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Development and Evaluation of Hydroalcoholic Extract of *Alhagi Camelorum* Loaded Liposomes

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Abstract
The extract of *Alhagi camelorum* (AC) has poor solubility and bioavailability. The present work aimed to develop an AC loaded liposome to improve the solubility and bioavailability. A liposome was prepared using thin film hydration method, and the optimized liposomal formulation was prepared using soya phosphatidylcholine (PC) and cholesterol (CH) and AC in the molar ratio (7:3:5). Formulation optimization was done using central composite design of response surface methodology. Based on preliminary experiment, the three independent variables X1, X2, X3 where, sonication time (X1; mins), ratio of Phospholipid: Cholesterol (X2; mg), Temperature (X3; degree) and dependent variables are Y1, Y2 where, Particle size (Y1; nm), Entrapment efficiency (Y2;%). *In vitro* drug release of AC extract and AC loaded liposome were assessed and found that 49.45% of extract from liposomes was released at pH 7.4 within 48 hours. The AC loaded liposome had particle size (101.2±2.19). The drug encapsulation efficiency of AC loaded liposome was 75%. The internal morphology of optimized liposomal formulation was seen by transmission electron microscopy and the surface morphology was done by scanning electron microscopy. This confirmed the usefulness of the liposomal delivery system for to improve solubility and bioavailability.

Keywords: *Alhagi camelorum,* novel drug delivery system, liposomes, particle size, entrapment efficiency

1. Introduction

The liver is responsible for breaking down toxins and converting them into less harmful substances that can be excreted from the body. It also plays a crucial role in drug metabolism, ensuring that medications are processed and eliminated efficiently. The liver is the main organ involved in the metabolism and excretion of a wide range of contaminants from the environment as well as medicinal medicines. These xenobiotic may negatively affect the structural and functional integrity of this important organ, either directly or indirectly (after bio activation), which can manifest clinically as inflammatory, non-inflammatory, or degenerative hepatic diseases. Therefore, exposure to high levels of environmental toxins or prolonged use of certain medications can lead to liver damage and disease. Alternative therapies, such as herbal medicine and acupuncture, have been explored as potential treatments for hepatic diseases, but more research is needed to determine their safety and efficacy. Additionally, lifestyle changes such as maintaining a healthy weight and avoiding alcohol and certain medications can help prevent and manage hepatic diseases. In modern medicine, symptomatic relief is provided by corticosteroids or immunosuppressive drugs, which, due to their high concentration of a single active ingredient (in virtually all cases), carry a toxicological burden, that may or may not be readily apparent to the recipient. On the other hand, herbal medicine represents a more multifaceted approach to health care because it addresses a multi-factorial approach to restoring health, seeks equilibrium between mind, body, and spirit, and often utilises a combination of plant-based ingredients that work synergistically to promote healing and prevent disease. Additionally, herbal medicine has a long history of use in various cultures and is often considered to be more natural and sustainable than conventional medicine. and environment, and places a greater emphasis on the multidimensional elements of health than on pathology alone. A variety of herbal medicines (with claimed liver-protective properties) are marketed across the world; but, with a few exceptions, scientific confirmation of these claims is not available [1-3].
However, phytotherapeutics needs a scientific approach to deliver the components in a sustained manner so as to increase patient compliance and avoid repeated administration. This can be achieved by designing novel drug delivery systems for herbal constituents. Novel drug delivery systems not only reduce the repeated administration (due to its sustained-release properties) to overcome non-compliance, but also help to increase the therapeutic value by reducing toxicity, increasing the bioavailability, stability, and targetability to a specific cell or organ (due to its sub-cellular size) [4-6]. Recently, pharmaceutical scientists have shifted their focus in designing a drug delivery system for herbal medicines using a scientific approach. For a long time, herbal medicines were not considered for development as novel formulations owing to lack of scientific justification and

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processing difficulties, such as standardization, extraction and identification of individual drug components in complex polyherbal systems. However, modern phytopharmaceutical research solves the scientific needs for herbal medicines as in modern medicine, which gives way for developing novel formulations such as nanoparticles, microemulsions, matrix systems, solid dispersions, liposomes, solid lipid nanoparticles and so on [7-10]. Moreover, these formulations can improve the solubility and permeability of poorly soluble herbal compounds, which can increase their absorption and distribution in the body. This can lead to better clinical outcomes and reduce the required dosage of the herbal medicine. These novel formulations enhance the therapeutic efficacy, bioavailability and stability of herbal medicines. They also provide targeted drug delivery and controlled release of active compounds, making them more effective and safe for use. Furthermore, the use of these formulations can also help in reducing the potential side effects associated with traditional herbal medicines. This makes them a promising alternative for patients who are looking for natural remedies with fewer adverse effects.

2. Materials and Methods

2.1. Materials

Alhagi camelorum were procured from local market. phosphatidylcholine and cholesterol were procured from Sigma Aldrich (New Delhi, India). Double distilled millipore water was used for formulation and evaluation. All the chemicals and reagents were of analytical grade and were used as received.

2.2. Optimization of Formulation Using Response Surface Methodology

Based on the preliminary experiment, the independent variables, which have relatively great influence on the entrapment efficiency and particle size dependent variable were chosen. While sonication time (X1), phosphatidylcholine and cholesterol ratio (X2) and temperature (X3) were chosen. The experiment procedure named central composite design (CCD) of response surface methodology is used for optimization. Briefly, these three factors were evaluated and experimental trials were performed at all 20 possible combinations. The regression model coefficient for the percent drug entrapment efficiency and the particle size of AC loaded liposomes were evaluated [11-15].

Table 1: List of variables used in the formulation optimization designing and their levels

Variable	Coded	Coded Level					ΔX
		(-α)	-1	0	1	(+α)	
Sonication time (min)	X1	1.18	2	4	6	6.82	2
Ratio of PC:CH (7:3)	X2	0.49	0.33	2.33	4.33	5.15	2
Temp. (ºC)	X3	37.18	38	40	42	42.82	2

2.3. Formulation of *Alhagi Camelorum* Extract Loaded Liposomes

AC extract loaded liposome was prepared by thin lipid film hydration or solvent evaporation method. Briefly, the 25 mg/ml stock solution of soya-phosphatidylcholine and cholesterol was dissolved in chloroform, similarly the stock solution of 5mg/ml of AC extract in ethanol was also prepared. Experimentally, predetermined value according to the formulation ratios of all excipient and drug were pipetted out in a 10ml of round bottom flask to remove the organic solvents and made a thin lipid film. The flask was vacuum dried using rotary evaporator 100 rpm and 40°C bath temperatures to form a thin film, then film was freeze dried to ensure complete removal of organic solvents. The formed lipid film was further hydrated overnight with PBS (pH 7.4). The obtained dispersion was sonicated using probe sonicator with (30% amplitude, 10 secs on off pulse) for 2 to 6 mins, to reduce the size of the vesicles [16-17].

2.4. Evaluation and Characterization

i). Evaluation

- **Entrapment Efficiency**

For entrapment and loading efficiency study, the liposomes were ultra-centrifuged at 15000 rpm for 60 minutes at 4ºC by using eppendorf centrifuge 5417R to separate the free extract from suspension. A clear solution of supernatant and pellets of liposomes were obtained. The supernatant was collected and absorbance was taken against same blank solution in UV visible spectrophotometer at 285nm for actual entrapped and loaded extract concentration in liposomes. The entrapment and loading efficiency were calculated using eq.2 & eq.3 [18, 19].

$$\%EE = \frac{\text{Total drug-Free drug}}{\text{Total drug}} * 100 \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \text{E.q.1}$$

ii). Percent Extract Release

In-vitro drug dissolution study was performed in USP dissolution apparatus (EDT-08Lx) using dialysis bag (MW cut off 14000 Da). Liposomal formulation containing extract and their respective blank solution was enclosed in dialysis bag and the end of bags were sealed neatly and tightly and bags were immersed in baskets, dissolution jars were filled up with 200 ml media solution DMSO: PBS (0.5:9.5) of pH 7.4 and the assembly was maintained at 37°C with continuous stirring at 150rpm, 2 ml sample from each jar were withdrawn and exchanged with fresh media solvent at a predetermined time interval 0,15,30,60 mins, 2, 4, 6,8,20,24,48hrs, the sink condition of dissolution jar was maintained with exchange of fresh media in every withdrawn of sample. Finally, the samples were suitably diluted and measured by using u.v. visible spectroscopy at 285nm [20].

- **Characterization of Formulation: C**haracterization of formulation with used excipients and AC extract was done to study the morphology of liposomal vesicle and internal structure.
- **Scanning Electron Microscopy (SEM):** SEM analysis was used to detect surface morphology of vesicles. Briefly, prior to examine, a drop of sample was fixed on brass stub using a pipette and excess amount of sample were squeezed out with a syringe and dried overnight. The sputter was coated with gold-palladium. Stub was completely dried, and the pictures were taken using instrument Quanta 250, the instrument was set at different magnifications for the sufficient detail of the surface morphology of samples [21].
- **Transmission Electron Microscopy (TEM):** Using the technique of transmission electron microscopy, the internal shape and particle size of the liposome loaded with AC extract were evaluated. On a copper grid with a 200-mesh mesh, a drop of liposome was applied, allowed to adsorb, and any extra was scooped up with filter paper. After adding a drop of 2% (w/v) uracyl acetate, the sample was kept exposed to it overnight. Before imaging the

vesicles at various magnifications with a TEM running at a voltage of 200kv, the sample was dried at room temperature [22].

3. Result and Discussion

3.1. Optimization of AC Extract Loaded Liposomal Formulation

The experimental data were fitted to a quadratic polynomial equation which is as follows:

Y1(nm)=93.79+13.53*X1-16.39*X2-1.91*X3-12.65*X1*X2 1.52*X1*X3+3.53 *X2*X3+13.95*$X1^2$+22.28*X22+4.16*X32.....Eq 2

Entrapment efficiency Y2 (%) = 77.74 + 0.89*X1 + 2.57 * X2 + 2.91*X3+0.93*X1*X2 1.07*X1*X3-1.05*X2*X3-0.66*X12-2.22*X22- 2.69*X32……. ……..Eq.3

The positive value in equations (2) and (3) demonstrated that altering the independent variables will result in a significant alteration of the manufactured liposomes' particle size and entrapment efficiency. The model is likely important because of its f-value of 24.02. Only 0.01% of the time could noise be the cause of a significant model f-value. In this scenario, X1, X2, X1X2, X12, and X22 values larger than 0.1000 indicate that the model terms are not significant. Values of probe>f less than 0.0500 suggest that the model terms are significant. The pred r-squared of 0.8477 and ade r-squared of 0.9160 are reasonably in accord. In this study, the signal to noise ratio of 14.536 shows an appropriate signal; a ratio of larger than 4 is preferable. This model can also be utilized to explore the design space. The greater positive value in the equation was noticed during the verification experiments. The value of 6 (+22.28) shows that the particle size value is highly influenced by the PC: CH ratio, that temperature has no effect on formulation particle size, and that the value of time in the equation is (-1.91). The model's f-value of 6.99 suggests that it is significant, and there is only a 0.27% possibility that noise may result in a large f-value. In this scenario, X2, X3, X22, and X32 are significant model variables because the values of prob>f smaller than 0.0500 indicate that they are. The model terms are not important if the value is bigger than 0.1000. The lack of fit f-value of 0.71 indicates that the lack of Fit is not substantial when compared to the pure error, and a large lack of fit value has a 64.36% likelihood of being caused by noise [23]. The signal-to-noise ratio is measured with adequate precision. This approach can be used to navigate the design spaces. A ratio greater than 4 is preferred, and this ratio of 7.348 suggests an adequate signal. Three-dimensional (3D) response surfaces and two-dimensional (2D) contour diagrams are displayed in Fig 1 & 2. The interaction between process variables, as shown by contour plot, are found to be very prominent as reflected in counter plot plotted between X1X2 (Elliptical plot; Fig. 1(A)).

Table 2: Central composite design for formulation optimization

S. No	Coded value			Actual value			Y_1 (nm)	Y_2 (%)
	X1	X2	X3	$X1^2$ (in min)	$X2^2$ (in mg)	$X3^2$ (in °C)		
1	-1.00	-1.00	-1.00	2	0.33	35	133.2	65.92
2	1.00	-1.00	-1.00	6	0.33	35	177.6	66.61
3	-1.00	1.00	-1.00	2	4.33	35	115.1	70.03
4	1.00	1.00	-1.00	6	4.33	35	120.5	77.82
5	-1.00	-1.00	1.00	2	0.33	45	118	72.33
6	1.00	-1.00	1.00	6	0.33	45	167.9	72.1
7	-1.00	1.00	1.00	2	4.33	45	125.6	75.59
8	1.00	1.00	1.00	6	4.33	45	113.3	75.72
9	-1.68	0.00	0.00	1.18	2.33	40	104.7	74.97
10	1.68	0.00	0.00	6.82	2.33	40	162.6	77.24
11	0.00	-1.68	0.00	4	0.49	40	187.4	67.88
12	0.00	1.68	0.00	4	5.15	40	127	75.52
13	0.00	0.00	-1.68	4	2.33	32.95	107.3	63.12
14	0.00	0.00	1.68	4	2.33	47.05	104.6	77.62
15	0.00	0.00	0.00	4	2.33	40	110.6	79.58
16	0.00	0.00	0.00	4	2.33	40	101.2	79.64
17	0.00	0.00	0.00	4	2.33	40	90.6	78.47
18	0.00	0.00	0.00	4	2.33	40	89.6	78.42
19	0.00	0.00	0.00	4	2.33	40	90.6	77.95
20	0.00	0.00	0.00	4	2.33	40	80.6	72.32

Table 3: Summary of ANOVA for response parameters of particle size (Y1) in CCD

Source	Sum of Squares	Df	Mean Square	F value	p-value Prob>F
Model	16785.90	9	1865.10	24.02	<0.0001
X1	2500.00	1	2500.00	32.20	0.0002
X2	3666.85	1	3666.85	47.22	<0.0001
X3	50.04	1	50.04	0.64	0.4408
X1X2	1280.18	1	1280.18	16.49	0.0023
X1X3	18.60	1	18.60	0.24	0.6351
X2X3	99.41	1	99.41	1.28	0.2843
$X1^2$	2805.57	1	2805.57	36.13	0.0001
$X2^2$	153.04	1	7153.04	92.12	<0.0001
$X3^2$	249.31	1	249.31	3.21	0.1034
Residual	776.51	10	77.65		
Lack of fit	235.67	5	47.15	0.44	0.8084
Pure Error	540.83	5	108.17		
Cor Total	17562.40	19			

Table 4: Summary of ANOVA for response parameters of Entrapment Efficiency (Y2) in CCD

Source	Sum of Squares	Df	Mean Square	F Value	p-value, Prob>F
Model significant	401.78	9	44.64	6.99	0.0027
X1	10.89	1	10.89	1.71	0.2207
X2	89.95	1	89.95	14.09	0.0038
X3	115.67	1	115.67	18.11	0.0017
X1X2	6.96	1	6.96	1.09	0.3212
X1 X3	9.20	1	9.20	1.44	0.2576
X2X3	8.90	1	8.90	1.39	0.2650
X12	6.28	1	6.28	0.98	0.3446
X22	70.88	1	70.88	11.10	0.0076
X32	104.12	1	104.2	16.30	0.0024
Residual	63.86		10	6.39	
Lack of Fit	26.45	5	5.29	0.71	0.6436
Significant pure Error	37.41	5	7.48		
Cor Total	465.64	19			

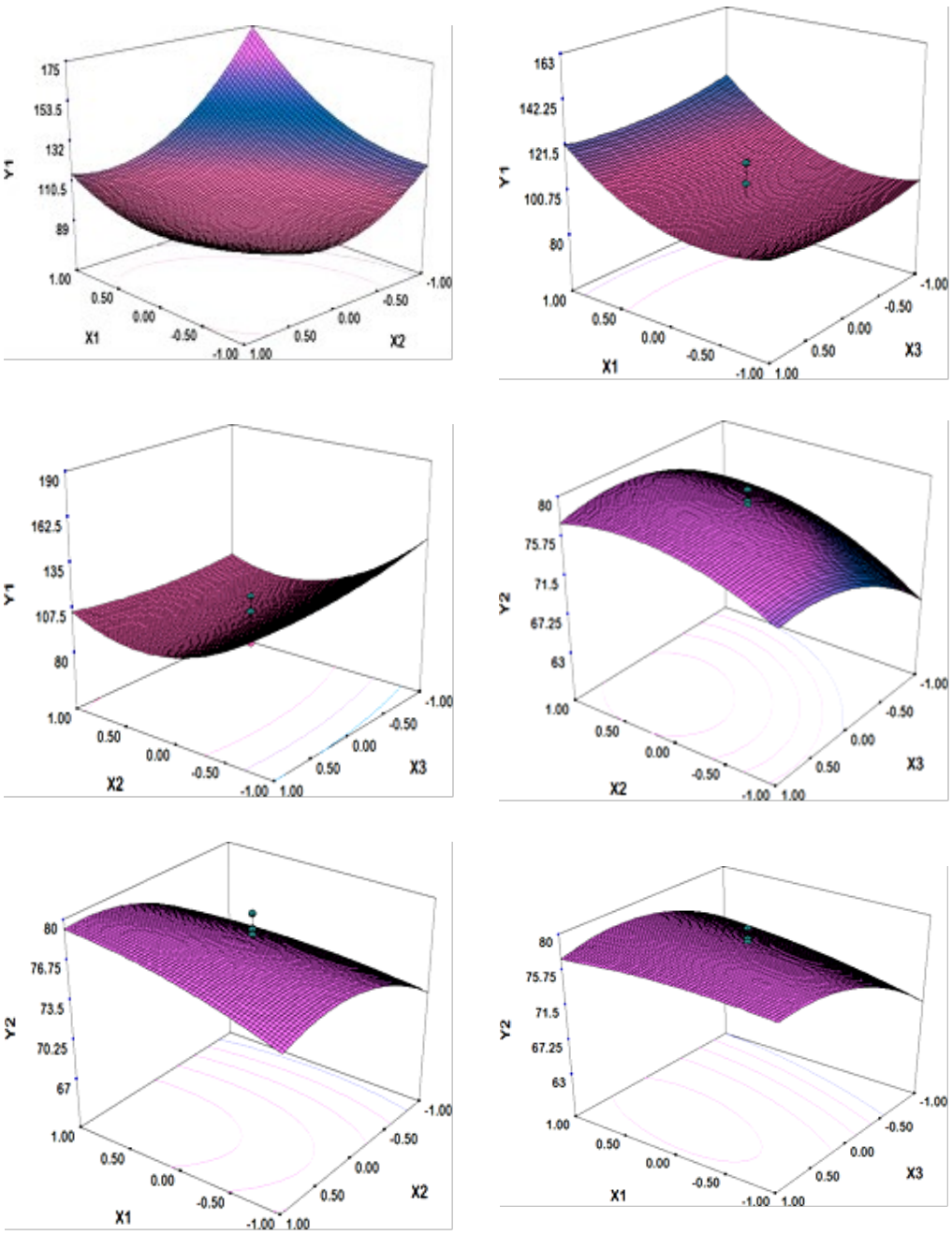

Fig 1: 3D graphs of independent variables (i) Particle size [Y1] (ii) Entrapment efficiency [Y2]

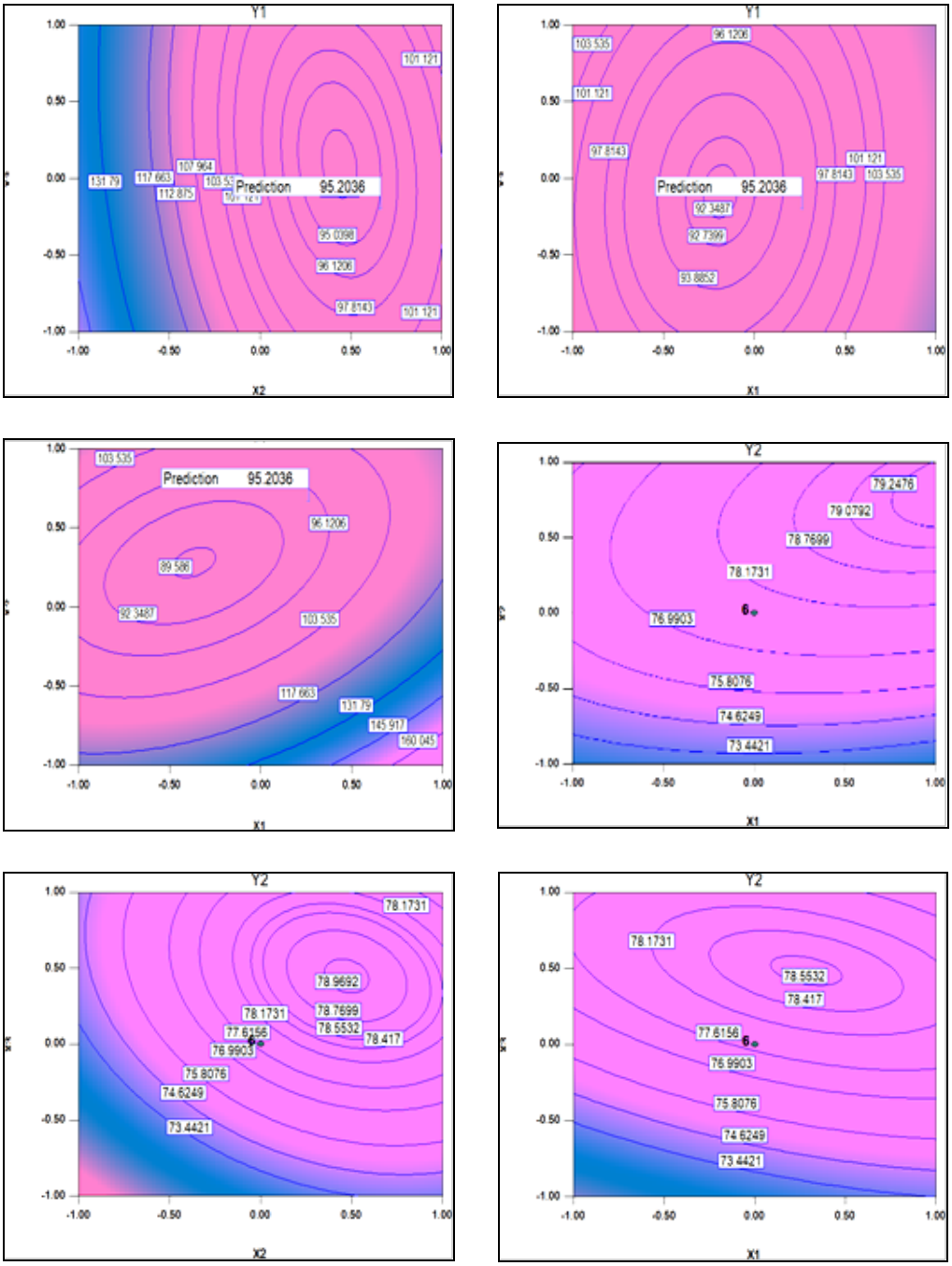

Fig 2: 2D graphs of independent variables (i) Particle size [Y1] (ii) Entrapment efficiency [Y2]

3.2. *In-vitro* Extract Release from Liposomal Formulation in PBS pH7.4 [24-26]

In-vitro drug release at PBS pH 7.4 was sustained and controlled in manner. Within 4 hours of study 50% of AC extract from drug solution was released and during the 48 hours' study 77.96% total extract was released whereas 55.9% of extract released from optimized liposomal preparation during 48 h which was the sustained release and could be due to fluidity of lipid membranes on vesicles due to cholesterol which increases the vesicles rigidity and elasticity and decreased vasculation from membrane. The graph of % drug release can be seen in Fig.3.

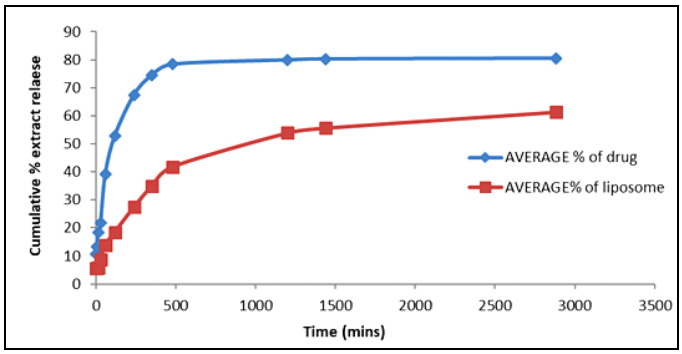

Fig 3: *In vitro* release study of pure extract and extract loaded optimized liposomal formulation.

3.3. External and Internal Morphology of Optimized Formulation

The photographs of the SEM microphotographs indicated the presence of spherical or sphere like structure of the liposomes while TEM clearly reveal that AC extract is uniformly distributed in lecithin polymer (as shown in Fig. 4) [27-29].

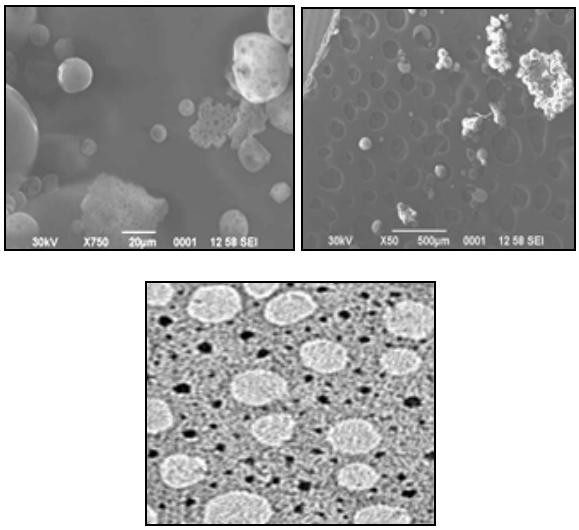

Fig 4: Photomicrographs of (a) Scanning electron microscopy (b) Transmission electron microscopy

4. Conclusion

In present investigation, AC extract loaded liposomes was successfully formulated and optimized using thin film hydration method. The small particle size of liposomes was capable to reduce the complications such as low solubility and poor bioavailability. Moreover, *in-vitro* release study of formulated liposomes showed sustained release which improves its bioavailability. Vesicles surface was found to be spherical and smooth in SEM, and TEM results clearly shows the entrapped extract into the vesicles. It can also be concluded that optimized liposomal formulation showed improved solubility and stability.

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