Enhancement and Extended Release of the Anti-hypertensive Drug Carvedilol using Optimized Ethosomal Gel via Transdermal Route

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Research

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Abstract

Background

Carvedilol, a popular anti-hypertensive drug, when orally administered has very poor bioavailability on the account of undergoing hepatic metabolism and therefore it becomes primal to explore an alternative drug delivery route for carvedilol. For a drug to be delivered by undergoing the least number of stages of metabolism and achieve high target specificity, transdermal delivery is the most preferred route. Hence, a study was conducted to test the potential of ethosomes as a candidate for transdermal delivery of carvedilol. A statistical study by using Central Composite Design (CCD) was also conducted for optimizing the quantity of the primary constituents present in the ethosomes. The optimized ethosomal formulation was then incorporated into a hydrogel to prepare the ethosomal gel.

Results

The optimized formulated ethosomal suspension and the ethosomal gel were undergone physicochemical, compatibility and in-vitro drug release studies along with characterization studies. The incorporation of the ethosomes into the hydrogel proved to be effective for skin application thereby ensuring better transdermal delivery. The optimized ethosomal gel has showed credible physical appearance, spreadability, viscosity and in-vitro drug release. The pharmacodynamic studies conducted on Wister rats revealed that the anti-hypertensive action was gradual and sustained lasting up to a period of 24 hours. The stability studies conducted also showed that prepared formulations maintained its consistency within the range for the measured parameters of physical appearance, rheological properties and entrapment efficiency for a period of 3 months.

Conclusions

The incorporation of the drug loaded into hydrogel and its effect on regulating systolic blood pressure in a sustained way lasting 24 hours proved to be better than the present available marketed formulation which has a rapid action with the anti-hypertensive effect lasting only for 10 hours. The chosen route for delivering the drug transdermally hence proved to be effective with better enhancement and permeation capability and shows the high potential of ethosomes to be considered for novel delivery of other anti-hypertensive drugs.

1. Background

Transdermal drug delivery poses a better advantage over other established routes of delivery because the concerned drug does not undergo pre-systemic metabolism resulting in better bioavailability and better patient compliance. The primary challenging part for transdermal drug delivery is crossing the barrier made by the stratum corneum (SC). One of the primary reasons is that SC is tightly connected which limits the drug permeation(1). To improve transdermal drug penetration, various physical and chemical methods have been tried and tested which include iontophoresis, microneedles and nanocarriers like liposomes and nanolipid particles (2). Microneedles have been used for successful transdermal delivery of cisplatin for synergistic chemo-therapy of breast cancer (3). Even though lipid nanoparticles have garnered attention in recent times, their mechanism of penetration and transdermal properties have not been fully understood (4). Moreover, conventional liposomes with their poor penetrating capability are not ideal candidates for transdermal delivery(5).

In the recent years, specially designed vesicular drug carriers, other than liposomes, have been developed and characterized for effective delivery of the drug involved by crossing the barriers of the skin layers(6, 7).One among such vesicular carriers is ethosomes. Ethosomes are lipid based vesicular drug carriers in which the concentration of ethanol is high. The spherical shape of binary ethosomes is well defined and has an enclosing of a lipid bilayer. They can

penetrate through SC with ease and therefore has been established as an effective candidate for transdermal drug delivery of a variety of drugs(2, 8). The size of the synthesized ethosomes is usually smaller in size and exhibit better stability than liposomes. The predominant presence of ethanol in high concentration in ethosomes impart them the ability to modify the highly dense alignment of the lipid bilayers in the SC thereby ensuring deeper penetration and moreover the low-toxicity and less irritability on the skin also promote the use of ethosomes for transdermal delivery(9).

Carvedilol, a popular anti-hypertensive drug, when administered orally, has low bioavailability mainly because of its lipophilicity and hence undergoes hepatic metabolism. It also has low dissolution capability belonging to the class II category in biopharmaceutical classification system(8, 10). However, its high lipophilicity and low molecular weight make the probability of transdermal drug delivery better. One of the feasible ways to increase the bioavailability of carvedilol is its incorporation into lipid based carriers. Some of the lipid based carriers include microspheres and solid lipid nanoparticles which have been administered via routes which escape hepatic metabolism. As mentioned earlier, ethosomes with its various favourable characteristics, can be considered to be a better choice among the available lipid based carriers for transdermal delivery.

Through this study, as shown in Fig. 1 successful entrapment of carvedilol loaded ethosomes in hydrogel for transdermal delivery was possible and the anti-hypertensive effect of the drug was tested on Wister Rats by tail-cuff method. Liposomes in the form of gel have been used to deliver drugs transdermally for psoriasis treatment (11). Hence, in this study hydrogels were tried and tested to ensure better stability and deposition of the intended drug on the skin(12). The use of hydrogels also made sure the involved drug was of proper consistency in its final stages when administered.

Beyond a certain limit, the content of ethanol in the formulated ethosomes has the tendency of making the vesicular membrane leaky which can result in low entrapment efficiency and stability (13, 14). Hence, the optimal formulation of ethosomes was the solution to tackle this problem. For proper optimization of ethosomal formulations statistical design studies have to be conducted under a given set of conditions. Based on the designs available, the central composite design (CCD), a robust form of response surface methodology (RSM), is the most preferred for determining the best possible formulation. The CCD model is very much efficient for the estimation of the extent of effectiveness of many individual variables(15, 16). Therefore, in this study besides the in-vitro and in-vivo studies, the focus was also given on determining statistically the various factors involved in ethosomal gel formulation of carvedilol(17, 18).

2. Results

The efficacy of transdermal drug delivery is highly dependent on the extent of drug solubility. In this study, the solubility of the drug carvedilol in ethanol was found to be very effective, the solubility increased as the concentration of ethanol in the buffer medium was increased. This trend can be seen from Table 1. However, when the concentration of ethanol was increased to 45% (v/v) during ethosomal preparation, the drug-loaded ethosomes could not be obtained.

Table 1
Solubility studies of Carvedilol in different media (Mean ± S.D, n = 3)

S.No.	Medium Composition (Ethanol: Transcutol : Buffer)	Solubility (mg/ml)
1	20:0:80	0.22 ± 0.01
2	30:0:70	0.34 ± 0.08
3	40:0:60	0.57 ± 0.06
4	50:0:50	0.76 ± 0.11
5	20:5:75	0.82 ± 0.07
6	30:10:60	1.05 ± 0.08
7	40:5:55	1.24 ± 0.09
8	50:10:40	1.53 ± 0.13

When the ethosomes were observed under SEM (at 12 kV and 30.0 kV), the ethosomes were found to be nearly spherical and properly dispersed with minimal aggregation as shown in Fig. 2. TEM was used to examine the periphery of the ethosomes formed it was seen that ethosomes formed were multilamellar and smooth surfaced as in Fig. 3.

As mentioned before, to find a statistical relationship among the various components involved in ethosomal formulation, CCD was used. The determined independent variables (X_i) were the amount of lipid (X_1), ethanol (X_2) and propylene glycol (X_3) added. The levels or extremities decided for each independent variable can be seen from Table 2. The dependant variables (Y_i) chosen were vesicle or particle size (Y_1), entrapment efficiency (%EE) (Y_2), cumulative drug release (CDR) (Y_3). The desired response for each dependent variable can be seen from Table 3.

Table 2
The upper and lower limits of the independent variables for CCD

Independent Variables	Levels		
	Low	Medium	High
X1 = Lipid (%)	2%	3.5	5%
X2 = Ethanol	20	30	40
X3 = PG	5	7.5	10

Table 3
The desired response of the dependant variables for CCD

Dependent Variable (Response)	Desirability Constraints
Y1 Particle Size (nm)	Minimize
Y2 (%EE)	Maximize
Y3 (%CDR)	Maximize
PDI	Minimize
Zeta Potential (-mV)	Maximize

The software DoE was used and from the regression equations, it was observed that a quadratic relation between the dependent and independent variables is more suitable with a better co-relation among the variables. This can be seen in Table 4. To imply a good and effective co-relation, the R^2 value should be at least 0.80. The observed R^2 values of vesicle size, %EE and %CDR for the dependent responses are 0.9890, 0.9887 and 0.9662. The adjusted R^2values of 0.9790 for size (Y1), 0.9785 for % EE (Y2), and 0.9358 for % CDR (Y3),were high enough to indicate the significance of the model (Table 4). The predicted R^2values, 0.9203 for size (Y1), 0.9318 for % EE (Y2), and 0.9025 for % CDR (Y3), given by the DoE, indicated a good correlation between the predicted and observed values. From the ANOVA analysis done, the responses indicated that the quadratic regression model was significant and valid for each of the responses Y1 ($p < 0.0001$), Y2 ($p < 0.0001$) and Y3 ($p < 0.0001$) and hence was appropriate as represented in Table 5.The 3D surface plots using RSM showing the co-relation between the dependent and independent variables are shown in Fig. 4

Table 4
Regression values of the selected responses during optimization

	Y1 (Vesicle size)			Y2 (% EE)			Y3 (% CDR)		
Model	R^2	Adjusted R^2	Predicted R^2	R^2	Adjusted R^2	Predicted R^2	R^2	Adjusted R^2	Predicted R^2
Linear	0.6611	0.5976	0.4484	0.3675	0.2489	0.0766	0.5597	0.4771	0.2359
2FI	0.7666	0.6588	0.5264	0.6574	0.4993	0.3302	0.8434	0.7711	0.6347
Quadratic	0.9890	0.9790	0.9203	0.9887	0.9785	0.9318	0.9662	0.9358	0.9025
p value	0.0001			0.0001			0.0001		

Table 5
ANOVA of optimized quadratic model of the novel ethosomal formulation

Parameter	Source	DF	Sum of squares	Mean of squares	F Value	*p* Value
Vesicle size	Model	9	2.051	2.279	99.65	< 0.0001
	Residual	10	22866.43	2286.64		
	Lack of fit	5	21497.1	4299.42		0.0045
	Pure error	5	1369.33	273.87		
% EE	Model	9	5869.50	652.17	97.24	< 0.0001
	Residual	10	67.07	6.71		
	Lack of fit	5	50.24	10.05		0.0029
	Pure error	5	16.83	3.37		
% CDR	Model	9	2090.19	232.24	31.76	< 0.0001
	Residual	10	73.13	7.31		
	Lack of fit	5	16.61	3.32	16.85	0.0105
	Pure error	5	56.52	11.30		

Table 6
Composition and Characterization of carvedilol ethosomal formulations (Mean±S.D; n=3)

Formulation Code (EF)	Lipid(%)	Ethanol (%)	PG (%)	Vesicle Size (nm)	%EE	PDI	Zeta Potential (mV)	%CDR
1	2	20	5	130 ± 1.72	99.12 ± 2.96	0.230 ± 0.03	-31 ± 1.8	97.89 ± 3.7
2	3.5	13.9	7.5	280 ± 1.89	89.56 ± 2.35	0.272 ± 0.05	-29 ± 1.56	95.87 ± 3.5
3	5	40	10	600 ± 1.96	94.09 ± 2.89	0.264 ± 0.09	-32 ± 1.46	98.74 ± 3.6
4	5	20	10	200 ± 1.51	95.82 ± 2.91	0.281 ± 0.31	-34 ± 0.45	98.82 ± 3.6
5	3.5	30	3.3	550 ± 2.82	94.41 ± 2.87	0.254 ± 0.46	-41 ± 0.12	87.5 ± 2.8
6	3.5	30	7.5	321 ± 1.94	95.24 ± 2.90	0.157 ± 0.43	-35 ± 0.46	93.35 ± 3.1
7	3.5	46.8	7.5	1200 ± 2.95	52.67 ± 1.58	0.238 ± 0.91	-29 ± 0.59	71.08 ± 1.7
8	5	20	5	550 ± 2.82	71.82 ± 1.98	0.268 ± 0.06	-28 ± 0.99	88.23 ± 2.7
9	3.5	30	7.5	345 ± 1.51	98.87 ± 2.89	0.312 ± 0.01	-29 ± 1.17	92.52 ± 3.0
10	3.5	30	7.5	315 ± 1.42	94.25 ± 2.87	0.170 ± 0.03	-31 ± 1.15	90.58 ± 2.8
11	3.5	30	7.5	318 ± 1.42	95.46 ± 2.88	0.135 ± 0.04	-34 ± 1.25	98 ± 3.6
12	2	40	5	1050 ± 2.01	56.74 ± 1.65	0.235 ± 0.09	-39 ± 0.48	69.29 ± 1.2
13	0.97	30	7.5	800 ± 1.91	55.45 ± 1.62	0.240 ± 0.01	-41 ± 1.23	85.26 ± 2.4
14	3.5	30	7.5	311 ± 1.95	95.78 ± 2.89	0.235 ± 0.05	-44 ± 0.85	99.24 ± 3.8
15	6	30	7.5	632 ± 1.92	81.79 ± 1.98	0.421 ± 0.06	-40 ± 1.23	98.51 ± 3.4
16	2	20	10	130 ± 1.72	99.08 ± 2.96	0.123 ± 0.01	-37 ± 0.90	99.89 ± 3.9
17	3.5	30	7.5	350 ± 1.54	93.56 ± 2.77	0.284 ± 0.05	-36 ± 1.45	96.07 ± 3.5
18	5	40	5	868 ± 1.98	90.53 ± 2.21	0.184 ± 0.46	-28 ± 0.85	89.28 ± 3.1
19	2	40	10	1086 ± 2.01	44.82 ± 1.38	0.294 ± 0.09	-29 ± 0.34	64.89 ± 0.9

Formulation Code (EF)	Lipid(%)	Ethanol (%)	PG (%)	Vesicle Size (nm)	%EE	PDI	Zeta Potential (mV)	%CDR
20	3.5	30	11.7	190 ± 1.86	98.01 ± 2.85	0.431 ± 0.13	-27 ± 1.22	99 ± 3.9

A PDI value of less than 0.5 is an indicator of homogenous size distribution of vesicles(19). From Table 6 it can be seen that for every formulation, the PDI value was less than 0.45 which ensured the homogenous distribution of vesicles. The value of zeta potential, an indicator of the stability of the suspension formed, should always have negative value to show that the suspension has good stability. All the formulations had a negative value for zeta potential.

For the sake of clarity and conformation, the FT-IR spectra (Fig. 5) of each component namely carvedilol, phospholipid, cholesterol, ethosomal suspension, carbopol 934 and ethosomal gel were taken. In carvedilol, all the characteristic peaks of the primary functional groups with the wavenumbers being 3342.89 cm^{-1}(-N-H stretching), 2922.72 cm^{-1}(-C-H stretching), 1443 cm^{-1}(C-C stretching), 1630 cm^{-1} and 1607 cm^{-1}(C = C stretching) and 1347 cm^{-1} to 1251 cm^{-1}(C-N stretching) were present in the prepared ethosomes as well as in the ethosomal gel which ensured that the molecular integrity of the drug was maintained when the drug got loaded in the ethosomes and then in ethosomal gel.

Regarding in-vitro release of the drug, in all the 20 formulations, the reduction in the release of carvedilol can be observed when the amount of phospholipids was increased from 2–5% (w/v) while the release increased with increase in the ethanol up to 40% (v/v). The increase in cholesterol content had a negating effect on the drug release even though the structural stability of the ethosomes was enhanced. The increase in PG content up to 10% (v/v) enhanced the permeability of the vesicles thereby increasing the drug release. The enhanced permeability can be accounted to the synergistic effect of transcutol with ethanol occurring in the vesicular bilayers.

As far as the in-vitro drug release pattern from the various prepared ethosomal formulations (EF1-EF20) was concerned, from Fig. 6 we can see that almost all the formulations, had a linear profile release up to 10 hours and then the curve plateaued without much release. From the graph, it can be deduced that the formulation EF1 had a better sustained release which lasted more than 72 hours. Moreover, the other characteristics associated with EF1 namely vesicle size, zeta potential and entrapment efficiency were also credible. Therefore this formulation was chosen to be incorporated into various hydrogel formulations(20).

Similarly, an in-vitro drug release studies from different formulations (G1-G6) of the ethosomal incorporated hydrogels were also done and release profile for each batch can be seen in Fig. 7. The hydrogel formulation G7 was loaded with the pure drug was chosen as a control. For G7 (the control), an almost 100% release of the drug was observed within the first ten hours with a linear profile while for the batches G1-G6 a linear release profile was observed with 60–80% of the drug released. While for batch G2 only 50% was released but also showed a better sustained release. For G6, the release stopped after 25 hours with 80% release, while for G4 and G5 90% release was observed which stopped after 50 hours. For batches G1 and G3 90% release could be seen lasting up to 75 hours but G2 batch showed better sustained release. Hence, the batch G2 was selected for further in-vitro and ex-vivo permeation studies. The composition of each gel formulation can be seen from Table 6. The drug release from the hydrogel formulations depends on the presence of three-dimensional polymeric cross-links of hydrogels which in turn is governed by a converse relationship with the viscosity of the hydrogel(21).

Table 6
Composition of carvedilol ethosomal gel formulations

Ingredients	G1	G2	G3	G4	G5	G6	G7
Carvedilol pure drug (mg)	-	-	-	-	-	-	6.25
Carvedilol loaded in E1 ethosomal suspension (mg)	6.25	6.25	6.25	6.25	6.25	6.25	-
Carbopol-934 (%)	0.5	1	1.5	2	-	-	-

When it comes to the case of ex-vivo permeation studies, both the ethosomal suspension and ethosomal loaded hydrogel showed better permeation than the control or the pure drug loaded hydrogel. Both the ethosomal suspension EF1 and ethosomal gel G2 indicated considerably higher amounts of drug permeation through the skin and higher steady-state flux Jss (93.54 ± 6.45 and 89.64 ± 7.26 μg.cm^2) when compared to the C-G formulations (54.59 ± 6.21 μg.cm^2).

For a better understanding of the pharmacodynamic study, a comparative model was adopted and the study was done for both ethosomes and ethosomal gel along with the marketed formulation. The case studies were done for hypertensive effect induced by both SC solution and MP. The SC solution and MP were orally administered to the rats. From Fig. 8 and Fig. 9, it can be observed that in the group of rats, in which no drug of any kind was administered, the induced systolic BP remained constant. The marketed formulation of carvedilol exhibited a rapid decrease in the systolic BP and normalizing it within 10 hours. Meanwhile, in case of both ethosomes and the ethosomal gel, a gradual reduction in systolic BP was observed which was brought down to the normal in 24 hours. This exhibited the sustained and extended action or release of carvedilol in case of both ethosomes and ethosomal gel.

When it came to the physicochemical properties of the drug loaded ethosomal gel formulations, as seen in Table 7 the pH value was well within the expected range as that for skin (5.5–6.8) and hence would not cause skin irritation. The spreadability of the gels were found to be low thereby ensuring better localization of the loaded drug and better skin penetration when applied. In all the gel formulations, the assay was found to be above 90%.

Table 7
Physicochemical studies of carvedilol ethosomal gel formulations

Formulation Code	Viscosity(cP)	pH value	Spreadability (g.cm/sec)	Assay (%)
G1	1.11 ± 0.2	5.44	7.80 ± 0.28	94.57 ± 0.54
G2	1.80 ± 1.0	5.68	8.24 ± 0.32	99.82 ± 0.62
G3	2.66 ± 1.5	5.81	6.57 ± 0.17	98.43 ± 0.23
G4	2.98 ± 0.52	5.54	6.20 ± 0.33	97.81 ± 0.13
G5	9.34 ± 1.0	5.93	6.82 ± 0.48	98.24 ± 0.44
G6	20.1 ± 1.5	5.84	5.37 ± 0.12	99.21 ± 0.46
G7	26.0 ± 5.7	5.61	7.90 ± 0.36	99.50 ± 0.25

Regarding the skin retention studies, both the chosen ethosomal suspension and ethosomal gel showed better retention capacity (12.31%±1.34 and 10.86%±3.21 respectively) than the control gel (4.63% ±1.23). The stability studies for both ethosomes and ethosomal gel revealed that the properties taken in consideration like the physical appearance, rheological properties, entrapment efficiency all remained intact for 3 months. The stability studies were conducted at both refrigeration and room temperatures (4 ± 2 °C & 27 ± 2 °C respectively). The change in the % EE was negligible (Table 8).

Table 8
Stability Studies showing the negligible change in %EE

Time (weeks)	Temperature	Carvedilol (%EE)
0	Refrigeration Temperature (4 ± 2^0C)	99
3		99
6		98
9		98
12		98
0	Room Temperature	99
3	(27 ± 2^0C)	98
6		97
9		97
12		97

3. Discussion

The effect of ethanol on increasing the solubility of carvedilol can help to avoid drug precipitation and hence we can prepare better stable ethosomes which will exhibit enhanced drug entrapment efficiency(22). This can be attributed to the fact that disruption of lipid molecules involved which then will affect the structural integrity of the ethosomes and hence lead to drug precipitation. Moreover, there is an increase in the vesicular size of the ethosomes formed when the concentration of ethanol is increased thereby making the transdermal delivery of the drug less effective(22, 23).

The multi-lamellar nature of the formulated ethosomes can be reasoned to the fact that the presence of ethanol contributed to the enhancement in the flexibility as well as the fluidity of the phospholipids bilayers(24). Out of the 20 batches of ethosomes prepared, the formulation which consisted of 2–5% phospholipid, 20–40% ethanol along with the appropriate amount of water had multi-lamellar structure. This again shows the influential nature of ethanol presence in confirming the type of vesicular structure formed during the ethosomal preparation. Another point to be noted is that, ethosomal formation is confirmed when the concentration of the hydrophilic phase is increased, that is when the phospholipid molecules reorganize leading to an increase in turbidity of the preparation medium.

From the statistical data obtained it can be deduced that the ethosomal formation as well as their size are dependent on the amount of phospholipid, ethanol and PG used in the formulation which thereby influence the % EE and the % CDR. To ensure proper transdermal drug delivery, the preferred approximate ethosomal size is 300 nm. It can be seen that for the different formulations from EF1 to EF 20 the vesicular size increased as the addition of phospholipid increased. The increase in phospholipid content was intended to enhance the structural rigidity of the vesicles. At the same time, changes in ethanol concentration were also done to test the stability of the vesicles. The effect was that ethanol addition favoured a reduction in the vesicle size which is due to the steric stabilization of the net charge of the system and edge activation mechanisms(25). The increase in PG concentration in the range of 10% (v/v) also resulted in the decrease of vesicle size, which shows the ability of PG to interpenetrate the phospholipid layer which provided better flexibility to the ethosomes.

The change in zeta potential was noted whenever a change in the concentration of the additives was made. An increase in the zeta potential value was observed with the increase in concentration of ethanol and PG which indicate that the

polar nature of ethanol and propylene glycol boosted the net surface charge hence resulting in strong electrostatic repulsion among the ethosomes. This phenomena prevented vesicle aggregation and therefore better stability and uniformity for the suspension(26).

Similarly, the % EE was also affected by the change in concentrations of the additives during ethosomal formulations. The amount of phospholipid had a directly proportional relation for %EE, suggesting the increase in phospholipid bilayer formed around the vesicle and hence better holding capacity of the lipophilic drug. However, the effect of ethanol showed a different trend. Up to a concentration of 40%(v/v), the %EE increased which can be attributed to the increased fluidity of the ethosomal membrane and also due to increased solubility of the lipophilic drug in the inner polar ethosomal core. But when ethanol concentration was above 40%(v/v), the %EE decreased due to increased solubilisation of the drug in ethanol causing disruptions in the vesicular membrane(27, 28), This was a contradicting the effect from the normal, which can be reasoned to increase in the fluidity of the membranes thereby the entrapped drug leaching out (29).

The decrease in the in-vitro release of carvediliol when the amount of phospholipids was increased can be because of the increased rigidity in the assembly of phospholipid bilayer as concentration increased. While the increased drug release when ethanol levels were raised can be attributed to the fluidity enhancement of the ethosomal membrane along with an increase in carvedilol solubility in the hydro-ethanolic core. The negating effect in drug release upon increase in cholesterol addition is because of the low permeability of ethosomal vesicles and hence a decrease in the formation of transient hydrophilic holes. On the contrary, the influence of the increase in PG content up to 10%(v/v) enhanced the permeability of the vesicles thereby increasing the drug release. The enhanced permeability can be accounted to the synergistic effect of transcutol with ethanol occurring in the vesicular bilayers.

The ethosomal suspension had better permeation capability than the corresponding hydrogel because of the influential role played by ethanol in fluidizing the lipids present at both in the vesicles and at the stratum corneum hence providing better malleability for the ethosomes. The presence of phospholipids also helped in providing better penetrative effect for the ethosomes by ensuring an effective mixing of the vesicles with the skin lipids thereby leading to the opening of the stratum corneum. The lower penetration by the drug loaded gel can be reasoned to the inherent high viscosity of the gel.

For skin retention studies, both the ethosomal suspension and ethosomal gel showed better retention capability than the pure drug loaded hydrogel. This occurred due to the combined effect of ethanol in strengthening the penetration effect of carvedilol by its high solubility in ethanol along with improving the elasticity of the prepared vesicles which allowed them to pass through the skin pores even though the skin pores have much smaller diameter than that of vesicles.

4. Conclusions

From the studies conducted, it can be brought to light that nano-sized ethosomal suspension loaded into hyrdogelcan be considered for transdermal delivery of the anti-hypertensive drug, carvedilol. A statistical study using CCD helped to know better the influence of the factors that directly affect the synthesis as well as the entrapment efficiency of ethosomes. The resulting size and morphology of the ethosomes were well within the acceptable range to be considered for transdermal delivery. The ethosomes and ethosomal gel exhibited the ability to penetrate the skin layers to a greater extent. It was also shown that successful incorporation of the drug-loaded ethosomes into hydrogel was possible without compromising the molecular integrity of the drug involved. The in-vitro and ex-vivo release of the drug showed a sustained release pattern and the amount of encapsulated drug released was in the expected limit. The pharmacodynamic studies revealed that the ethosomes and ethosomal gel had gradually decreased the systolic BP and

the anti-hypertensive action lasted for 24 hours while for the present marketed formulation, the action was rapid and lasted only for 10 hours. The skin irritation studies conducted showed that the ethosomal gels were safe to use. Moreover, the stability of the ethosomes and ethosomal gel was credible lasting up to3 months. Overall, the use of ethosomes as a drug delivery vehicle for an anti-hypertensive drug was found to very effective.

5. Materials

5.1 Materials

The drug, carvedilol, was procured from Chandra Laboratories, Hyderabad India. Soy lecithin, ethanol, PG, tri-ethanolamine and Carbopol-934 were purchased from Research Lab Fine Chem Industries, Mumbai India while cholesterol was obtained from Merck Ltd., Mumbai, India. The purchase of ultrapure water was done from Cortex Laboratories, Hyderabad, India. Centrisart filters with molecular cut off at 20000 were purchased from Sartorius Research Lab Fine Chem. Industries, Mumbai, India. The remaining chemicals used were of analytical grade and solvents were of HPLC grade.

5.2 Preparation of carvedilol loaded ethosomes

The ethosomes were prepared using ethanol [(20–40% (v/v)], PG [10% (v/v)], 2–5% (v/v) soy phospholipids and 0.005% (v/v) cholesterol in ultrapure water. The soy phospholipids, cholesterol, carvedilol drug solution, and PG were added to ethanol gradually followed by vigorous stirring to solubilise properly. The mixture was heated to 30 ^{o} C and distilled water was added slowly drop wise while the mixture was being stirred magnetically at 700 rpm. After addition of water, the stirring was carried out for an additional 5 minutes. The formed ethosomal suspension was then sonicated for reduction of the vesicular size to the desired extent(30). The final step was refrigeration of the suspension at 4 ℃(31).

5.3 Solubility studies

The extent of solubility of carvedilol was tested using ethanolic solutions in water of varying concentrations – 20, 30 and 40% (v/v). Before centrifugation, in each vehicle (2 ml centrifuge tube) an excess of amount of 1.5 ml of carvedilol was taken. After vortexing, the centrifuged tubes were kept for incubation in an orbital shaker (Remi Electrotechnik Ltd, Mumbai, India) for 48 hours at an ambient temperature of 25 °C to ensure proper solubilisation (32, 33). For removal of the excess undissolved drug, the incubated samples were undergone centrifugation at 3000 rpm. The supernatant taken at regular intervals were quantified for determining the drug concentration using RP-HPLC method.

5.4 HPLC quantification of dissolved drug

The HPLC unit used for the drug quantification (Shimadzu, Japan) had the following specifications: LC-10AT solvent module with SPD-10A column, PDA detector with LC10 software. The RPCL column had the specifications of C18 (150 × 6 mm) with 5 µm packing material. The mobile phase used was acetonitrile and 15 mM of ortho-phosphoric acid in the volumetric ratio 37:63 with the addition of tri-ethylamine in the concentration of 0.25%(v/v). The pH of the mobile phase was adjusted to 2.5 using ortho-phosphoric acid. A 20 µl of each sample was injected and the eluent detection was done at a wavelength of 242 nm. The flow rate was maintained at 1 ml/min with a runtime of 12 minutes (34).The final step was refrigeration of the suspension at 4 ℃(31).

5.5 Preparation of carvedilol loaded ethosomal hydrogel

Using various concentrations of the polymer Carbopol 934, the hydrogel was formulated, the concentrations of Carbopol being 1% (w/w), 1.5% (w/w) and 2% (w/w). Accurately weighed quantities of the polymer were dissolved in specific quantities of the prepared ethosomal suspension using a magnetic stirrer at 1000 rpm. The process was continued until smooth lump-free homogenous gels were attained. Appropriate quantity of tri-ethanol amine was added to adjust the pH of the prepared gel. The pH was adjusted to 5.5. The final semi-solid gel was stored overnight at room temperature.

5.6 Characterization of Ethosomosal Formulation

5.6.1 Assay of the encapsulated drug in ethosomes

The diluent used for dissolving the prepared ethosomal formulations was chloroform and methanol in 1:1 (v/v) ratio and diluted with the mobile phase. The HPLC parameters used and assay determination was done the same way as for quantification of the dissolved drug earlier(35).

5.6.2 Drug entrapment efficiency

Firstly, the prepared ethosomal formulation was undergone centrifugation at 8000 rpm for 30 minutes. The centrifuge tubes used were Centrisart tubes. The free unencapsulated drug concentration present in the supernatant was determined by HPLC and entrapment efficiency was calculated using Eq. 1

5.6.3Particle sizing and distribution

The average vesicle size, PDI and zeta potential of the ethosomes were determined by using the DLS method. To avoid the error due to multi-scattering action, a 2 ml quantity of each sample was undergone dilution with distilled water by proper mixing. The diluted sample was then injected into a clean disposable zeta cell and measurements were recorded using a zetasizer (Malvern Nano-ZS90).

5.6.4 FT-IR studies

FT-IR studies were conducted for the pure drug, the excipients used, the ethosomal suspension and the ethosomal hydrogel to determine the occurrence of any physio-chemical interactions and compatibility between the drug and the excipients used. The K-Br pellet technique was used. The scanning range and the resolution were kept at 400–4000 cm^{-1} and 4 cm^{-1} respectively(36). The FT-IR instrument used was of making Bruker Optics Germany Model-200.

5.6.5 Particle morphology

SEM was used to examine the surface morphology of the prepared ethosomes. After adhering the ethosomal suspension onto the carbon-coated stubs, they were sputtered with platinum using a coating machine (Auto Fine Coater, JFC-1600, JEOL, Japan) and then observed under the SEM in high vacuum atmosphere(37, 38). The SEM used was of the make JSM-6501LA, JEOL, Japan.

5.6.6 Particle appearance

The shape determination and overall appearance of the prepared ethosomes were observed using TEM. The sample preparation was done by placing a drop of the diluted ethosomal suspension on a carbon coated grid and followed by addition of a drop of aqueous 2% phosphotungstic acid solution. After the removal of excess liquid, the suspension was air-dried and TEM imaging was done at an acceleration voltage of 100 kV(39).

5.6.7 Physical Examination and pH measurement of ethosomal gel

The physical characteristics of the prepared ethosomal gels were determined by visual examination. The gel samples were visually examined to determine the homogeneity, consistency, phase separation and appearance of any aggregate formations. The pH was measured by using a digital pH meter (Remi, Hyderabad, India). For proper measurement, it was ensured that the glass electrode of the pH meter was completely dipped into the gel system (40).

5.6.8 Viscosity measurement of gels

The viscosity was measured using a viscometer (Brookfield Viscometer, CAP 2000L) under high torque and low-temperature mode. The cone used was of No. 1 type. About 500 mg of each sample was taken for analysis. 5 minutes of prior settling time was ensured before viscosity determination (41).

5.6.9 Spreadability of the gels

The extent and degree of the gel Spreadability were measured using the glass slide apparatus with the help of a modified wooden block. Using a glass side, a quantity of gel of known weight was placed on the movable pan using a glass slide and then placed on the fixed glass slide to make sure the gel was properly sandwiched between the glass slides for 5 minute duration. The excess gel exiting from the sides was continuously removed. The Spreadability was determined using Eq. 2.

5.6.10 Skin irritation test of the ethosomal gel

All the animal studies were conducted on Wister rats after obtaining permission from CPCSEA with the wide permission being documented as No.51/01/C/CPCSEA/2013/13. Using a clipper, the hair from the dorsal portion of nine rats was removed and the ethosomal gel was applied on the blank skin portion. Before the application, the rats were divided 3 groups with each group consisting of 3 members. Each group had a characteristic based on the application of the gel as follows

Group 1 - No application of gel on the rats

Group 2 - The prepared ethosomal gel was applied on the rats

Group 3 – Blank (without drug) ethosomal gel was applied on the rats

Each time the amount taken for the application was 500 mg with uniform spreading over the blank skin portion of area 4 cm^2. Any sign of erythema or redness of skin was observed after every 24 hours up to 72 hours. The time is measured from the point of gel application.

5.6.11 In-vitro drug release studies

The dialysis bag method was used to carry out the in-vitro release studies of both the ethosomes and ethosomal gel. Before the test, it was made sure that the membrane of the dialysis was properly hydrated with complete wetting of the membrane(24). The hydration medium used was PBS of pH 6.8 and the hydration was carried out for 2 hours. The samples of both ethosomal suspension and ethosomal gel each containing the drug were transferred to the dialysis bags with both ends sealed. The bags were then suspended in bottles containing 200 ml of the buffer solution and rotated at 100 rpm in a thermostatically temperature-controlled water bath shaker. The temperature was maintained at 37 ± 0.5 °C throughout the process. For each sample, 1 ml of the aliquot was taken at pre-determined time intervals. For the first 6 hours, the aliquot was taken on an hourly basis and then after the sample was taken after 8, 16, 24, 48 and 72 hours. The drug concentration after each time interval, was determined at 242 nm spectrophotometrically.

5.6.12 Ex-vivo skin permeation studies

The ex-vivo skin permeation studies were carried for both ethosomal suspension and ethosomal gel. For both the suspension and gel, the batch which showed the most promising results in terms of physical studies, entrapment efficiency and in-vitro drug release studies was selected. After sacrificing the rats, the skin from the abdominal portion was chosen for conducting the studies. The hair from the skin was removed thoroughly using a razor blade and the skin was separated from the connective tissue diligently using a scalpel to prevent perforations or incisions. After removal, the skin was then washed thoroughly with double distilled water and stored at − 18 °C to retain its metabolic efficiency. The skin was then hydrated overnight at 25^{o}C in PBS (pH 6.8 and containing 0.02% sodium azide as a preservative). The overnight hydration was done to ensure the removal of extraneous debris and leachable enzymes(42, 43).

A skin sample of appropriate size was fixed at the ends of a diffusion cell ensuring a permeation area of about 5.3 cm^2 was available. The SC portion of the skin layer faced the donor compartment while the dermis side of the skin met the receptor compartment. A 200 ml of a solution of transcutol, ethanol and PBS at pH 6.8 in the ratio 10:40:50 (v/v) was used as the hydration medium. Such a hydration medium was chosen to ensure proper sinking during the permeation studies (20). The diffusion cells were maintained in a thermostatically controlled water bath shaker at 37 ± 1^{o}C at 100 rpm. At pre-determined time intervals (0, 1, 2, 3, 4, 5, 6, 8 and 24 hours), a 5 ml sample of the receptor medium was withdrawn, and then filtered using a nylon syringe filter of 0.22 µm size. Every time a sample was taken, an equivalent amount of fresh receptor medium was added to maintain the volume constant. The assay of the drug in the sample taken was determined at 242 nm using spectrophotometry. For the selected sample batches, the cumulative drug permeation through the skin was plotted against time to see the release pattern. The steady state flux (J_{ss}) was also determined. The measurements were taken in a triplicate manner and compared with those of a control batch.

5.6.13 Pharmacodynamic study

The pharmacodynamic study was conducted for both ethosomal suspension and ethosomal gel. A comparative study was done with control as well as the marketed formulation. One group of rats were not administered with any drug whatsoever. In another groups of rats, in which the pure drug or its various formulations were administered, the hypertensive effect was induced using sodium chloride solution and MP separately. The sacrificed rats weighed in the range of 220–250 g and were fed ad libitum as per the standard procedure. After two weeks from inducing hypertensive effect, the rats in which the mean systolic BP was 150–160 mm Hg were selected and the drug and its various formulations were administered. The marketed formulation was administered orally (10 mg/kg of body weight) while the rest of the formulations were administered transdermally (10 mg/kg of body weight). Before the blood pressure

measurement was done, the rats were properly trained to stay calm and non-aggressive in the cages. The systolic BP was measured by the tail-cuff method (Bio-pack system Inc., Santa Barbara, USA) at pre-determined time intervals after the drug administration (1, 2, 4, 6, 10, 12, and 24 hours) for all the groups(44, 45).

5.6.14 Stability studies

The stability studies were conducted for both ethosomal suspension and ethosomal gel. Two batches were used for each of the formulations, one was stored at 4 °C and the other at room temperature at 23–30 °C. The parameters determined for stability studies were mean vesicle size, PDI, zeta potential, entrapment efficiency (EE%) and assay using HPLC. The stability studies were conducted at 0, 1, 2, 3 and 6 months(46, 47).

5.6.15 Statistical studies for optimizing the formulation of ethosomes

The CCD modelwas implemented to optimize the correct formulation of the carvedilol-loaded ethosomes. The software used for the study was DoE (Version 11, Stat-Ease Inc., Minneapolis, USA). For CCD modelling, the variables are chosen to be either dependable or independent. The independent variables were the different constituents of ethosomes namely amount of phospholipids (X1), amount of ethanol (X2) and amount of PG (X3). While the dependent variables (responses) were the vesicle size (Y1), % EE (Y2), and % CDR (Y3). Based on the experimental setup and number of factors involved in the formulation, a quadratic relation between the factors was chosen governed given by Eq. 3. The ANOVA method was used to know the significant effect of the factors and their interactions.

List Of Abbreviations

SC: Stratum Corneum
RSM: Response Surface Methodology
CCD: Central Composite Design
PG: Polyethylene Glycol
TRA: Tri-ethanol amine
% EE: Entrapment Efficiency
PDI: Polydispersity Index
DLS: Dynamic Light Scattering
SEM: Scanning Electron Microscopy
TEM: Transmission Electron Microscopy
CPCSEA: *Committee for the Purpose of Control And Supervision of Experiments on Animals*
PBS: *Phosphate buffer solution*
MP: *Methyl Prednisolone*
BP: *Blood Pressure*
% CDR: Cumulative Drug Release
ANOVA: Analysis of Variance

List Of Equations

Equation 1

$$\% \text{ EE} = \left[\frac{A_{total} - A_{unentrapped}}{A_{total}}\right] \times 100$$

where A_{total} = total amount of carvedilol in ethosomal suspension; $A_{unentrapped}$ = unentrapped carvedilol in ethosomal suspension

Equation 2

S = M/T

where S = spreadability in g/s, M = mass in g, T=time in sec

Equation 3

$$Y = B_0 + \sum_{i=1}^{2} B_i X_i + \sum_{i<j}^{2} B_{ij} X_i X_j + \sum_{i=1}^{2} B_{ij} X_i^2 + €$$

where Y = a response applicable for the vesicle size, % EE and % CDR; X_i = terms include independent variables (X= A (amount of lipid), B (amount of ethanol), and C (amount of PG) ranging from ($-1 \leq X \leq 1$). B_i terms are the equation coefficients related to the main factor. € is the experimental error

Declarations

Ethics approval and consent to participate

All the animal studies were conducted on Wister rats after obtaining permission from *Committee for the Purpose of Control And Supervision of Experiments on Animals*, India with the wide permission being documented as No.51/01/C/CPCSEA/2013/13.

Consent for publication

Not applicable

Availability of data and materials

All data and materials used are all available in the manuscript.

Competing Interest

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.

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Authors' contributions

A. Padmanabha Rao: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Data curation, Writing - original draft.

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Noufel Samed: Conceptualization, Writing - review & editing.

Alle Madhusudhan: Conceptualization, Writing - review & editing.

Jin-Chul Kim: Supervision, Project administration, Writing - review & editing

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Figures

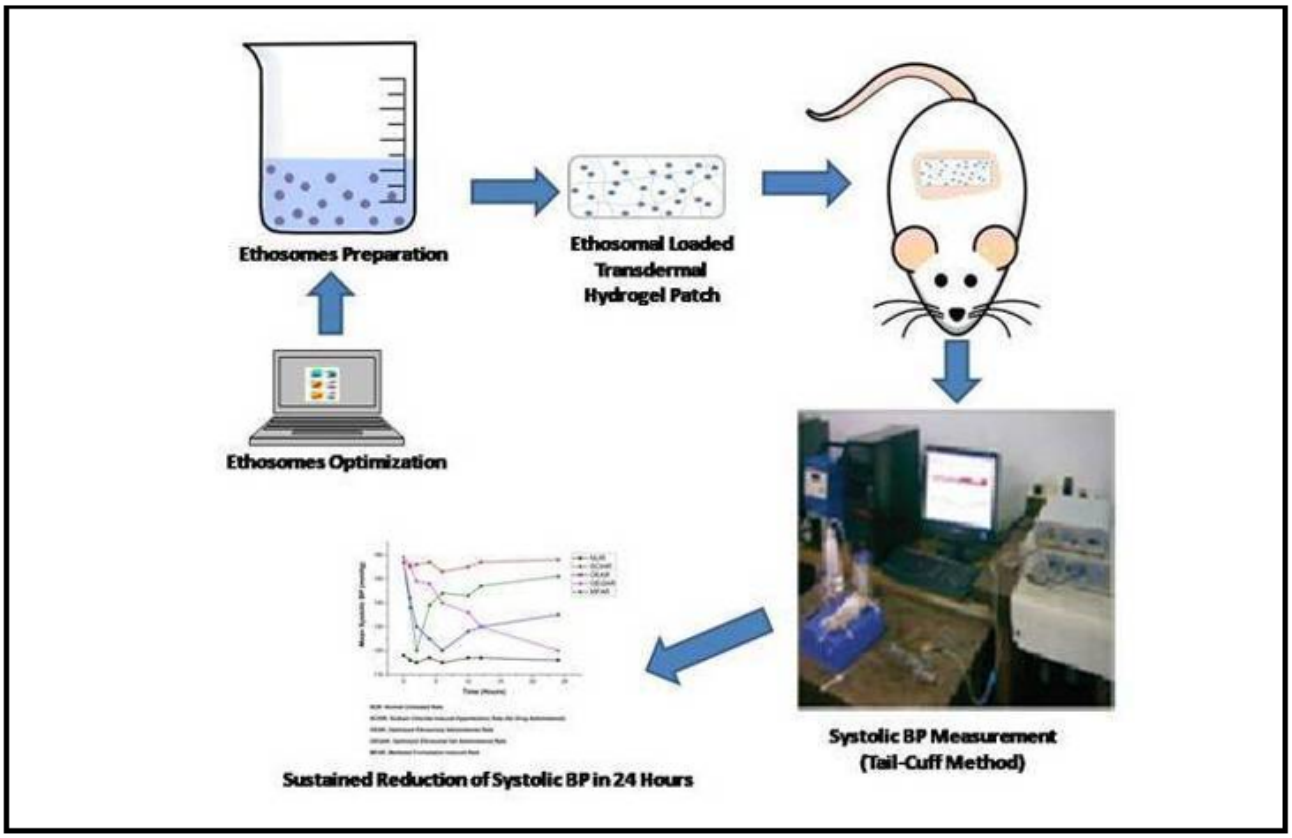


Figure 1

Schematic showing the preparation of optimized ethosomes and its subsequent loading on to hydrogel followed by pharmacodynamic study for examining the anti-hypertensive effect of the drug carvedilol

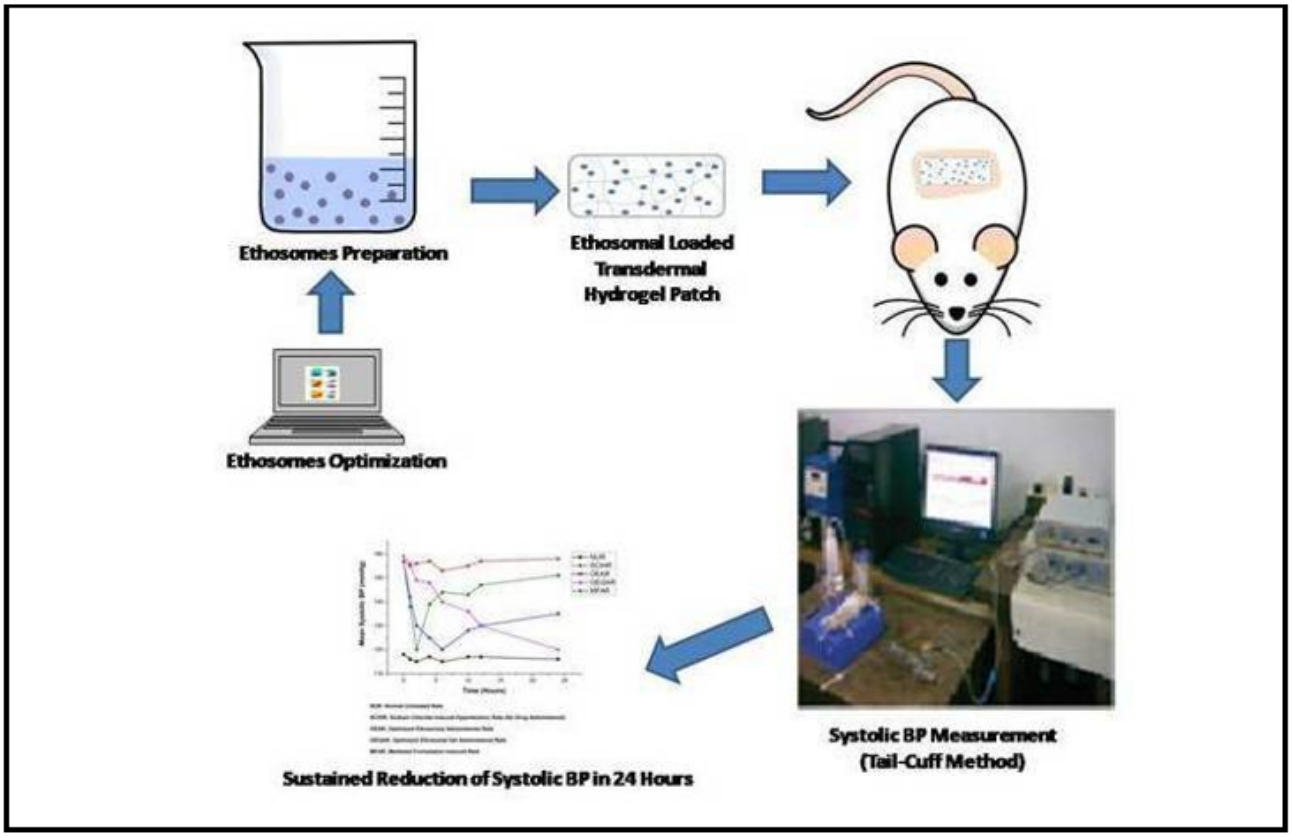


Figure 1

Schematic showing the preparation of optimized ethosomes and its subsequent loading on to hydrogel followed by pharmacodynamic study for examining the anti-hypertensive effect of the drug carvedilol

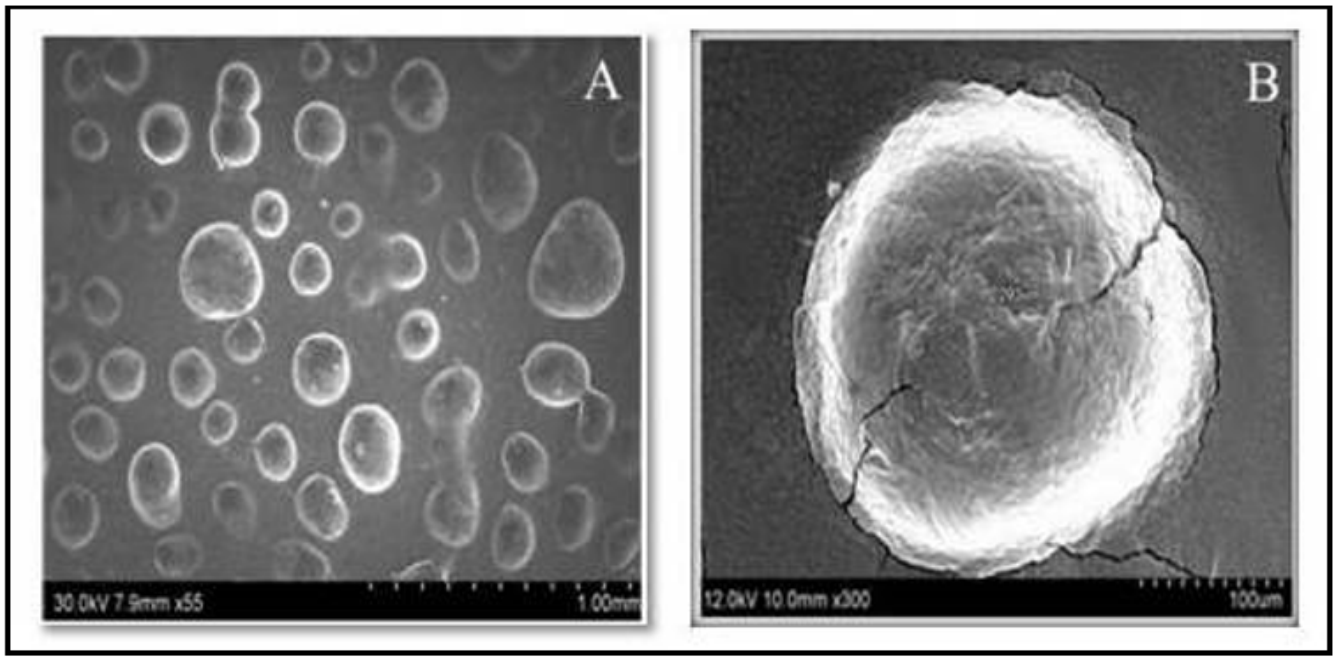
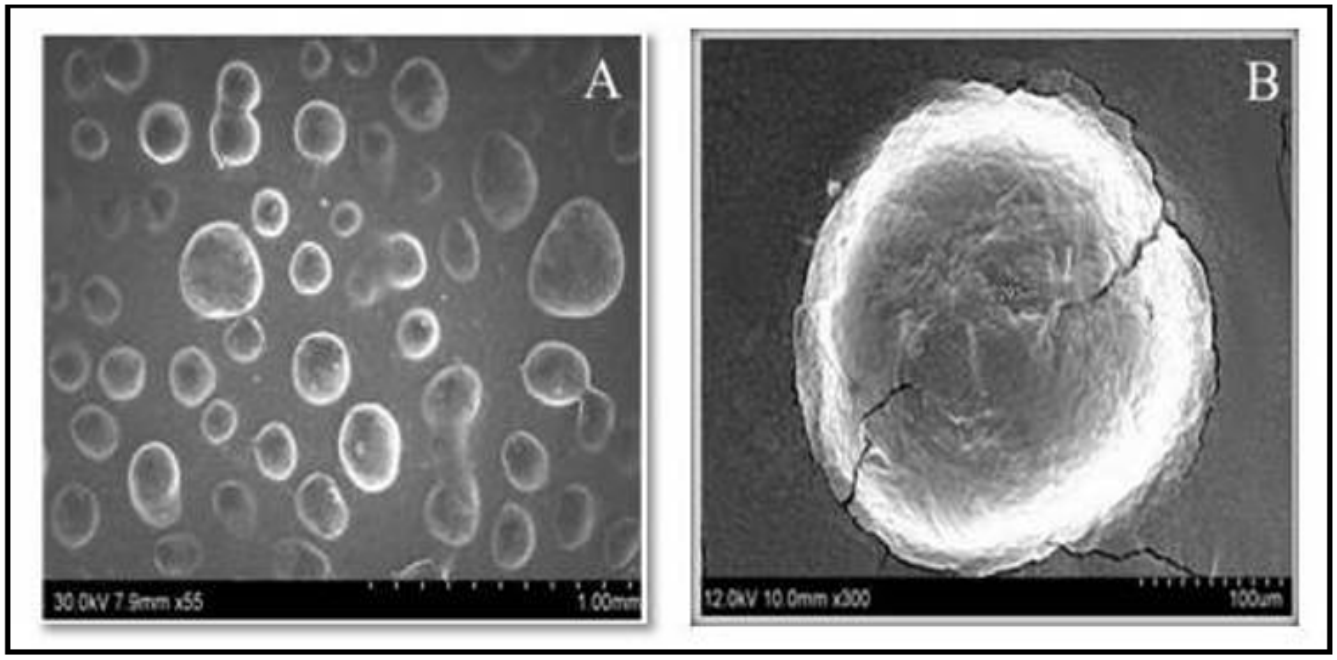


Figure 2

SEM images of prepared ethosomes



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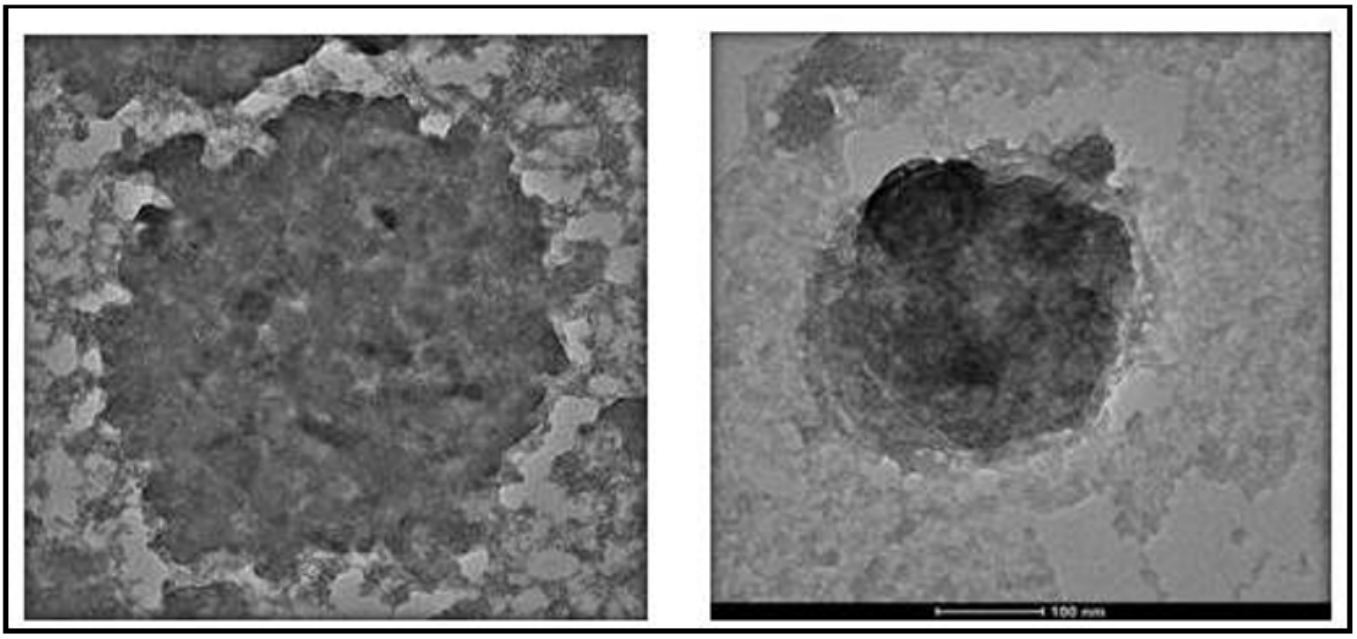
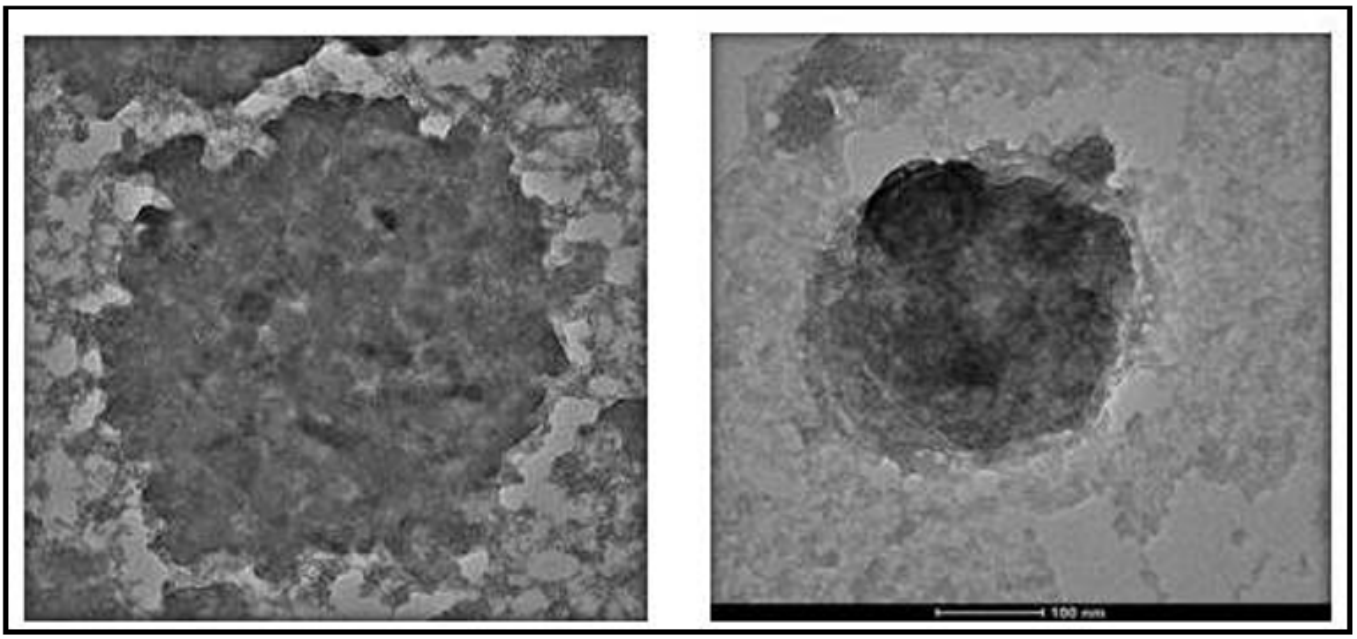


Figure 3

TEM images showing the bilamellar layer of the ethosomes



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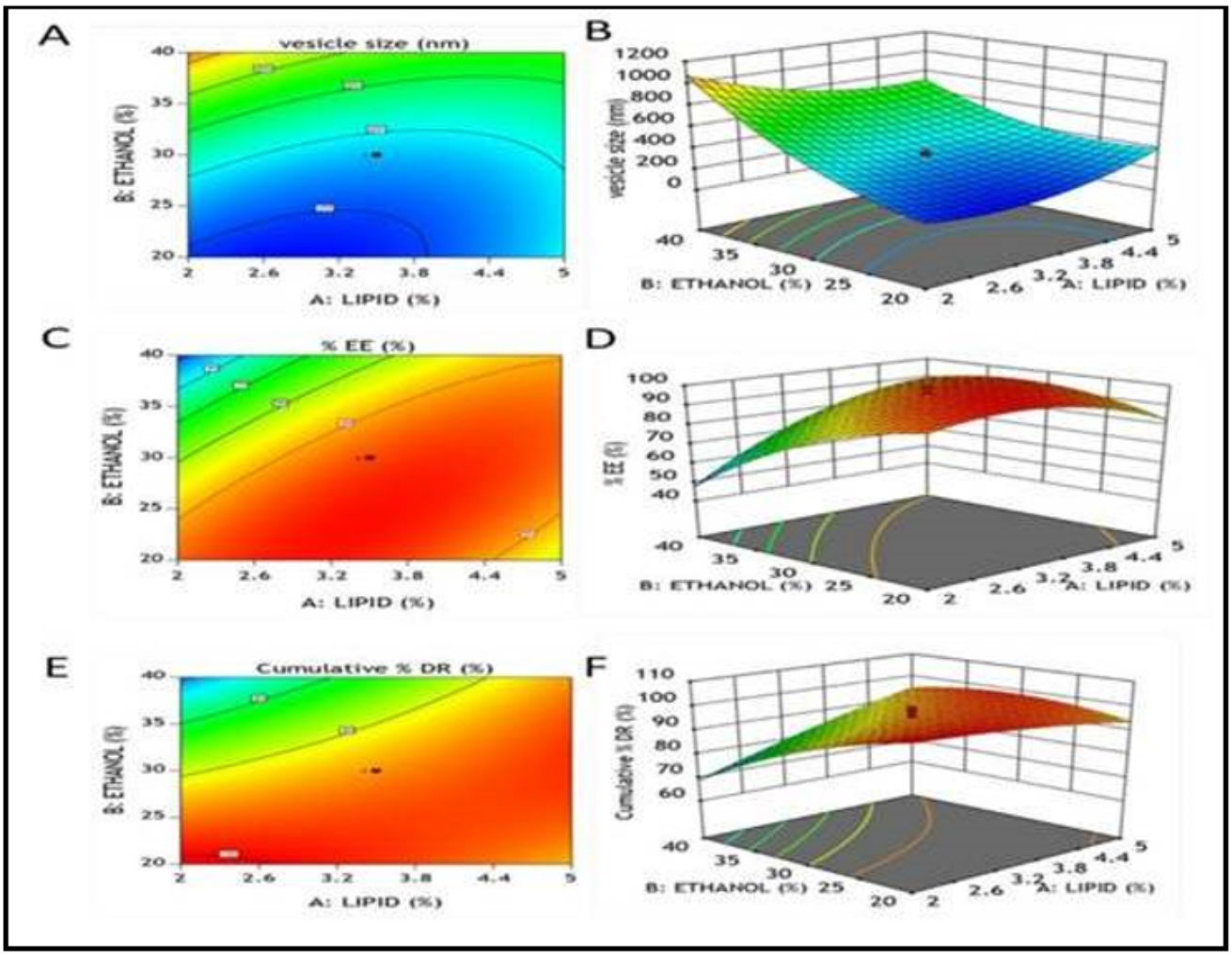
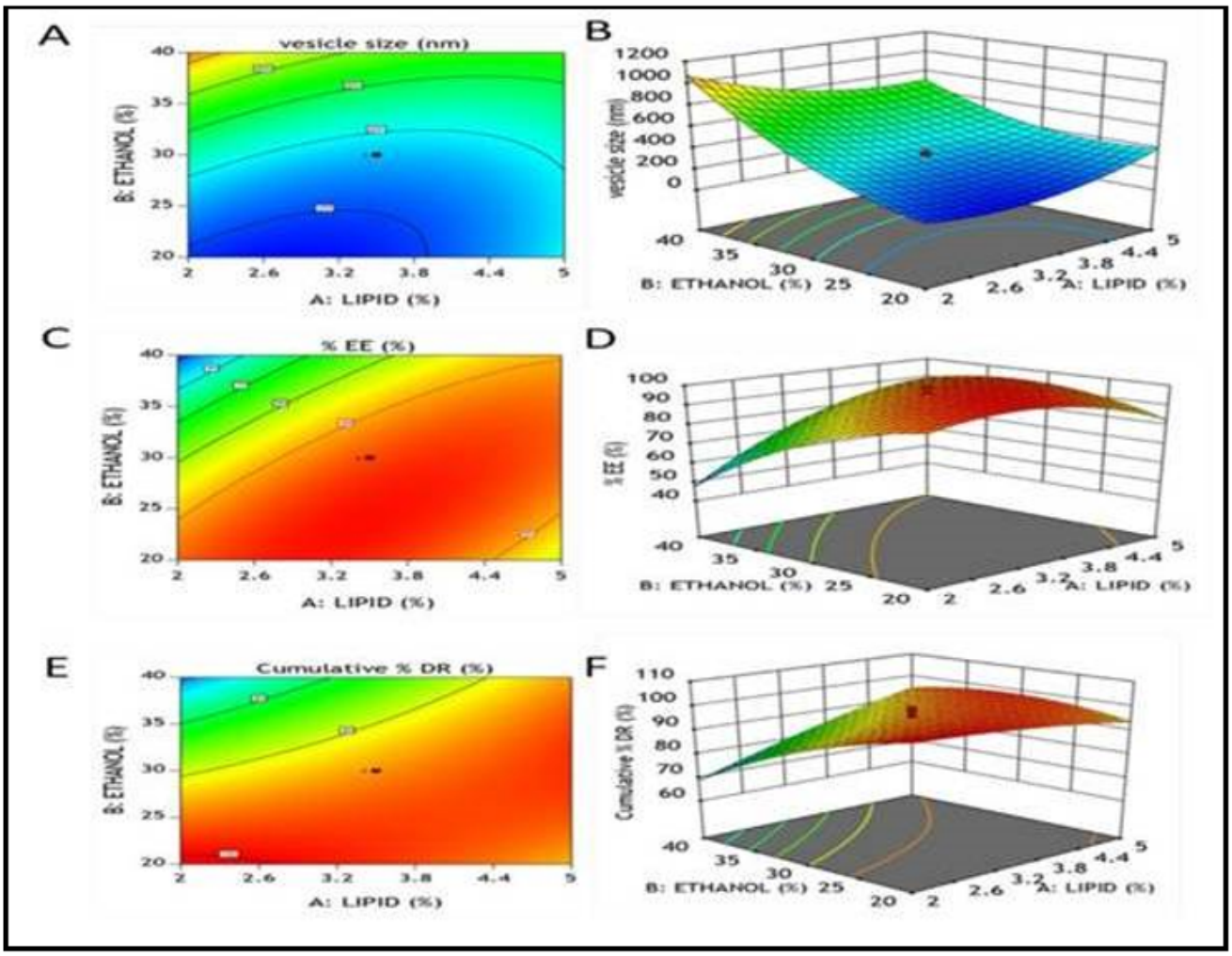


Figure 4

Contour plots of responses showing the interactive effects of amount of lipid and amount of ethanol on vesicle size (Y1) (A&B), %EE (Y2) (C&D), and %CDR (Y3) (E&F)



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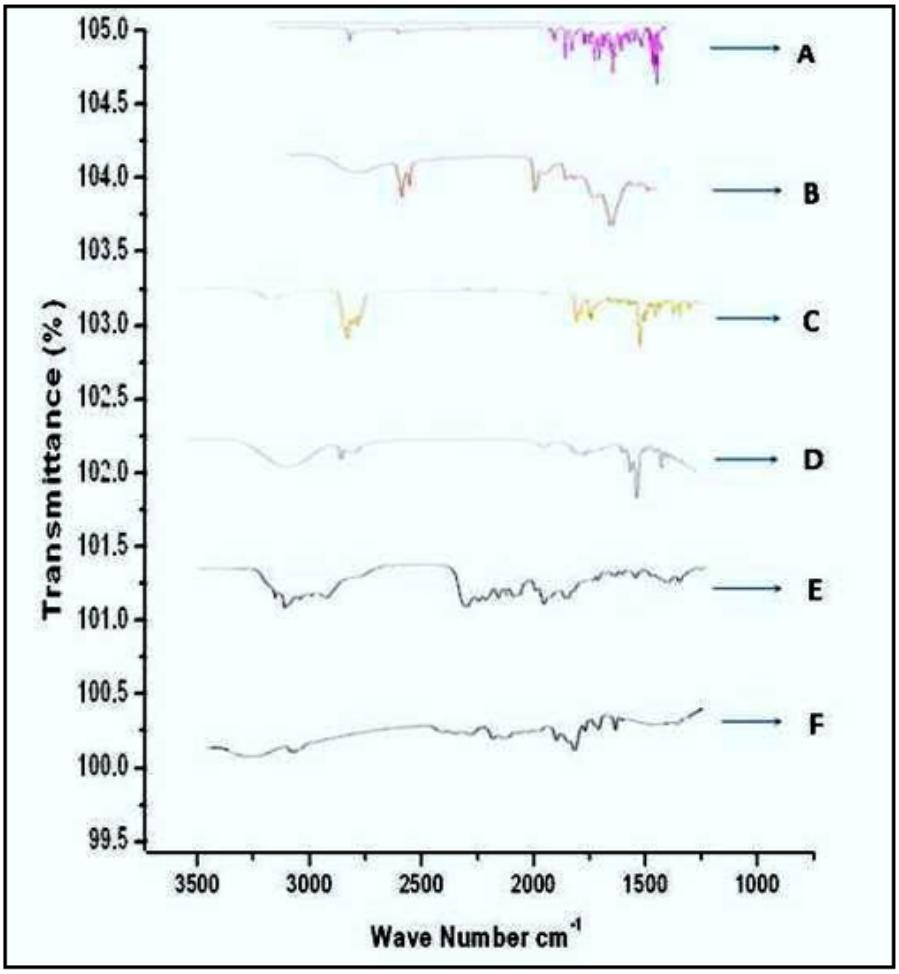
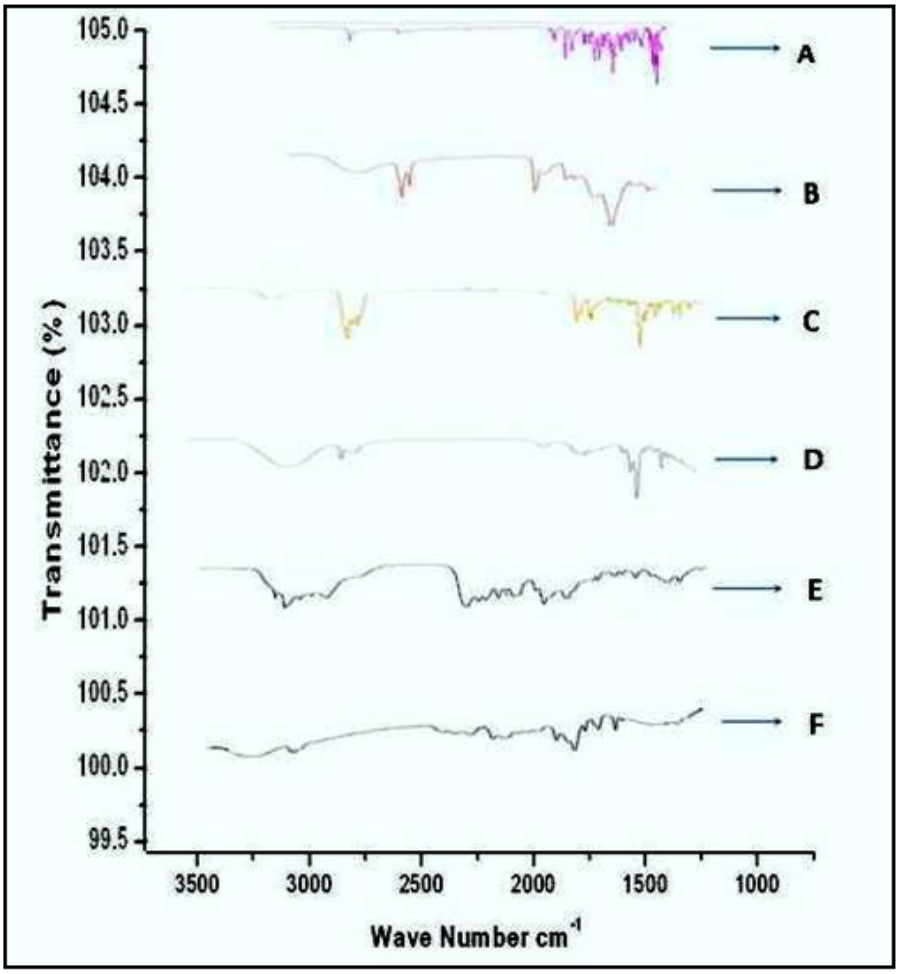


Figure 5

FT-IR spectrum of carvedilol (A), phospholipid (B), cholesterol (C), ethosomal suspension (D), Carbopol-934 (E), and ethosomal gel (F)



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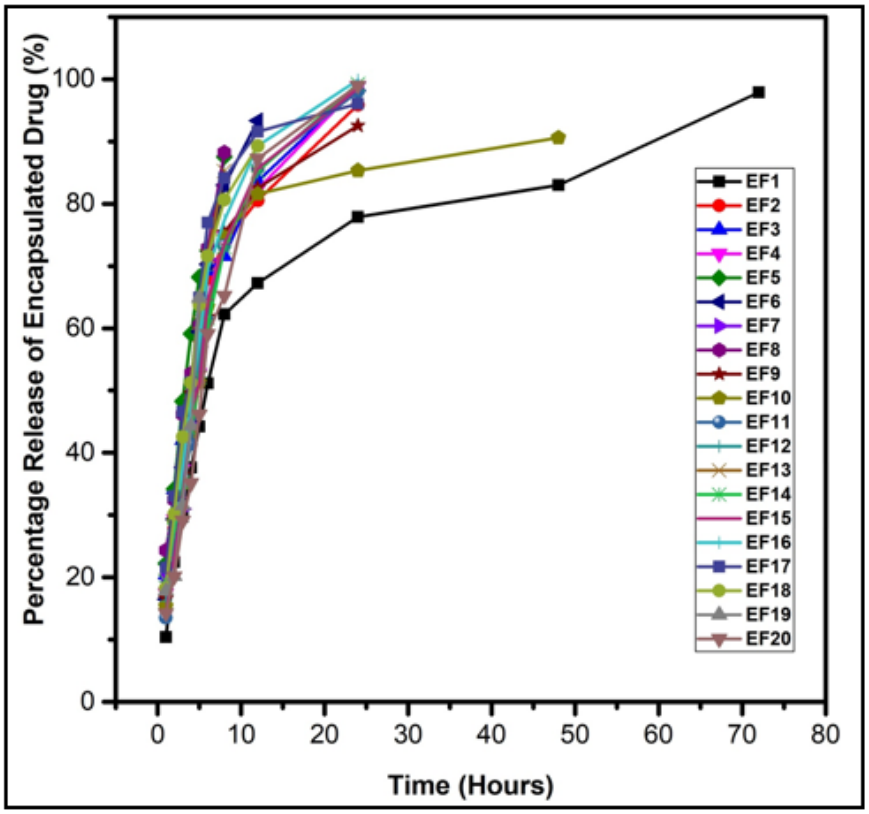
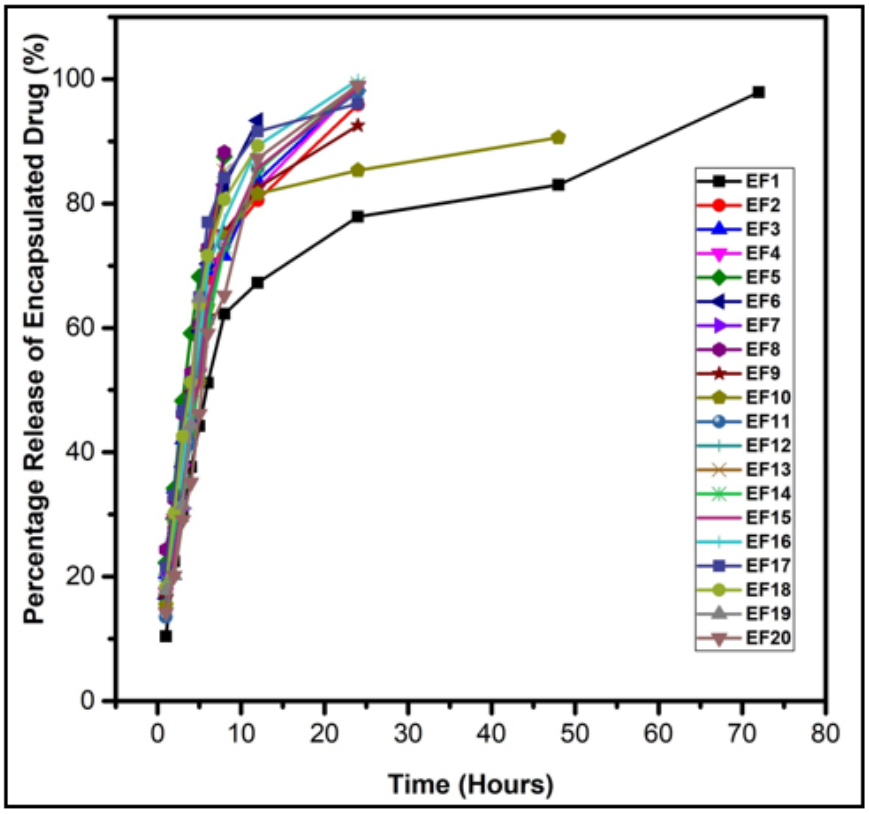


Figure 6

In-vitro drug release studies of formulation (EF1-EF20) (EF-Ethosomal formulation)



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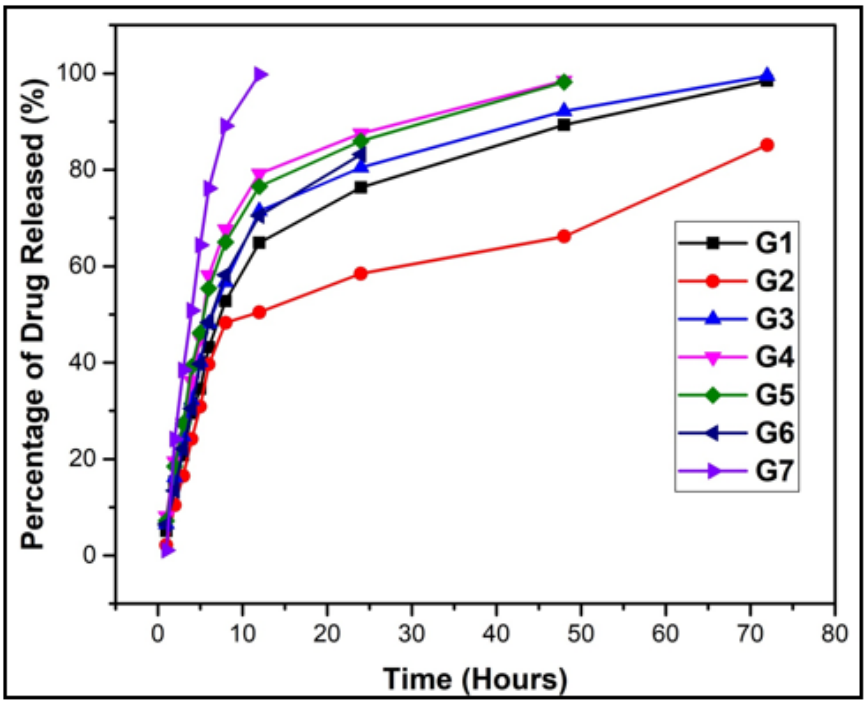
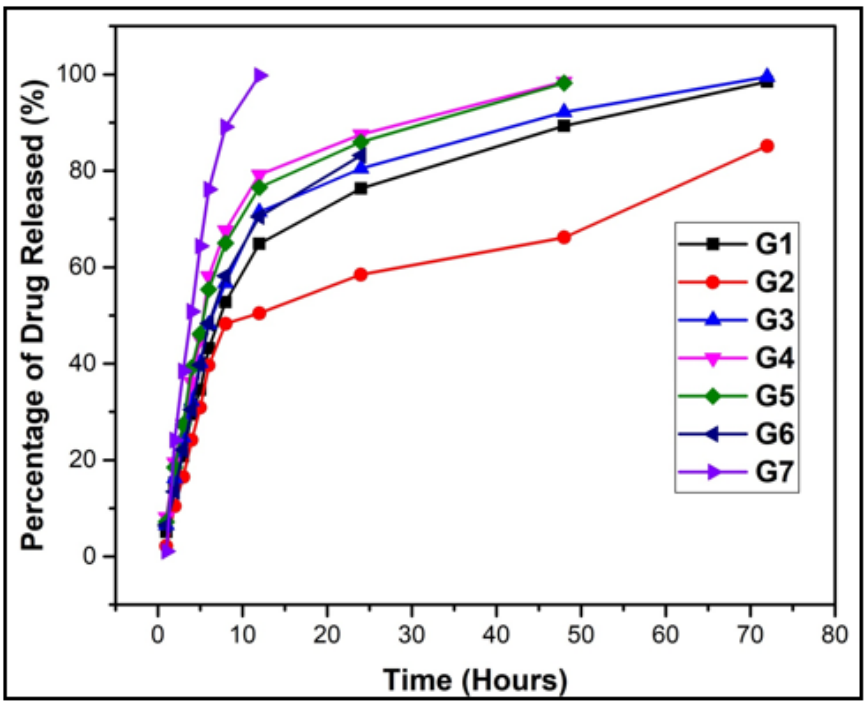


Figure 7

In-vitro drug release studies of gel formulation (G1-G7) (G- Ethosomal Hydrogel)



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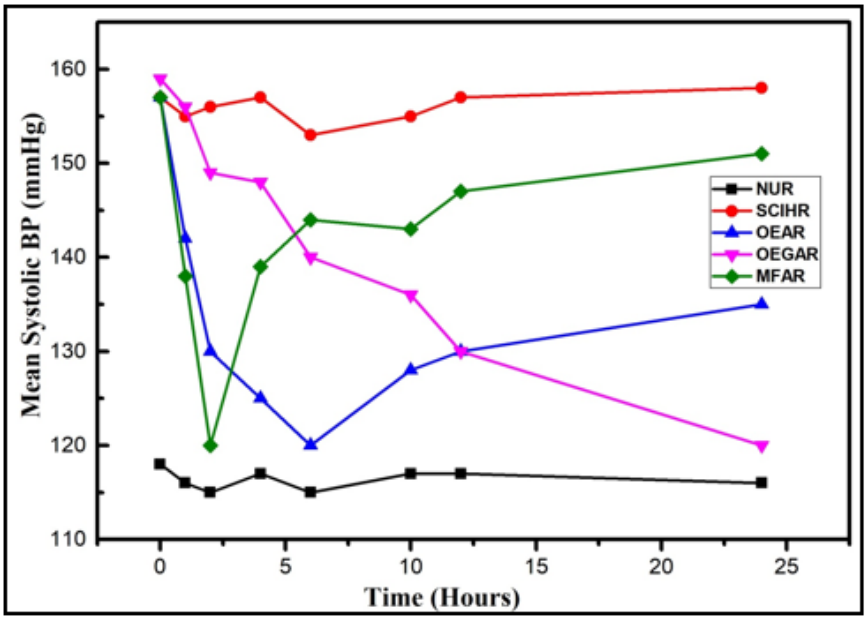
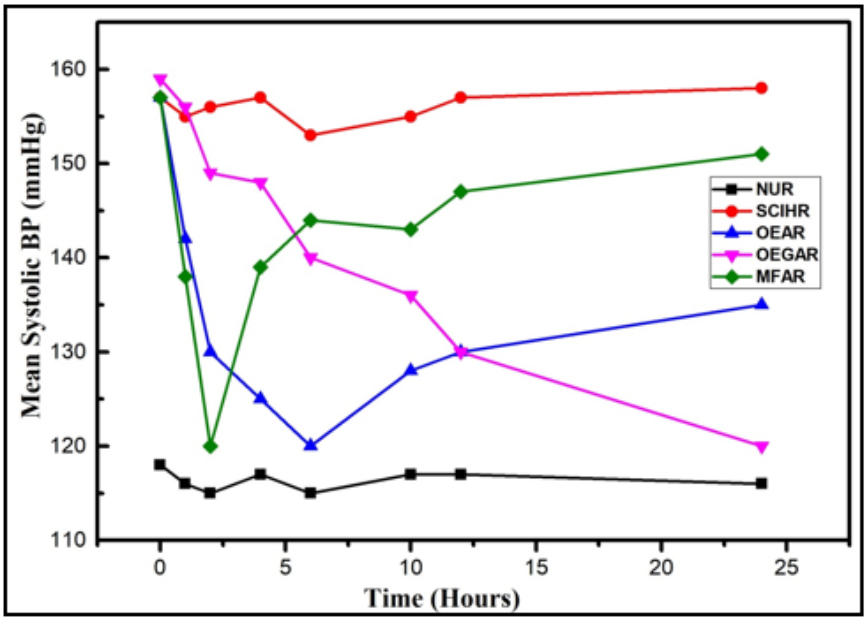


Figure 8

Pharmacodynamic Study (Sodium chloride Induced) NUR- Normal Untreated Rats SCIHR- Sodium Chloride Induced Hypertension Rats OEAR- Optimized Ethosomes Administered Rats OEGAR- Optimized Ethosomal Gel Administered Rats MFAR- Marketed Formulation Administered Rats



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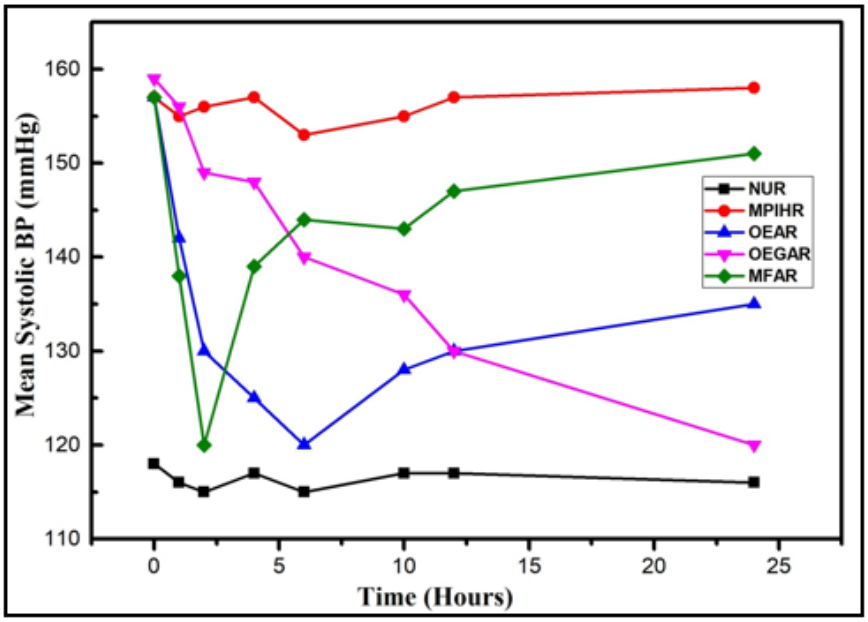
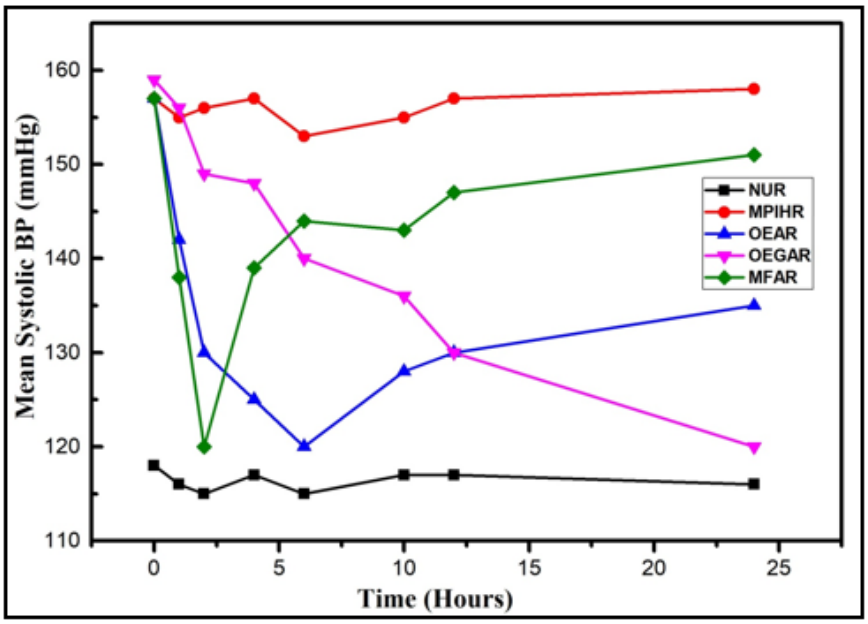


Figure 9

Pharmacodynamic Study (MP Induced) NUR- Normal Untreated Rats MPIHR- Methyl Prednisolone Induced Hypertension Rats OEAR- Optimized Ethosomes Administered Rats OEGAR- Optimized Ethosomal Gel Administered Rats MFAR- Marketed Formulation Administered Rats



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