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Method of Development and Characterization of Pterocarpus Marsupium Loaded Liposomes.

Rajat Singh, Garima Garg, Shivam

Abstract: ***The main motive behind this study was to formulate an herbal drug loaded nanosized carrier which enhances the permeability of a hydrophilic herbal drug through the membrane and increases the drug concentration into the systemic circulation. And second motive is to provide a cheap formulation to the future consumers which will be deal with the diabetes on that time. An alternative of that drug was silver nanoparticles present but they are very costly at that time. The effectiveness of the formulation was considered on the basis of the In-Vitro inhibition study of the alpha-amylase by Pterocarpus marsupium loaded liposomes and compared with a positive control Acarbose and it was shows that high concentration of drug was as effective as acarbose to inhibit the alpha – amylase.***

Keywords: ***the drug concentration into the systemic circulation. And second motive is to provide a cheap formulation to the future consumers which will be deal with the diabetes on that time.***

I. INTRODUCTION:

Diabetes have the status of a budding epidemic in India, more than 62 million people were currently diagnosed with this disease. Basically, it is a bundle of chronic disorder of fat, Carbohydrate and protein metabolism which can cause increased fasting and post prandial blood sugar levels. Globally diabetes is approximated to raise from 4% in 1995 to 5.4% by the year 2025.Diabetes mellitus is also known as diabetes because it is a group of various metabolic disorders and their symptoms are high blood sugar include with frequent urination, increased hunger and increased thirst. When they leave untreated, they will cause many obstacles like diabetic ketoacidosis, hyperosmolar hyperglycemic state, or death and some sever long-term stumbles which are cardiovascular disease, stroke, chronic kidney disease, damage to the eyes, and foot ulcer. ("Diabetes Fact sheet N°312". WHO 2013)

There are basically three types of diabetes mellitus which are as follows:

1. Type 1 Diabetes Mellitus
2. Type 2 Diabetes Mellitus
3. Gestational diabetes

Use of Herbal medication for treatment purpose:

Due to the increased focus of people towards herbal substance and the traditional evidences of the herbal plants and herbs for the treatment of various diseases. For the treatment of diabetes there are various plants are available in which some of the following as follows.

- Holy Fruit tree (Aegle marmelos)
- Onion (Allium cepa) & Garlic (Allium Stivum)
- Japanese angelica tree (Aralia elata)
- Neem (Azadirachta indica)
- Orchid tree (Bauhinia candicans & B. forficte)
- Little tree plant (Biophytum sensitivum)
- Black Mustard (Brassica nigra)
- Cinnamon (Cinnamomum zeylanicum)
- Huanglian (Coptis chinensis)
- Guar (Cyamopsis tetragonoloba)
- Java plum (Eugenia jambolana)
- Banyan (Ficus bengalenesis)
- Potato (Grewia asiatica)
- Gurmar (Gymnema sylvestre)
- Henna (Lawsonia inermis)
- Purple lossestrife (Lythrum salicaria)
- Alfalfa or Lucerne (Medicago sutivu)
- Bitter Melon (Momordica charantia)
- Mulberry (Morus alba)
- Curry leaf (Murraya koeingii)
- Holy basil (Ocimum sanctum)
- Ginseng root (Panax ginseng)
- Kutki (Picrorrhiza Kurroa)
- Rhizoma (Polygonati odorati)
- Guava (Psidium guajava)
- Vijayasar (Pterocarpus marsupium)
- Gaduchi (Tinospora cordifolia)
- Fenugreek Seeds (Trigonella foenumgraecum)
- Ginger (Zingiber officinale)
- Jamun (Syzygium cuminii) etc.

II. MORPHOLOGY OF VIJAYASAR

For the rate of the minimization of the blood sugar level in the blood Vijayasar (Pterocarpus marsupium) is to be selected for the research purpose because less work is to be done on this drug.

First the all pharmacognostic description the drug are as follows:

Synonyms:

Vijayasar, Vijaysaar, Vijaysar, Bijasal, Indian Kino Tree, Malabar Kino Tree, Bija saar, Bila, Asana, Beejaka, Petaca, Bandhukavriksha, Venga etc.

Botanical name:

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Pterocarpus marsupium

Family name:

Fabaceae

Pterocarpus marsupium Roxb. is grown in the deciduous and evergreen forests of central, western and southern regions of India. It is found mostly in the states of Gujrat, Madhya Pradesh, Bihar, Orissa. The heart wood, leaves, flowers and bark have functional medicinal properties. The heart wood of Pterocarpus marsupium is astringent, bitter, acrid, anti-inflammatory, anthelmintic and anodyne. It is used for the treatment of elephantiasis, leukoderma, diarrhoea, dysentery, rectalgia, cough and greyness of hair. It is also reported that an aqueous infusion of the wood is of use in diabetes and water stored in vessels (glass) made of the wood, called tumbler, is reputed to have anti-diabetic qualities. The bark is used as an astringent and also for relieving toothache. The blaken leaves are contemplately utility as an external application for boils, sores and skin diseases. Pterocarpus marsupium is widely used in "Ayurveda" as "Rasayana" for management of various metabolic disorders including hyperlipidemia. The bark of Pterocarpus marsupium may be considered as good source of natural anti-oxidants for free radical mediated ailments. (BHUPENDRA, C. AND AMRENDRA, K.C 2012)

Chemical Constituents:

Researches in the past have established the genus Pterocarpus to be the rich sources of polyphenolic compounds. All active principles of Pterocarpus marsupium are thermostable. The plant contains pterostilbene 4-5%, alkaloids 0.4%, tannins 5%, protein, pentosan, pterosupin, pseudobaptigenin, liquiritigenin, isoliquiritigenin, garbanzol, 5-de-oxykaempferol, P-hydroxybenzaldehyde, Beudesmol, erythrodirol-3-monoacetate, l-epicatechin, marsupinol, irisolidone-7-O-A-L-rhamnopyranoside, have been obtained mainly from the heartwood and root.

Gum kino from the bark provides non-glucosidal tannins.

- Kinotannic acid
- Kinonin (C28H24O12)
- Kino-red (C28H22O11)
- Pyrocatechin
- Pyrocatechin acid & small quantities of resin, pectin and gallic acid.

Aqueous extract of the heartwood of Pterocarpus marsupium contains 5 new flavonoids C-glucosides namely 6-hydroxyl-2-(4-hydroxybenzyl)-benzo-furan-7-C-â-D-glucpyranoside, 3-(á-methoxy-4-hydroxybenzylidene)-6-hydroxybenzo-2(3H)-furanone-7-C-â-D-glucopyranoside, 2-glucopyranoside, 8-(C-â-D-glucopyranosyl)-7,3,4-trihydroxyflavone and 1,2-bis (2,4-dihydroxy, 3-C-glucopyranosyl)-ethanedione and two known compounds C-â-D-glucopyranosyl-2,6-dihydroxyl benzene and sequiterpene were isolated. (GAIROLA, S. et al 2010)

III. METHOD OF FORMULATION DEVELOPMENT

Preparation of Blank Liposomes: The preparation of liposomes based on the bath sonication method. For the preparation of liposomes Cholesterol, Chloroform, Phospholipids, & Phosphate buffer saline having a pH 7.4 was taken and weighed accurately as described in table and then mix cholesterol, Phospholipids and cholesterol in a clean beaker uniformly and then add this mixture into the Phosphate buffer saline having pH 7.4 with continues steering and then transfer the mixture into rotary flash evaporator at 60oC to evaporate the whole chloroform to make SUV type of liposomes but the vesicle size may be big so that for the reduction of size bath sonication for 30 min was done.

Fig 1: Blank liposomes

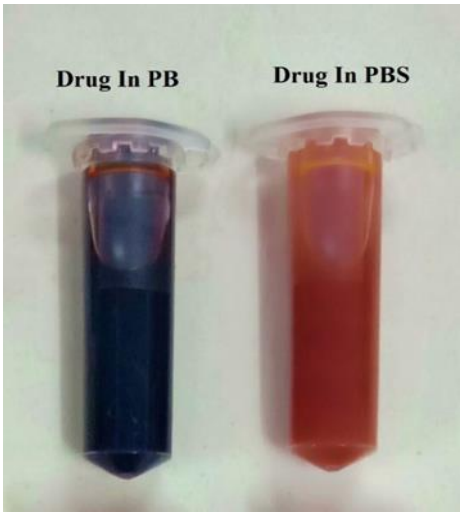

Fig 2: Drug incorporation in PB and PBS solutions.

Preparation of Drug Loaded Liposomes: It was also based on the bath sonication method. For the preparation of the drug loaded liposomes extract of Pterocarpus marsupium, Cholesterol, Chloroform, Phospholipids, & Phosphate buffer saline and Phosphate buffer both having pH 7.4 was taken and weighed accurately as described in the table and then two batches of the liposomes was prepared in which one was prepared in Phosphate buffer saline and second was prepared in Phosphate Buffer having pH 7.4 in this extract of Pterocarpus Marsupium, cholesterol, chloroform, Phospholipids mixed in two different beakers and then add into both buffer solutions respectively with continuous stirring and then transfer both batches one by one into the rotary flash evaporator at 60oC to evaporate the whole chloroform to make SUV type of liposomes but the vesicles size may be big so that for the reduction of size was done by Bath Sonicator for 30 min.

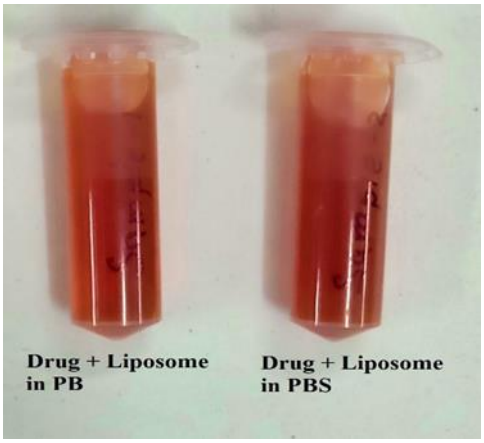

Fig 3: Developed batches of liposomes of Pterocarpus marsupium in PB and PBS solutions respectively.

Table 1: Batch preparation list for formulation development

S.No.	Formulation	Lecithin	Cholesterol	Chloroform	DOPC	DPPC	Drug	PB solution	PBS Solution
1	Blank	134mg	67mg	7ml	8.3mg	8.3mg	-	100ml	-
2	Drug in PB	-	-	-	-	-	2gm	100ml	-
3	Drug in PBS	-	-	-	-	-	2gm	-	100ml
4	F1	134mg	67mg	7ml	8.3mg	8.3mg	4gm	150ml	-
5	F2	134mg	67mg	7ml	8.3mg	8.3mg	4gm	-	150ml

Inference: According to this table various formulations was developed for the treatment of diabetes which was evaluated by using various evaluation parameters.

Characterization of Pterocarpus marsupium loaded liposomes:

FTIR spectroscopy of blank liposomes:

50 mg of dried form of liposomes mixed with 100 mg of spectral grade KBr and pressed into disc under hydraulic pressure. Then FTIR spectra were recorded in the 4000-400 cm-1 range.

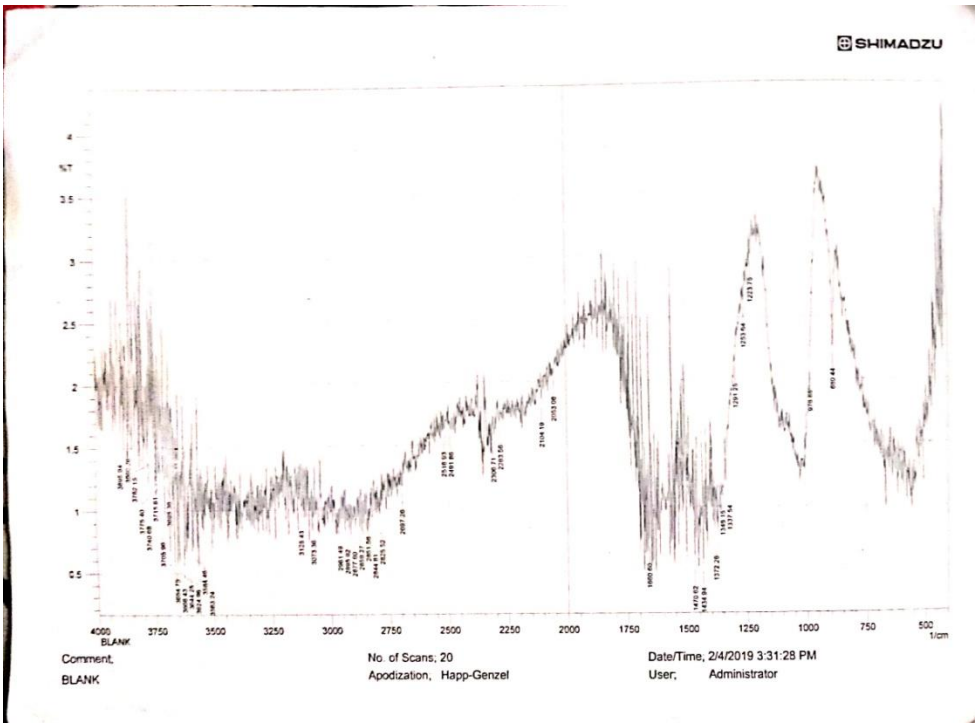

Fig 4: FTIR spectra of blank liposomes.

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Interpretation of Blank liposomes:

Functional Groups	Wavenumber (cm^{-1})
≡C-H-	2851.58
C=C-H-	3073.36
C-H Aromatic	3073.36
O=C-H- Aldehyde-	2697.26
-OH- Phenols-	3624.96-3644.25
-OH -Hydrogen bonded alcohol-	3230-3390.98
≡ N-H- Amines	3350-3501.38
-C-O- Alcohols, ethers-	1223.75-1291.25
≡C-N-Amines-	1223.75-1348.15
>C=C<Alkenes-	1660.60
-NO2-Nitro compound-	1337.54-1470.62
C-H- Stretching-	2851.56-2961.49

Inference: According to this spectral analysis there are long chain polymers was present with some aldehyde, amines, alcoholic ethers, OH bonds, Nitro compounds etc. In this spectra various double and triple bonded carbon molecules in chain form was present.

FTIR spectroscopy of Pterocarpus marsupium extract loaded liposomes:

Both batches were taken for the FTIR spectral. 50 mg of dried form of both samples of Pterocarpus marsupium extract loaded liposomes mixed with 100 mg of spectral grade KBr and pressed into disc under hydraulic pressure. Then FTIR spectra were recorded in the 4000-400 cm-1 range

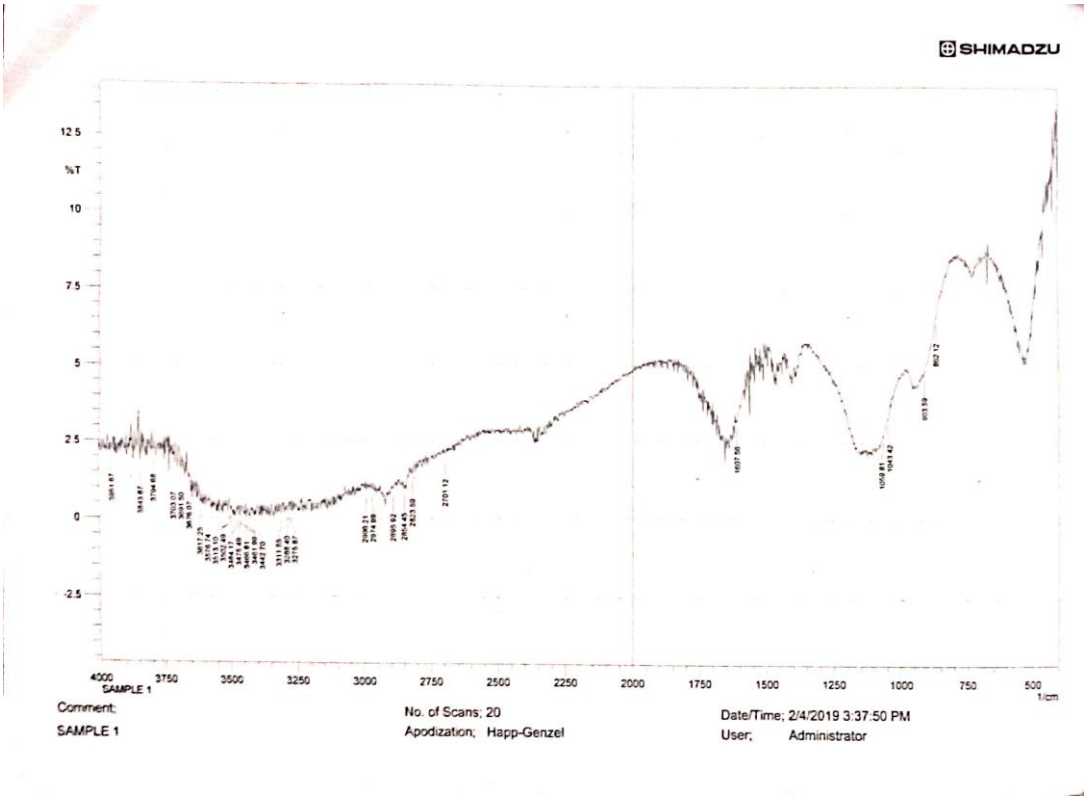

Fig 5: FTIR spectra of the drug loaded liposomes in PB solution.

Interpretation of Drug loaded liposomes in PB

Functional Groups	Wavenumber (cm-1)
≡C-H-	2854.59- 2895.92
C=C-H-	3030-3095.98
C-H Aromatic	3030-3095.98
O=C-H- Aldehyde-	2666.53-2854.45
-OH- Phenols-	3617.25
-OH -Hydrogen bonded alcohol-	3275.87-3311.55
≡ N-H- Amines	3311.55-3502.49
-C-O- Alcohols, ethers-	1043.42-1059.81
C-H- Stretching-	2895.92

Inference: According to this spectral analysis some basic components like aromatic ring aldehyde, phenolic compounds, OH bonds, amines etc was matched with the normal drug spectra and some traces of the long chain molecules were present in it. It was show that there was no interaction between drug and polymer.

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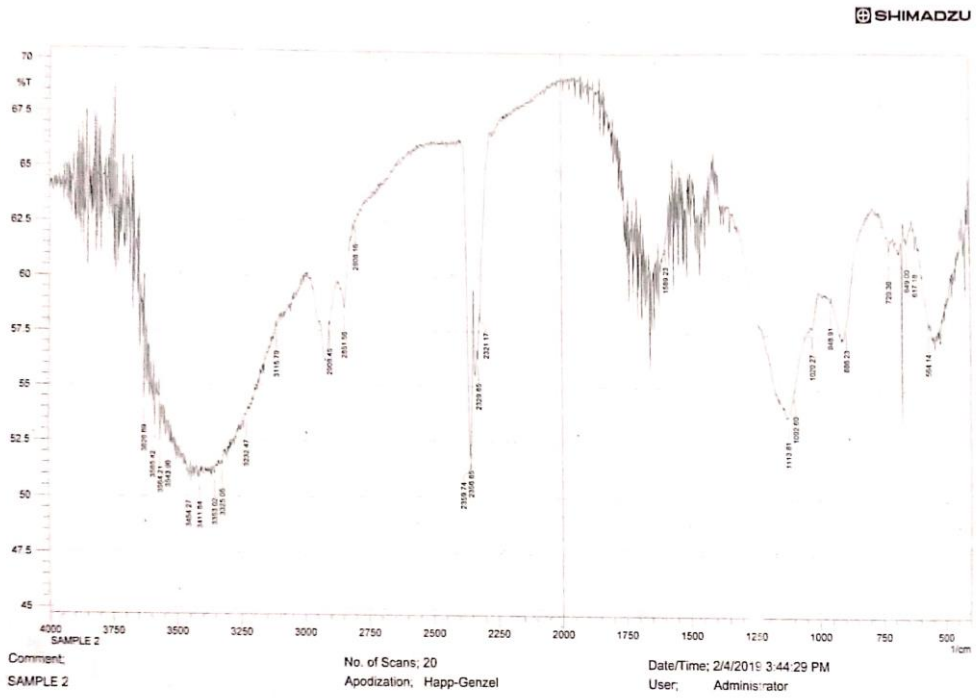

Fig 6: FTIR spectra of drug loaded liposomes in PBS solution.

Interpretation of Drug loaded liposomes in PBS:

Functional Groups	Wavenumber (cm-1)
≡C-H-	2823.59
=C-H-	3030.93-3083.96
C≡C-H-	3300.94
C-H Aromatic –	2988.49-3083.49
O=C-H- Aldehyde-	2767.66-2853.49
O-C-O- ketone-	1090.67
-OH Phenols-	3605.67
-OH- Hydrogen bonded alcohol-	3237.29-3419.56
≡N-H- Amines-	3300.94-3501.52
-C-C- Aromatic ring-	1515.39- 1565.83
C-H Stretching-	2823.59-2918.10

Inference: According to the spectral analysis of this sample was observed that it contains the aromatic, phenolic compounds with some aldehyde, ketones and amines which was matched with the drug spectra and some other elements like long chain acids and carbon double and triple bonds was present. It was show that there was no interaction between drug and polymer with respect to this spectral analysis.

Percentage of Drug Entrapped:

Total Amount of Pterocarpus marsupium extract = 8 gm which is divided into two equal parts 4 + 4 gm which were further divided **in** to 3 small batches of 1.3 gm each to make 6 different batches so that the percentage of drug entrapped in liposomes was

Liposomes in Phosphate buffer:

Table7.3: Percentage encapsulation of batch 1

S.No	Sample size(gm)	Wavelength	Conc in gm/150 ml
1	1.3	3.010	0.691
2	1.3	3.020	0.693
3	1.3	3.005	0.690
Mean			0.691

Amount of unbound drug in the phosphate buffer: 0.69gm/ 150 ml

So that the percentage of drug entrapment = $\frac{4 - 0.69}{4} * 100$

= 82.7%

Liposomes in Phosphate buffer saline:

Table7.4: Percentage encapsulation of batch 2

S.No	Sample size(gm)	Wavelength	Conc in gm/150 ml
1	1.3	2.552	0.585

2	1.3	2.563	0.588
3	1.3	2.545	0.583
Mean			O.585

Amount of unbound drug in Phosphate buffer saline: 0.59gm/150ml

So that the percentage of drug entrapment = $\frac{4 - 0.59}{4} * 100$

= 85.25%

Inference: This study shows that about 4 gm of the drug was incorporated into the liposomal formation in which about 0.69 and 0.59gm of drug was still remain outside the in PB and PBS respectively. So that about 3.31gm in PB and 3.41gm in PBS was founded to be bound.

Permeation study:

Table7.5: Permeation rate of drug through membrane with respect time of formulation 1 in PB

time (min)	first batch	second Batch	Third Batch	Mean
0	3.99	3.43	3.9	3.773333333±11.30999
30	12.79	12.65	12.81	12.75±11.30999
60	29.22	29	29.91	29.37667±11.30999
90	33.42	32.96	33	33.12666667±11.30999
120	27.46	28	27.66	27.70666667±11.30999
150	25.38	25.67	24.91	25.32±11.30999
Mean				22.00889±11.30999

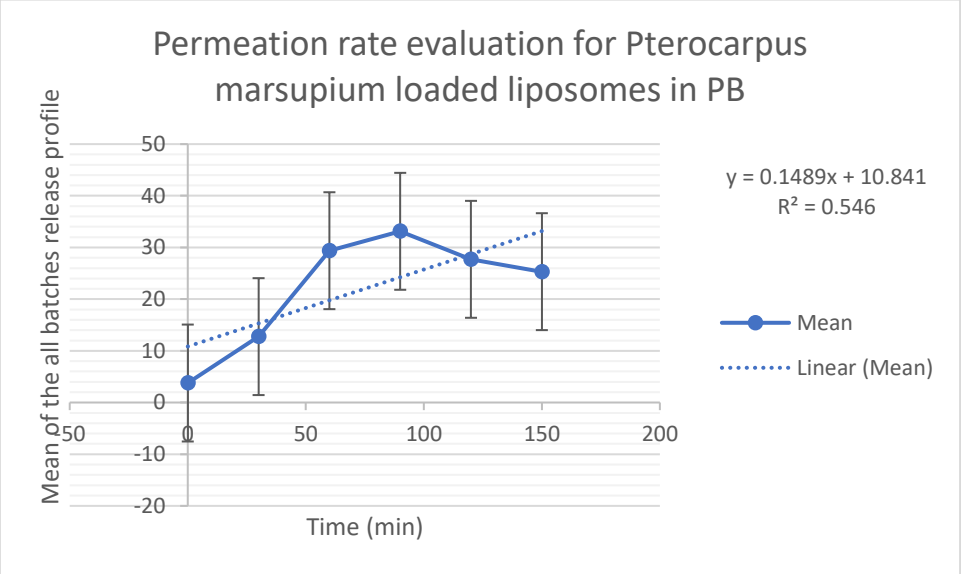

Fig7: Permeation rate of *Pterocarpus marsupium* liposomes in Phosphate buffer.

Table7.6: Permeation rate of drug through membrane with respect time of formulation 2 in PBS

time (min)	first batch	second Batch	Third Batch	Mean
0	2.99	2.54	2.9	2.81±11.41885
30	11.79	11.65	11.81	11.75±11.41885
60	27.22	27.66	27.91	27.59666667±11.41885
90	32.42	32.96	33.62	33±11.41885
120	26.46	27.56	26.66	26.89333333±11.41885
150	24.38	24.67	24.91	24.65333333±11.41885
Mean				21.11722222±11.41885

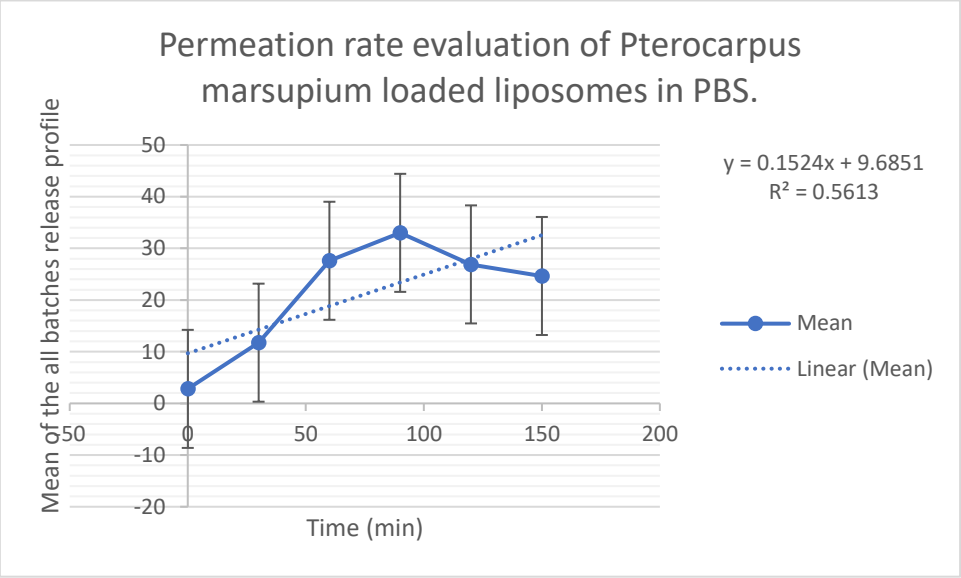

Fig 8: Permeation rate of *Pterocarpus marsupium* liposomes in Phosphate Buffer saline.

Inference: After taking the U.V. Spectra of the release profile show that at the initial time period the drug concentration was increased showing first order non-linear kinetics up to reaching a time period 1:30 hr. after that a liner decrease the drug concentration. The reason behind to perform this study to examine the enhancement of the permeation rate of the Pterocarpus marsupium in the liposomal suspension form membrane to achieve the better absorption of drug though membrane by enhancing permeation rate

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Determination of particle size:

The particle size of Pterocarpus marsupium loaded liposomes can be measured by under optical microscopy (Quasmo Phase contrast microscopy) to evaluate the formation of liposomes and the particle size was determined by Malvern zeta size analyser.

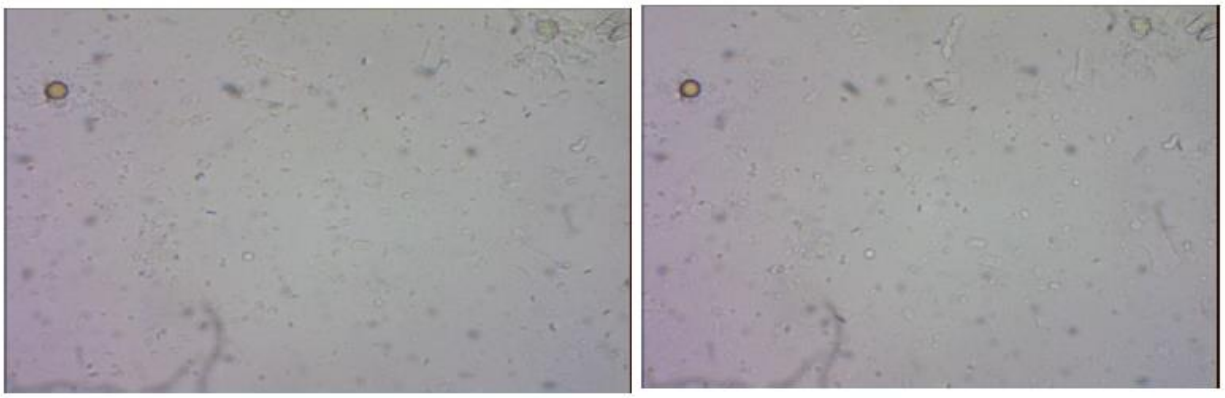

Fig 8: Microscopic detection at 45X magnification for Formulation 1 in PB

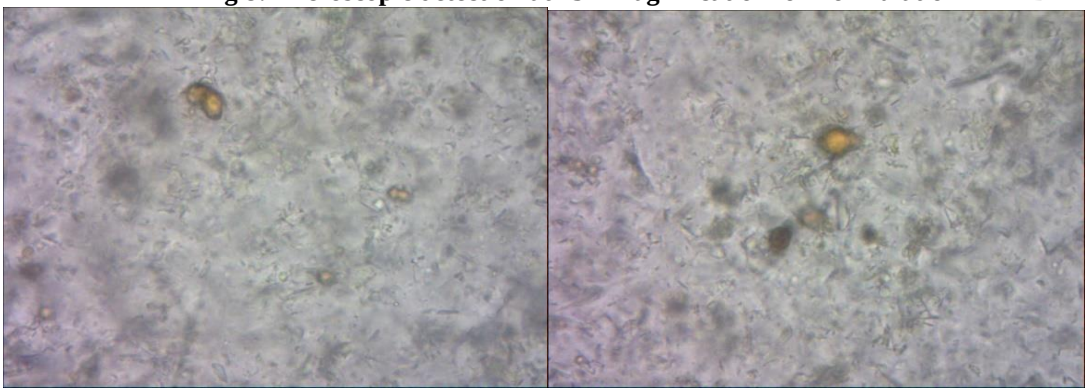

Fig 9: Microscopic detection at 45X magnification for formulation 2 in PBS.

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After taking a number of images and calculating the size of the liposomes which are able to see through the optical microscope being calculated by using stage micrometer size calculation method.

Table7.7: Calculation for size detection of liposomes by using Stage micrometer method.

Scope particle size	magnification factor	Actual size in um
11	15	165
15	15	225
10	15	150
10	15	150
10	15	150

But some liposomes were in the nano range which would be identified by using Malvern Zeta sizer which was as follows.

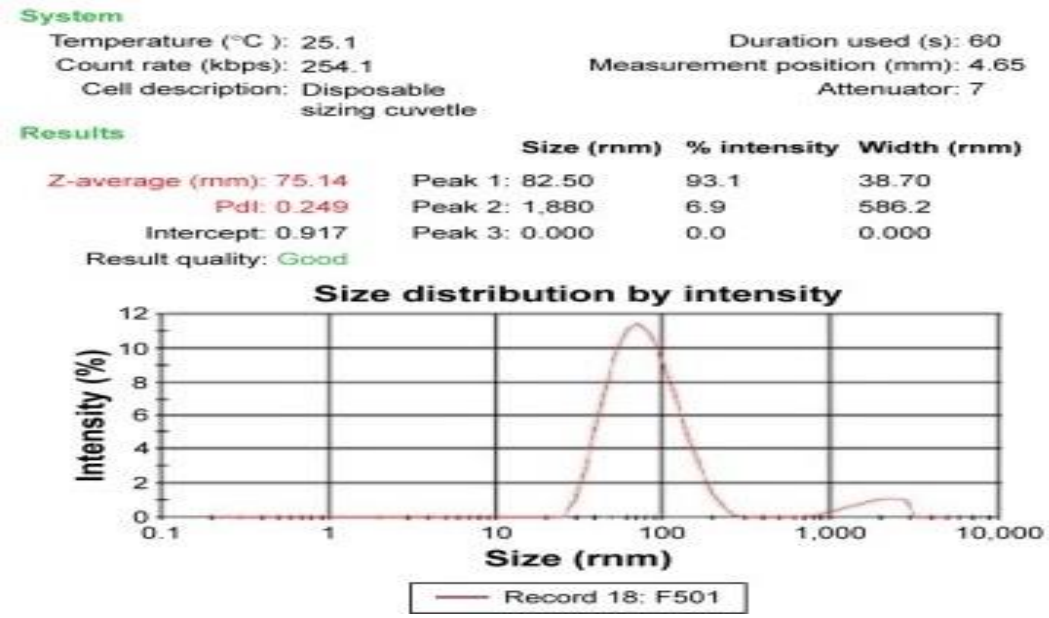

Fig 10: Malvern Zeta sizer graph for size detection of *Pterocarpus marsupium* loaded liposomes in PB solution.

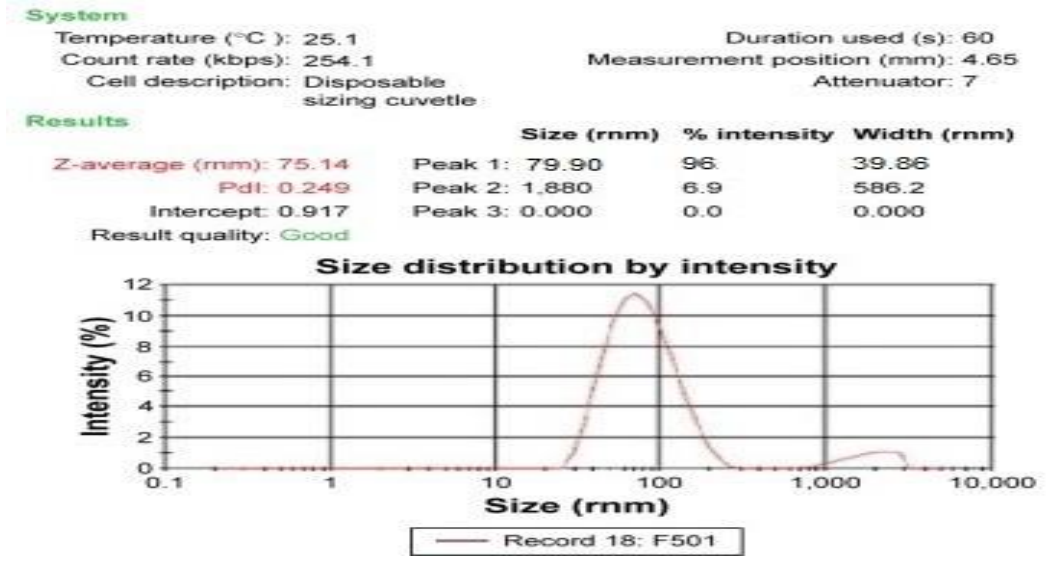

Fig 11: Malvern zeta sizer graph for size detection of the *Pterocarpus marsupium* loaded liposomes in PBS solution.

Inference: According to the Optical microscopy (45X magnification) of the both samples was observed 150-225 um range of some liposomal droplets but major liposomes were present in nano range which was calculated by Malvern Zeta sizer the peaks shows the first region of about radius 82.50nm and width about 38.70nm which shows the maximum range 93.1 % of liposomes in PB solution and According to Malvern Zeta sizer the peaks shows the first region of about radius 79.90nm and width about 39.86nm which shows the maximum range 96% of liposomes in PBS.

Determination of zeta potential:

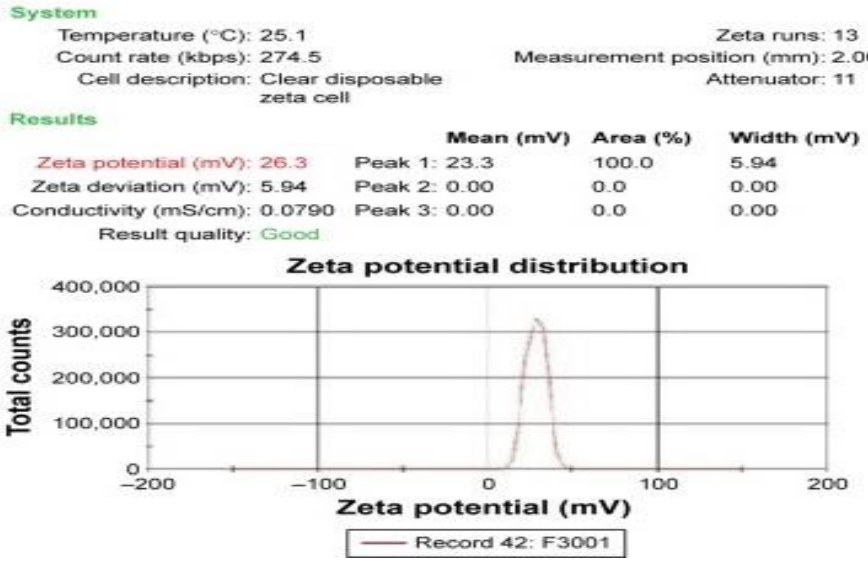

Fig 12 : Malvern Zeta potential for evaluating the potential of the drug loaded liposomes in PB solution.

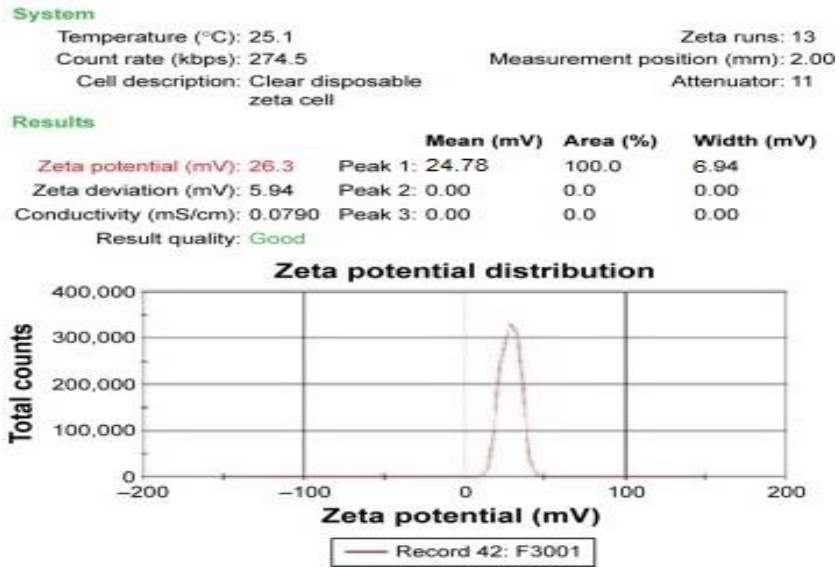

Fig 13: Malvern Zeta Potential for evaluating Potential of the drug loaded liposomes in PBS solution.

Inference: According to the Malvern Zeta potential the mean Potential of the sample 1 was 26.3mV and the mean first peak potential was 23.3mV and width was 5.94mV for liposomes in PB solution and according to the Malvern Zeta Potential the mean potential of the sample 2 was 26.3mV and the mean of the first peak was 24.78 and its width was 6.94mV for liposomes in PBS solution.

Stability Studies:

The stability of the Pterocarpus marsupium extract encapsulated liposomes was evaluated after storage at 2-8ºC, room temperature and 45ºC for three months. The particle size distribution and drug encapsulation efficiency of the samples were determined as a function of the storage time.

Batch One

Table7.8: Stability study of the *Pterocarpus marsupium* loaded liposomes in PB solution.

Parameters	Changes due to storage								
	Within 15 days			Within 1 month			Within 3 months		
	2-8ºC	RT	45ºC	2-8ºC	RT	45ºC	2-8ºC	RT	45ºC
Drug Remaining	3.4	3.4	3.4	3.4	3.4	3.4	3.3	3.3	3.3
pH	7.2	7.1	7.3	6.8	6.7	6.9	6.3	6.7	6.8
Color Change	No change	No change	No change	No change	No change	No change	No change	No change	No change

Batch Two

Table7.9: Stability study of Pterocarpus marsupium loaded liposomes in PBS solution.

Parameters	Changes due to storage								
	Within 15 days			Within 1 month			Within 3 months		
	2-8ºC	RT	45ºC	2-8ºC	RT	45ºC	2-8ºC	RT	45ºC
Drug Remaining	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
pH	7.4	7.4	7.4	7.2	7.3	7.3	7.1	7.2	7.0
Color Change	No change	No change	No change	No change	No change	No change	No change	No change	No change

Inference: In between the duration of stability study of the sample products there was some slightly changes in concentration were occur which was evaluated by using UV method and there were also change in pH but not change in color.

In-Vitro study of Pterocarpus marsupium loaded liposomes:

Table7.10: percentage inhibition of α Amylase by Acarbose

Concentration (ug/ml)	Absorbance	Percentage inhibition
50	0.832	41.44%
100	0.703	50.52%
200	0.625	56.01%
400	0.426	70.02%
800	0.226	84.09%

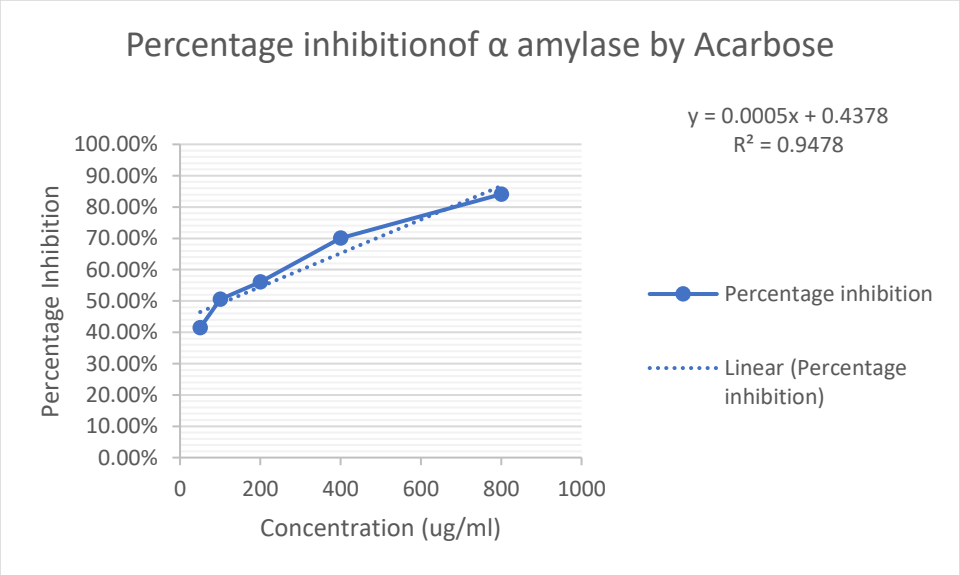

Fig 14: Graph of Percentage inhibition of α amylase by acarbose

Table7.11: Percentage inhibition of α Amylase by Pterocarpus marsupium loaded liposomes.

Concentration (ug/ml)	Absorbance	Percentage inhibition
100	1.11	21.88%
200	0.951	33.07%
400	0.642	54.82%
800	0.531	62.63%
1000	0.41	71.14%

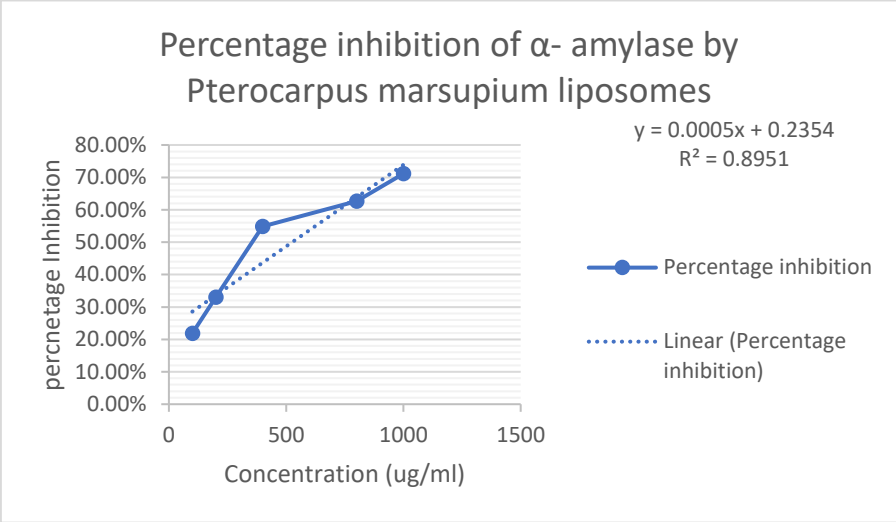

Fig 15: Percentage inhibition of α amylase by Pterocarpus marsupium loaded liposomes.

Inference: The IC50 value of positive control was found to be 180 µg/ml and that of biosynthesized *Pterocarpus marsupium* liposomes were 700 µg/ml. The percentage inhibition of Acarbose and *Pterocarpus marsupium* liposomes at lower and higher concentration was found to be 41.44% and 84.09% for positive control Acarbose and 21.88% and 71.14% respectively. Which was similar to the study performed on the Acarbose and *Pterocarpus marsupium* extract.

IV. CONCLUSION

In this study the liposomes of the *Pterocarpus marsupium*shows good results with comparison the synthetic antidiabetic drugs. It shows the good permeability, stability, In- Vitro Inhibition of the alpha- amylase with reference of acarbose and Zeta sizer and microscopic images shows the wide range of the vesicle size upto um- nm range.

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