

FORMULATION AND EVALUATION OF NOVEL DRUG DELIVERY SYSTEM FOR *PONGAMIA PINNATA* SEED EXTRACT

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ABSTRACT-

Purpose: Liposomes have been used to improve the therapeutic index of new or established drugs by modifying drug absorption, reducing metabolism, prolonging biological half-life or reducing toxicity. Liposomal formulations have also been successfully used in the treatment of a number of dermatological diseases. Various synthetic as well as herbal drugs have been incorporated into liposomes to improve its efficacy. Herbal extracts can be incorporated into liposomes in order to increase the drug permeability. Karanja (*Pongamia pinnata*) seed extract is well known by anti-inflammatory activity.¹ *Pongamia pinnata* contains flavonoids like karanjin and Pongamol that have excellent antioxidant and photoprotective and anti-inflammatory activity. It also helps to prevent wrinkles, premature skin aging and skin cancer. Karanjin is main constituent in *Pongamia pinatta* seed which have less absorption through skin; hence there is a need to formulate a novel form in order to increase its absorption through skin. This can be achieved by developing a transdermal drug delivery system. The aim of this study was to formulate effective and stable liposomes as a novel drug delivery system for *Pongamia pinatta* seed extracts and to evaluate its physico-chemical properties & anti-inflammatory activity.² Liposome's were prepared by incorporating different amount of ingredients together (given below) and a certain amount of the herbal extract. Hence, this extract was incorporated into liposomes to enhance its activity in transdermal delivery. Drug diffusion study was performed to determine the percent release of drug through skin. The objective of the present research work is to convert this age old miraculous herb into nanotechnology based formulations i.e. liposome. Also, formulated liposomes were evaluated for particle size, Zeta potential, encapsulation efficiency, Transmission electron microscopy, In-vitro drug diffusion study, Membrane stabilization study and determination of active constituents by HPTLC.^{3, 4}

Methods: *Pongamia pinnata* seed Extract was incorporated into liposomes by thin film hydration method. The batch having lipid ratio i.e. Soya lecithin: Cholesterol (4:1); *Pongamia pinnata* seed extract concentration 250mg with entrapment efficiency 78% was finalized.

Keywords: Karanja (*Pongamia pinnata*) seed extract, Thin film hydration, Soya lecithin, Cholesterol, in-vitro drug diffusion.

INTRODUCTION

Pongamia pinnata (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 tumours, piles, skin diseases, wounds and ulcer. 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. The present research work was aimed to formulation and evaluation of novel drug delivery system for *Pongamia pinnata* seed extract. The liposomes are deposited on the skin and begin to merge with the cellular membranes. In the process, the liposomes release their payload of active materials into the cells. Liposomal preparations reduce the skin roughness because of its interaction with the corneocytes and of the intercellular lipids resulting in skin softening and smoothening. Liposomes are used for higher concentration of drugs in deeper layers of skin and a reduction in percutaneous absorption and unwanted side effects. *Pongamia pinnata* possesse good anti-inflammatory activity. Among the hydro-alcoholic extract seed extracts of found to be more active towards the inflammatory activity.⁵

The objective of the present research work is to convert this age old miraculous herb into nanotechnology based formulations i.e. Liposomal.

Materials and methods-

Cholesterol and soya lecithin were purchased from Hi-media Laboratories Ltd (Mumbai). All materials and solvents (Acetone, Chloroform, Methanol) were used of AR Grade. The dried seed of the *Pongamia pinnata* was collected from Ahmednagar region, was identified and authenticated from Department of Botany, Guru Nanak Khalsa College (Mumbai). Karanjin (Marker) was obtained from Yucca Enterprises (Mumbai).

Preparation of extract-

The seeds were dried in shade and then ground to produce coarse powder. It was defatted with petroleum ether by soxhlet apparatus. And then extracted with 70% ethanol. The extract was filtered through muslin cloth. Concentrated on water bath at 80°C. The extract was kept in sterile bottle.

Phytochemical analysis-

The extracts of *Pongamia pinnata* seed obtained from soxhlet extraction were subjected to phytochemical screening separately using reagents and chemicals. Preliminary screening involved qualitative determination of alkaloids, glycosides, terpenoids, volatile oils, flavonoids, tannins and phenolic compounds, carbohydrates and proteins.

Formulation of liposome:

A lipid phase was prepared by dissolving accurately weighed quantities of *Pongamia pinnata* seed Extract, Soya lecithin and Cholesterol in the chloroform-methanol mixture (2:1 v/v) in 250 ml round bottom flask containing glass beads. The solvent mixture was removed from the lipid phase by rotary evaporation at 45-50°C (Superfit) under reduced pressure, to obtain a thin film of lipids on the wall of the flask and the surface of beads. The dry lipid film was hydrated with Phosphate buffer pH (6.6) at a temperature of 60± 2°C. Dispersion was left undisturbed at room temperature for 2-3 hours to allow complete swelling of the lipid film and hence to obtain vesicular suspension.⁶

Optimization of formulation:

The preparation of liposome includes optimization of various process variables such as ratio of Soya Lecithin: Cholesterol, various concentrations of *Pongamia pinnata* seed Extract, and effect of sonication time. An entrapment efficiency measurement was performed on UV-spectrophotometer. In order to quantify the content of *Pongamia pinnata* seed Extract in the supernatant and pellets in samples, series of standard solutions was prepared. To determine encapsulation efficiency of *Pongamia pinnata* seed Extract liposomes. Drug entrapment efficiency was calculated by using centrifugation method. The liposome suspension was taken and centrifuged at 1500rpm for 20min. Then, separate sediment & supernatant from centrifugation and then diluted with phosphate buffer pH 6.6. Then the absorbance was taken at 219nm. The drug entrapment efficiency was calculated from the following formula,^{7,8}

$$\% \text{ Drug Entrapment efficiency} = \frac{\text{Amount of Drug Entrapped}}{\text{Total amount of Drug}} \times 100$$

Characteristics and Evaluation of liposomal formulation⁹⁻¹²

Particle Size Analysis-

Liposomal suspension was exposed to ultrasonic irradiation with duration of 30 min in continuous sonication bath. The sample was left to cool down and placed in the fridge at 4 °C for 1 day prior to further test. The liposomal suspension was then tested for particle size analysis by microscopy. Sample of liposomal suspension was evaluated for particle size after suitable dilution. Optical microscopy was used with oil immersion lens. Diameters of 50 liposomes were measured and mean geometric diameter and standard deviation was calculated.

Transmission Electron Microscopy-

Transmission electron microscopy (PHILIPS, CM200) of liposome was done from Institute of chemical Technology, Mumbai. For Transmission Electron Microscopy (TEM), the samples were negatively stained with a 1% w/v aqueous solution of phosphotungstic acid (PTA) prior to use.

Zeta potential Measurement-

The zeta potential of a particle is the overall charge that the particle acquires in a particular medium. The zeta potential of a liposome preparation can help to predict the fate of the liposomes in vivo. Measurement of the zeta potential of samples in the Zetasizer Nano is done using the technique of laser Doppler velocimetry. In this technique, a voltage is applied across a pair of electrodes at either end of a cell containing the particle dispersion. Charged particles are attracted to the oppositely charged electrode and their velocity is measured and expressed in unit field strength as their electrophoretic mobility.

In-vitro Drug diffusion study-

Diffusion study is used for the topical formulations intended for systemic activity. The aim of the study is to determine percentage of active drugs release in the system for specific time interval. Liposomes are having topical activity therefore in the present study amount of the active drugs remain in the skin layers was determined. Lesser the active drug release from the skin longer is the period of action.

In-vitro Anti-inflammatory activity-¹³**Membrane stabilization****Procedure-**

The assay mixtures consisted of 2 ml of hypotonic saline (0.25% w/v sodium chloride), 1.0 ml of 0.15 M sodium phosphate buffer, pH 7.4, 0.5 ml of 10% v/v human erythrocyte suspension, 1.0 ml of drugs (standard and extracts) and final reaction mixtures was made up to 4.5 ml with isosaline (0.9% w/v sodium chloride). To determine the anti-inflammatory activity by human red blood cell membrane stabilization method, the following solutions were used. The reaction mixtures were incubated at 37°C for 30 min and centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant solution was measured spectrophotometrically at 560 nm. Each experiment was carried out in triplicate and average was taken. The percentage inhibition of haemolysis or membrane stabilisation was calculated using the following formula-

$$\% \text{ Inhibition} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

Where, A control is the absorbance of control sample

A sample is the absorbance of test sample or standard drug.

Heat induced haemolysis-

Procedure – The 2ml reaction mixture consisted of 1 ml of drug (standard and alcohol and aqueous extracts) solution and 1 ml of 10% RBC suspension. Instead of extracts only saline was added to the control test tube. Aspirin was taken as a standard drug. To determine the anti-inflammatory activity by heat induced haemolysis the following solutions were used. All the centrifuge tubes containing reaction mixtures were incubated in water bath at 56°C for 30 min. At the end of the incubation the tubes were cooled under the running tap water. The reaction mixture was centrifuged at 2500 rpm for 5min and the absorbance of the supernatant was taken at 560nm. The experiment was performed in triplicates for all the test samples. Percent inhibition of haemolysis was calculated by the formula mentioned below-

$$\% \text{ Inhibition} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

Where, A control is the absorbance of control sample

A sample is the absorbance of test sample or standard drug

Inhibition of protein denaturation-

The reaction mixture consisted of test extracts and 1% aqueous mixture of bovine albumin fraction. PH of the reaction mixture was adjusted using small amount of 1N HCl at 37°C. The sample extract were incubated at 37°C for 20 min and then heated to 51°C for 20mins. . After cooling the samples the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows –

$$\% \text{ Inhibition} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

Where, A control is the absorbance of control sample
A sample is the absorbance of test sample or standard drug

High performance thin layer chromatography (HPTLC) analysis¹³

HPTLC analysis of Hydro-alcoholic extracts of *Pongamia pinnata* seed and liposome.

Preparation of liposome sample for HPTLC fingerprinting

500mg of extract was added in 10ml methanol and sonicated for 15 minutes.

Quantitation of Karanjin-

Preparation of liposomal sample for quantitation of Karanjin

Sample preparation for Hydro-alcoholic remains the same as that for HPTLC fingerprinting

Standard solution – 10mg Karanjin was added in 10ml methanol (1mg/ml). 1ml from stock solution was pipetted out and volume was made up to 10ml with methanol (0.1mg/ml).

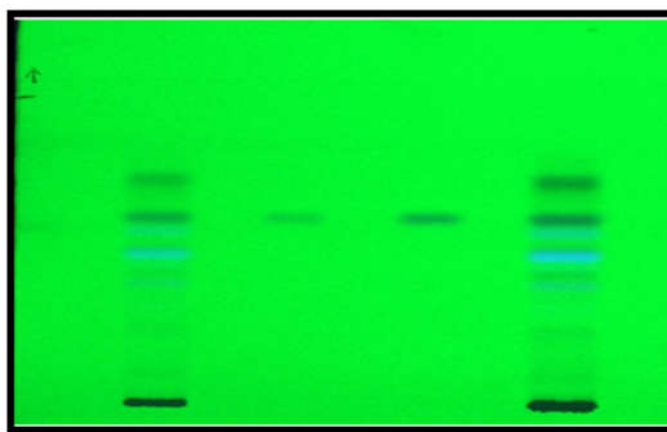
Mobile phase used for HPTLC of extracts and quantitation of quercetin

Toluene: Ethyl acetate (7:3)

Phytoconstituents responsible for anti-inflammatory activity are Karanjin for *Pongamia pinnata* respectively.

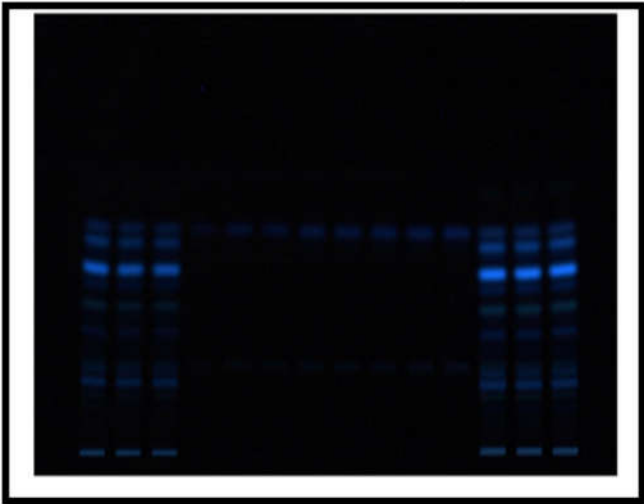
Extract	<i>Pongamia pinnata</i>
Mobile Phase	Toluene:ethyl acetate [7:3 v/v]

Mobile phases selected for *Pongamia pinnata* extract for HPTLC analysis.



Visualization of HPTLC plate at 254 nm

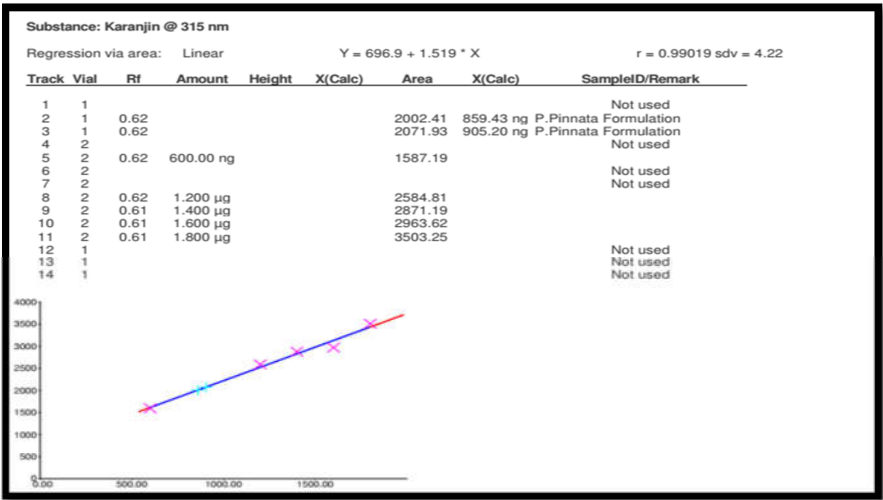
Picture shows that, lane 1st denotes Formulation of liposomes, 2nd denotes Karanjin



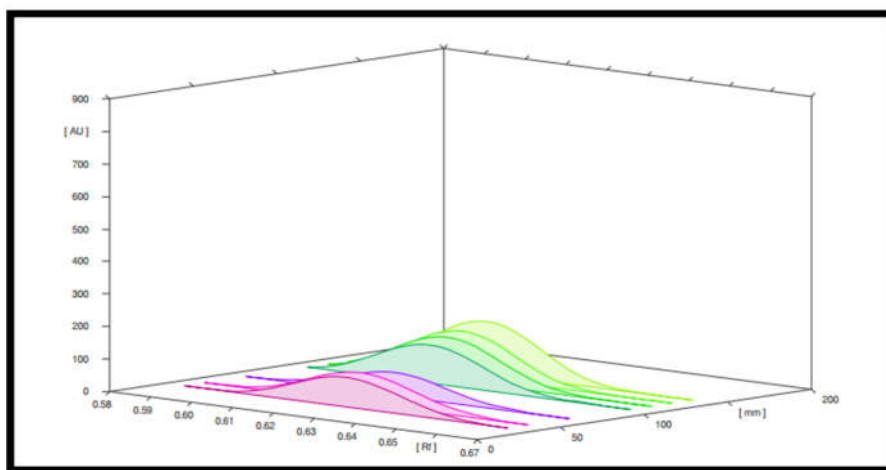
Visualisation of HPTLC plate at 366 nm

Quantitation of Karanjin-

The results of quantitation of Karanjin are as follows-



Standard graph of Karanjin



Three dimensional diagram showing peaks of Karanjin in the extracts of *P.pinnata* seed and in liposomal formulation

The Rf value of standard Karanjin matched with the Rf value of band seen in the hydro-alcoholic extract of *Pongamia pinnata*. This showed the presence of karanjin in hydro-alcoholic extract.

Results and Discussion-

The *Pongamia pinnata* seed extract obtained by maceration and sohxlet technique is observed for color, odor and appearance. Result and decription of *Pongamia pinnata* seed extract was found to be similar as mentioned in literature. The extract is thick semisolid, dark bown in color.

Phytochemical analysis-

Phytochemical screening was done using ethanol as solvent. Phytochemical screening confirmed the presence of various phytoconstituents like flavonoids, tannins, carbohydrate, and glycosides. These physical parameters was within the acceptable range.

Optimization of formulation-

Amount of Soya Lecithin (SL) and cholesterol (CH) was found to be critical in the preparation and stabilization of liposomes. Cholesterol is essential for lowering permeability, and imparting stability. Without cholesterol, prepared liposome shows non rigidity and irregular shape. Therefore the Soya Lecithin and cholesterol were used in different molar ratio for preparation of liposomes. Then the liposomes containing different concentrations of *Pongamia pinnata* seed extract was prepared using SL: CH (4:1). Based on the entrapment efficiency finalized the concentration. Hence, on the basis of entrapment efficiency the *Pongamia pinnata* seed extract concentration was finalized e.g. 250mg.

Evaluation of Liposomes¹⁴⁻¹⁶

Particle size analysis-

The particle size of drug loaded liposomes as determined by Horiba scientific was found to be 182 nm by rotary evaporator method.

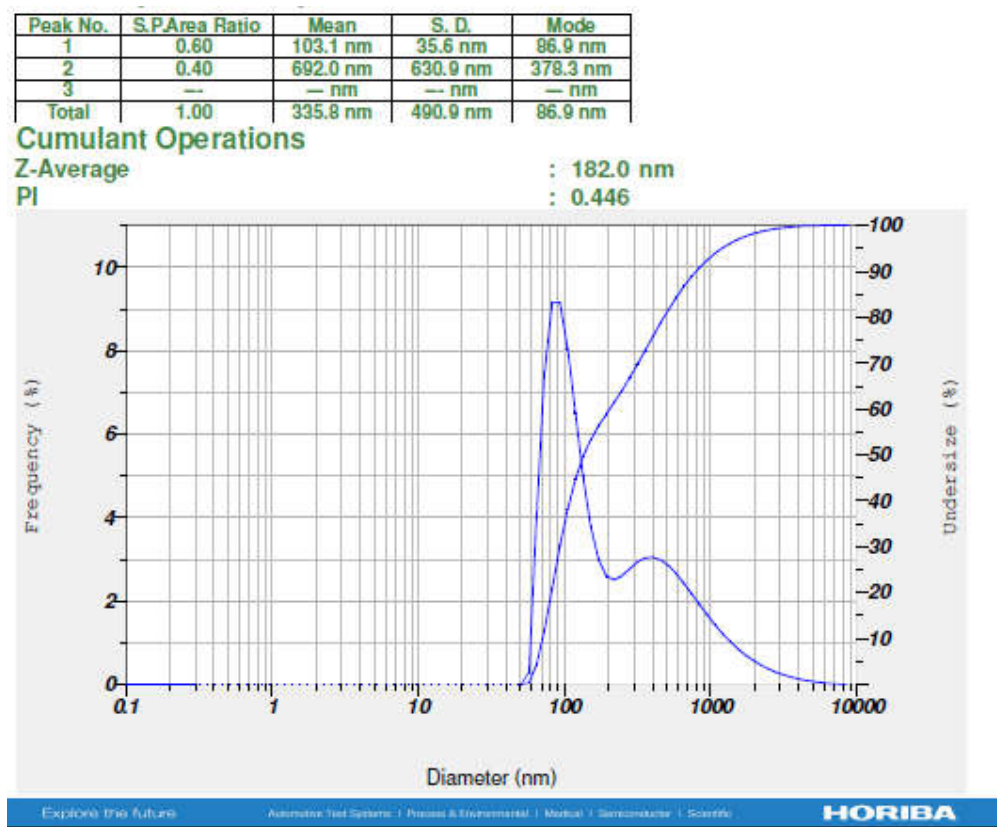


Fig. 1 Particle size analysis

Transmission electron microscope-

In TEM, spherical lamellar vesicles were observed in giving the liposomal suspension at 50 nm scale. TEM photographs shown in (Figure 2)

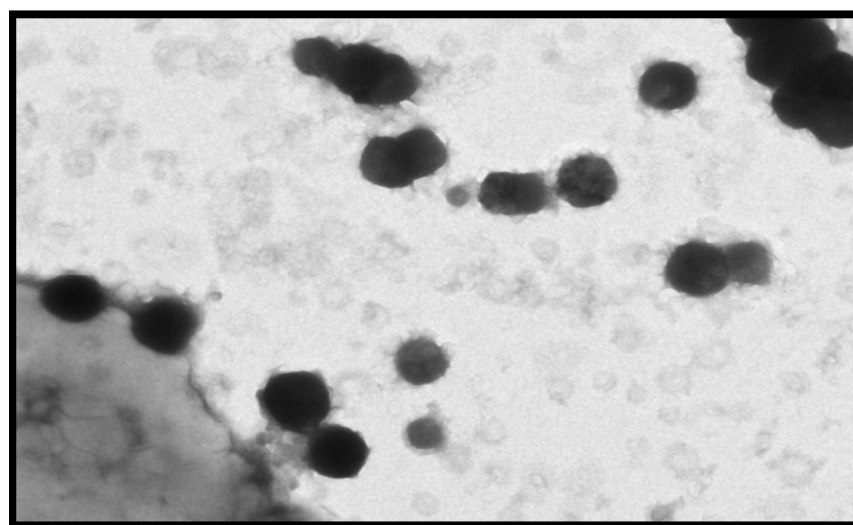


Fig.2 Transmission Electron Microscopy

Zeta potential-

Zeta potential of drug loaded liposomes as determined by Horiba scientific was found to be 66.7 mV.

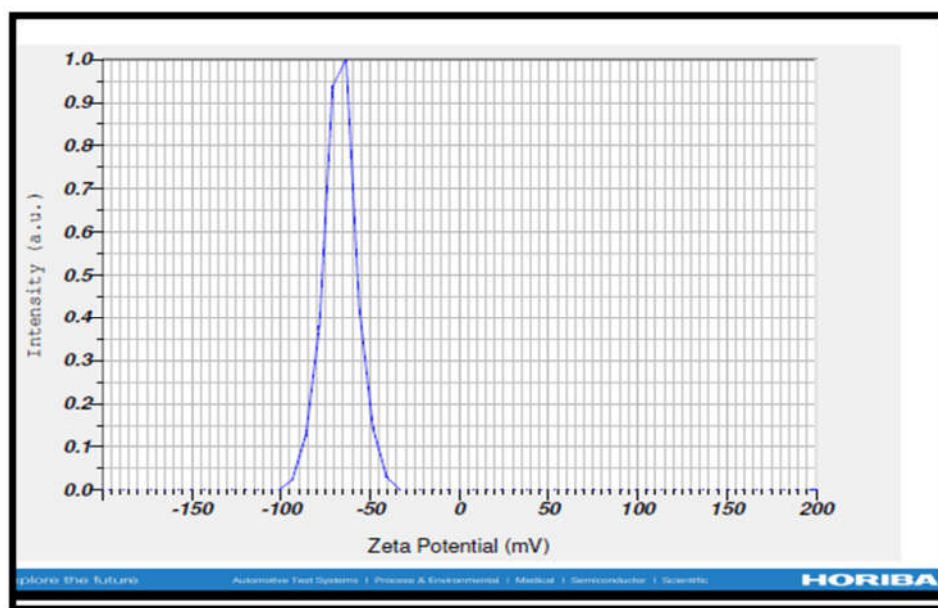


Fig.3 Zeta Potential

***In-vitro* drug diffusion study-^{17, 18}**

The in-vitro drug diffusion of optimized liposomal batch released at the end of 24 hour was found to be %. (Table 4) and extract was released found to be % (Table 5). The figure 4 shows the % drug release from liposomal formulation batch.

Time (hr)	Absorbance	Absorbance Conc.	Conc./1000 mg/ml	Conc. mg/20 ml	Cumulative release	%Cumulative release	Log% Cumulative release
1	0.110	4.3	0.0043	0.086	0.086	2.15	0.3324
2	0.127	6	0.006	0.12	0.206	5.15	0.7118
3	0.137	7	0.007	0.14	0.346	8.65	0.9370
4	0.160	9.3	0.009	0.18	0.526	13.15	1.1189
5	0.172	10.3	0.0010	0.21	0.736	18.4	1.2648
6	0.179	11.2	0.00112	0.22	0.956	23.9	1.3783
7	0.184	11.7	0.00117	0.234	1.190	29.75	1.4734
8	0.186	11.9	0.00119	0.238	1.428	37.7	1.5763

Table 4: Results of Drug diffusion study on Liposomal formulation

Time (hr)	Absorbance	Absorbance Conc.	Conc./1000 mg/ml	Conc. mg/20ml	Cumulative release	%Cumulative release	Log% Cumulative release
1	0.0820	1.5	0.0015	0.030	0.030	0.75	0.1249
2	0.086	1.9	0.0019	0.038	0.068	1.7	0.2304
3	0.091	2.4	0.0024	0.048	0.116	2.9	0.4623
4	0.093	2.6	0.0026	0.052	0.168	4.2	0.6232
5	0.095	2.8	0.0028	0.056	0.224	5.6	0.7481
6	0.112	4.5	0.0045	0.090	0.314	7.85	0.8948
7	0.118	5.1	0.0051	0.102	0.416	10.4	1.0170
8	0.122	5.5	0.0055	0.110	0.526	13.15	1.1189

Table 5: Results of Drug diffusion study on *Pongamia pinnata* seed extract (Drug)

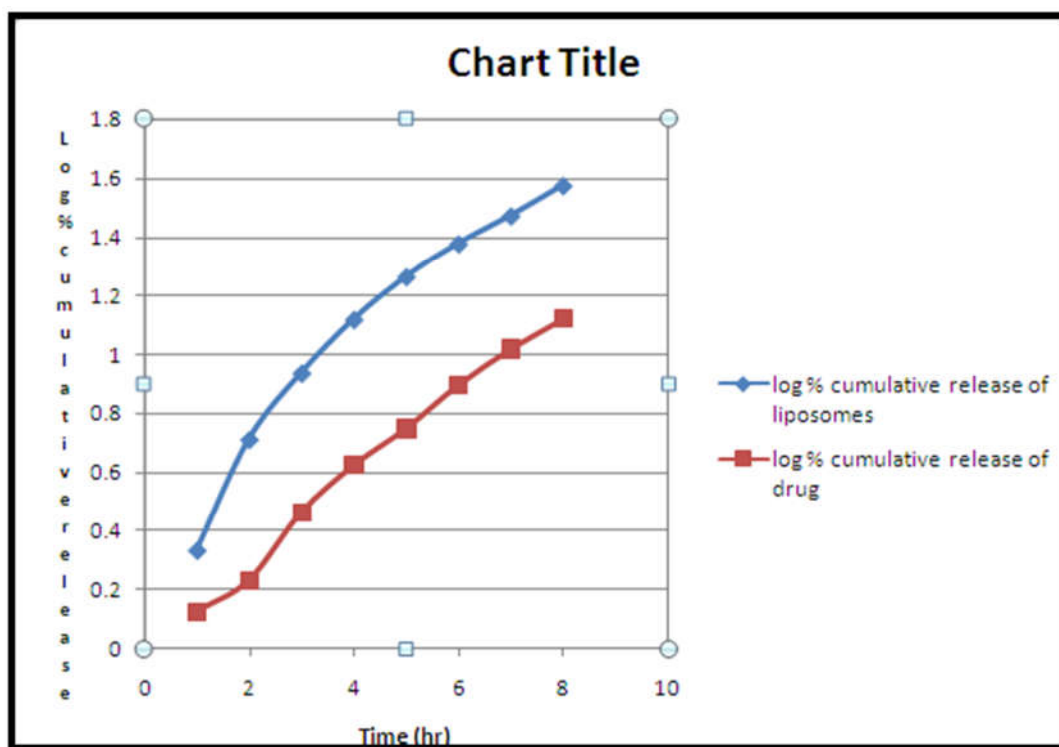


Fig.4: Percent Drug Release

Result: In vitro drug diffusion study, the cumulative percent drug release for formulation was found to be higher i.e. 37.7% and formulation batch shows smaller i.e. 13.45% after 24 hours.

***In-vitro* Anti-inflammatory study-**

In Anti-inflammatory activity optimized liposomal batch was gives more % inhibition in Protein denaturation and Membrane stabilization as compare to Heat induced haemolysis.

Dose	Membrane stabilization (% Inhibition)		
	Drug (Karnjin extract)	Liposome	Aspirin
100 µg/ml	34.98%	39.31%	47.58%
200 µg/ml	38.62%	46.20%	53.10%

Dose	Heat induced haemolysis (%Inhibition)		
	Drug (Karnjin extract)	Liposome	Aspirin
100 µg/ml	31.07%	28.68%	48.58%
200 µg/ml	39.53%	38.62%	51.93%

Dose	Protein denaturation (% Inhibition)		
	Drug (Karnjin extract)	Liposome	Aspirin
100 µg/ml	75%	85.29%	83.82%
200 µg/ml	78. 67%	88.2%	89.70%

Table 6: Percent Inhibition

Conclusion

The *Pongamia pinnata* seed extract was incorporated into liposomal drug delivery system to increase the the rate of permeation into the skin and also to decrease the adverse side effects of extract of karanja seed. The SL: CH ratio (4:1) is optimized for the preparation of liposomes. *Pongamia pinnata* seed extract concentration **250mg** with entrapment efficiency **78%** was finalized. The vesicle size was found **182nm**.

In vitro drug diffusion from liposomal was found to be **37.7%** and drug was found to be **13.15%** respectively.

In **Anti-inflammatory** activity % inhibition was found to be more in Membrane stabilization and Protein denaturation as compare to Heat induced heamolysis.

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