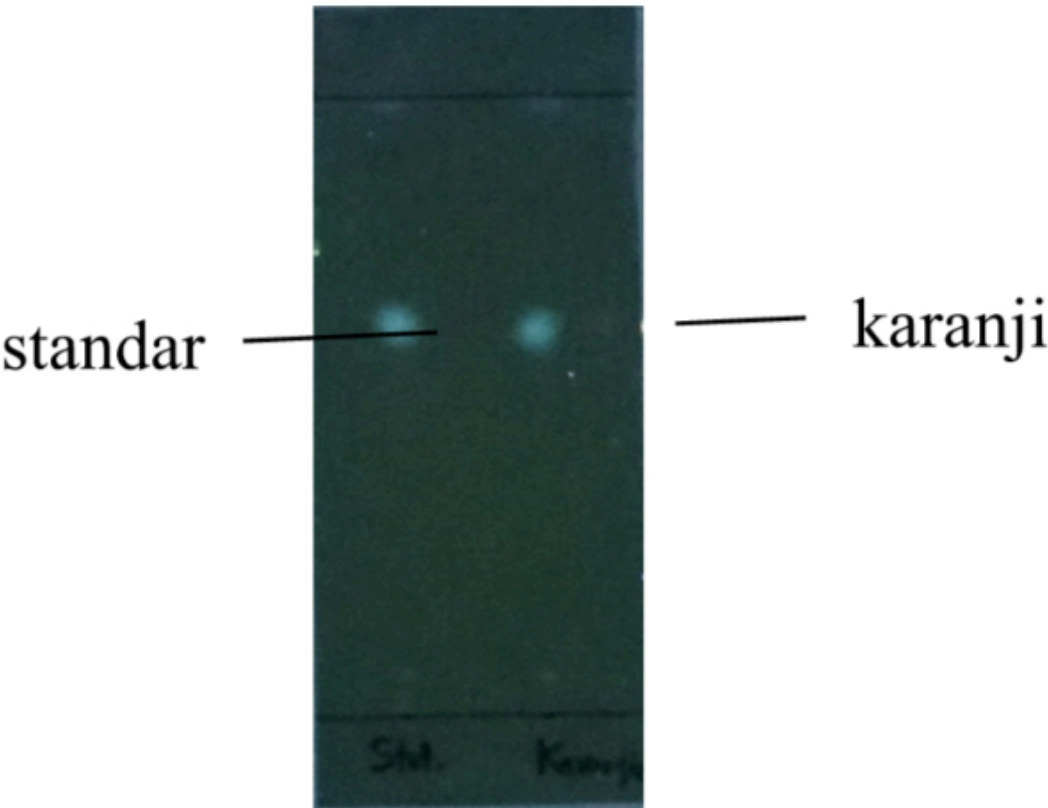


Figure 3

TLC of karanji with reference standard under UV Lamp at 366 nm

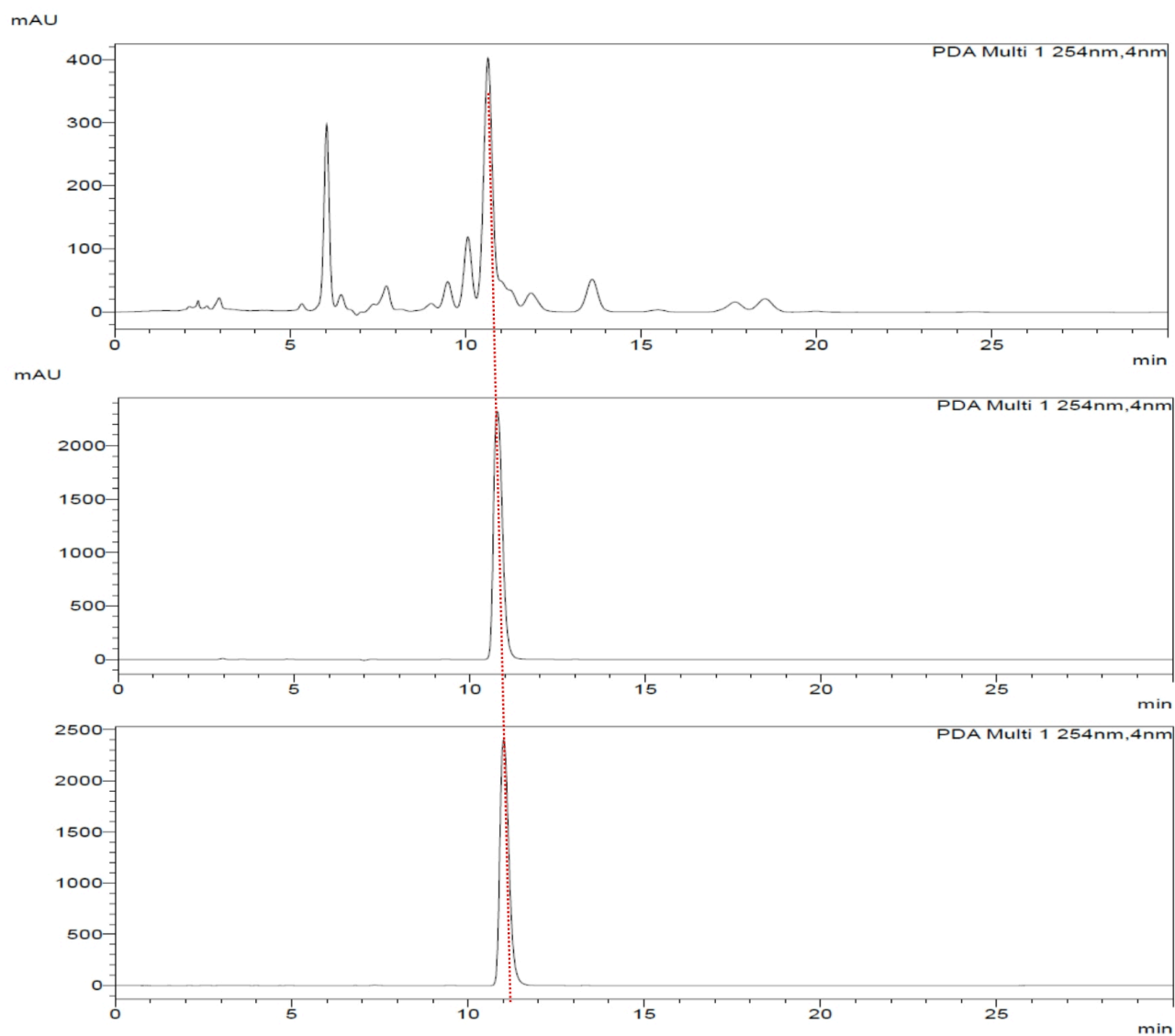


Figure 4

Chromatographic profile of the sample 'E', isolated and reference standard of karanjin

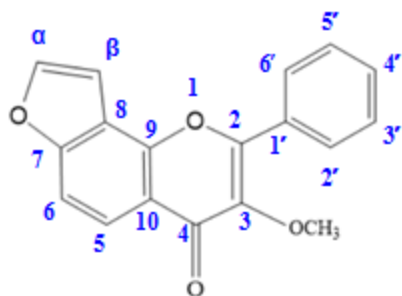


Figure 5

Structure of karanjin($C_{18}H_{12}O_4$)

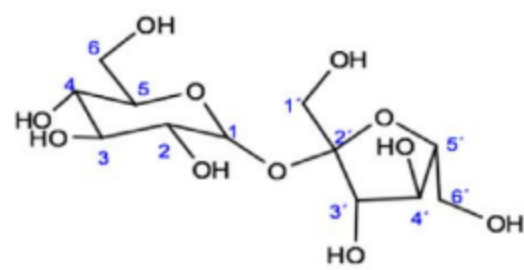


Figure 6

Structure of sucrose($C_{12}H_{22}O_{11}$)

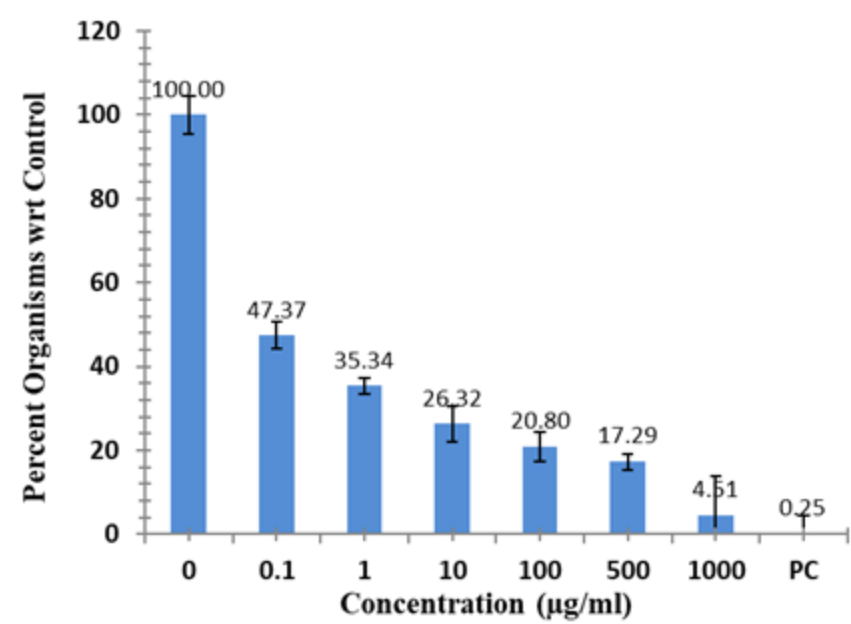


Figure 7

Antimicrobial activity of karanjin against *M. kansasii*