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FORMULATION AND OPTIMIZATION OF ETHOSOMES LOADED WITH *SPONDIAS PINNATA* BARK EXTRACT FOR TOPICAL DELIVERY BY USING 2^3 FACTORIAL DESIGN

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Abstract

Incorporation of herbal drugs into novel drug delivery systems like ethosomes may overcome the disadvantages associated with herbal drugs. Ethosomes formulation penetrates the drug in a deeper layer of skin and accessible in sufficient quantity in the required site. The present study aimed to formulate and evaluate ethosomes loaded with *Spondias pinnata* extract for topical delivery. Extraction of bark powder of *Spondias pinnata* done by Soxhlet extraction method. Phytochemical testing performed to identify the phytoconstituents. The anti-microbialscreening was performed by cup plate method. Ethosomes loaded with *Spondias pinnata* extract formulation were fabricated by using 2^3 factorial designs. Ethosomes were prepared by the cold method according and evaluated for vesicle size, entrapment efficiency, scanning electron microscopy, and transmission electron microscopy. *In vitro* drug permeation *study* of optimized formulation was performed through goatskin membrane and compare with extract solution. Different phytochemical constituents like alkaloids, saponins, phenols and flavonoids were present in the plant extract. The vesicle size, zeta potential, PDI and percentage entrapment efficiency of optimized ethosomal formulation was found to be 141.1nm, -54.1 mV, 0.321 and 71.24% respectively. Microscopic images showed presence of uniform spherical vesicles. *In vitro* permeability studies show that the flux of ethosomal suspension was 2.5 fold higher than extract solution, may be the attribution of ethanol. The extract had proficient anti-microbial activity. The factorial design (2^3) had the ability to obtain an optimized formula of ethosomes. Overall conclude that the ethosomal formulation could be abetter choice for the topical delivery of *Spondias pinnata* extract for its anti-microbial activity.

Key words : *Spondias pinnata,* Anti-microbial activity, Flux, design of experiment (DoE).

Introduction

Development of novel drug delivery systems has drawn one of the primary considerations in recent decades (Kulkarni, 2011). Novel drug delivery systems are used in delivering herbal drug at a predetermined rate at the site of action which minimizes side effects associated with increased bioavailability of drugs. Incorporation of novel drug delivery to herbal drugs reduces the first-pass metabolism of drugs, drug degradation, and dose dumping at non-targeted areas (Khare, 2007). Further, the delivery of drugs to the skin can be enhanced by formulating into lipid vesicles such as liposomes, ethosomes (Sundari *et al.,* 2017), niosomes etc.

Ethosomes are soft malleable vesicles that are composed mainly of varied sorts of phospholipids (phosphatidylcholine, phosphatidylserine, phosphatidic acid), ethanol (high concentration), and water. The size range of ethosomes varies from microns to nanometers. The ethanol present in the ethosomes in relatively high concentrations interacts with lipid bilayer thereby increasing the membranes fluidity (Pawar *et al.,* 2015).

The QbD approach emphasizes the understanding

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of various components of the system for improved control over desired output. Design of experiments (DoE) and multivariate statistical data analysis are essential elements of QbD, recognized by the recent International Conference of Harmonization Q8 guideline. These tools facilitate varying all the formulation variables simultaneously, allowing quantification and prioritizing the effects produced by these variables, along with any possible interaction between them, in the defined design space (Nadpara, *et al.,* 2012).

Spondias pinnata is a well-known plant indigenous to the South East Asian countries of the world. This plant has been known to possess anti-microbial, anti-diabetic (Mondal *et al.,* 2009), ulcer protective, anti-cancerous (Chaudhuri*et al.,* 2015) anti-diarrhoeal, anthelmintic (Mondal *et al.,* 2010), hepatoprotective (Shetty *et al.,* 2016) and cytotoxic activity. It is also reported that the different parts of the plant have also been used as antiemetic and anti-tubercular agent (Attanayake *et al.,* 2015). The plant is found to contain many phytoconstituents like flavonoids, saponins, phenols, polysaccharides, etc. out of which flavonoids contribute to the anti-microbial (Manik *et al.,* 2013) activity of the bark extract.

Material and Methods

Plant material and reagents

Spondias pinnata bark was collected from the local areas of Mangaluru, Dakshina Kannada. It was authenticated by Dr. Jyothi Miranda, Head of the Department of Botany, St. Aloysius College, Mangaluru, India. Soya phosphatidylcholine (SPC) was purchased from Hi-media laboratories, Mumbai. Cholesterol was purchased from Merck, Mumbai. Ethanol was purchased from Nice Chemicals, Kerala. Polyvinyl alcohol, polyvinyl pyrrolidone, and polyethylene glycol 400 were purchased from Loba Chemie Pvt Ltd, Mumbai. All other solvents and reagents used were of analytical grade.

Methods

Extraction of plant material (Soxhlet extraction)

50 g of the dried powdered bark weighed and kept inside the thimble. Then 500 ml of ethanol was added in the round bottom flask. The solution was heated at 50°C for 6-7 hours to complete 5 cycles per day. The apparatus switched off until 50 cycles completed. The collected extract was then dried at 90°C in a water bath for concentration, and percentage yield was calculated (Kamal *et al.,* 2015).

Prepared extract was subjected to different chemical tests according to the standard procedure in order to determine the presence of various phytoconstituents (Das *et al.,* 2011).

Fourier transform infrared spectroscopy (FTIR) study

FTIR spectroscopy of extract was carried out to find the principle peak and compared with the references standard. It was also performed to check the compatibility between constituent present in extract, soya phosphatidylcholine (SPC), and cholesterol in case of ethosomes. The FTIR spectra of the extract, and optimized ethosomal formulation were recorded on Alpha Bruker spectrometer. After obtaining the FTIR spectra for the samples, the principle peak was compared and analyzed for any incompatibilities.

Calibration of *Spondias pinnata* bark extract by UV-Visible Spectrophotometer

The ethanolic extract was dissolved in small amount of ethanol, followed by addition of phosphate buffer of pH 7.2 and then suitably diluted. The solutions were scanned for its specific UV-Visible range of absorbance maxima. Then the absorbance of the different serial diluted samples was measured at the ëmax, and a standard calibration curve was plotted with concentration against absorbance.

Anti-microbial screening of the extract

Cup plate diffusion method using nutrient agar was employed for the antibacterial activity testing. Nutrient agar plate prepared was inoculated with the organism *E. coli*. The agar plate was divided into five equal portions. Then five cavities were made one in each division. In four of the cavities, the different concentrations (2, 4, 6, & 8µg/ml) of the extract solution were filled, in the fifth cavity ethosomal suspension of extract was filled and sixth cavity Neomycin (standard) was placed using the holder. The agar plate was incubated at 37ºC for 24 hours and then the zone of inhibition was measured (Gupta *et al.,* 2010).

Experimental design

Based on the initial studies and reports published, the factors that influenced the formation of ethosomes were identified as the concentration of soya phosphatidylcholine, the concentration of ethanol and sonication time ((Dhiman *et al.,* 2013) which were taken at two levels (low and high) as shown in table 1, resulting in a 2^3 full factorial design comprising eight trials shown in table 2. The responses of the independent variables selected were vesicle size and percentage entrapment efficiency.

Preparation of ethosomes

Ethosomes were prepared by the cold method

Table 1: Formulation factors for the multilevel factorial design.

Independent variables	-1 (Low)	+1 (High)
Conc. of SPC (A, mg)	250	1250
Conc. of ethanol (B, %)	20	40
Sonication time(C, min)	6	9
Dependent Variables	Goal	
Vesicle size (nm) Y1	Minimum	
Entrapment efficiency (%) Y2	Maximum	

Table 2: Formulation composition batches of ethosomes as per 2^3 full factorial design for 25 ml formulations.

Formulation	SoyPhosphatidyl Choline (mg)	Ethanol (%)	Cholesterol (mg)	Extract (mg)	Propylene glycol(ml)	Sonication time(min)
F1	250	20	25	25	1	3
F2	1250	20	25	25	1	3
F3	1250	20	25	25	1	9
F4	250	40	25	25	1	3
F5	1250	40	25	25	1	9
F6	250	40	25	25	1	9
F7	250	20	25	25	1	9
F8	1250	40	25	25	1	3

(Supraja *et al.,* 2017) as per table 2. Drug, soya phosphatidylcholine (SPC), and cholesterol were dissolved in ethanol by using a magnetic stirrer. Propylene glycol was added to the solution and heated to 30°C. Water heated to the same temperature was added to the above solution and stirred for 10 minutes to allow the formation of vesicles. The size of ethosomal vesicles formed was then reduced by using probe sonicator.

Characterization of ethosomes

Vesicle size, PDI and zeta potential

The vesicle size, PDI and zeta potential of the prepared ethosomes were determined using Malvern zeta sizer which employs dynamic light scattering.

Percentage entrapment efficiency (%EE)

10 ml of the prepared ethosomes were taken in a Tarsus centrifuge tube having a capacity of 15 ml. It was then centrifuged for 12000rpm for 60 minutes at 4°C. The supernatant was collected, and the amount of extract present in the supernatant was analyzed at 209 nm (Indora *et al.,* 2015). The percentage entrapment efficiency of the extract was determined using the formula:

$$\%EE = \frac{\text{Amount of drug entrapped}}{\text{Total amount of drug}} \times 100$$

Amount of drug entrapped = Total amount of drug-total amount of the drug present.

Formulation and characterization of optimized formulation

The optimization of ethosomes was carried out by inputting the responses with minimum particle size and maximum entrapment efficiency. The software generated the solution with desirability $\geq$8 was selected as an optimized formulation. As per the solution generated by the software; 250 mg SPC and 20% ethanol and sonication time of 9 minutes were used to formulate the optimized formulation.

The optimized formulation was assessed for Vesicle size, zeta potential, and % entrapment efficiency. Further, the optimized formulation was evaluated for parameters like scanning electron microscopy, transmission electron microscopy, and in vitro drug release.

Scanning electron microscopy (SEM)

The shape of the vesicles formed is determined using Scanning Electron Microscopy. A drop of the ethosomes was mounted on a glass stub and air-dried. It was coated with Polaren E 5100 Sputter coater and then viewed by SEM.

Transmission electron microscopy (TEM)

Ethosomal suspension was dispersed in distilled water, and then 10 µL of this diluted suspension was placed on the carbon-coated grid, which then visualized using Jeol/JM 2100, source LaB6 electron microscope. An accelerating voltage of 200 kV is employed in the above method (Bendas *et al.,* 2007).

In vitro drug release studies

In vitro drug release studies of the optimized ethosomal formulation was done using a two-sided open tube with help of a dialysis membrane previously soaked in phosphate buffer of pH 7.4 (Bendas *et al.,* 2007). 2ml of ethosomal preparation loaded with the extract is placed in the donor compartment covered with the cellophane

membrane. The receptor compartment is filled with 30 ml of phosphate buffer of pH 7.4. The receptor medium was then stirred using a magnetic stirrer. The temperature of the medium was adjusted to 37±0.5°C. At definite intervals, 3 ml of the sample was withdrawn from the receptor compartment was checked for absorbance at 209 nm. The receptor compartment was replaced with exact same amount of phosphate buffer of pH 7.4 to maintain the sink conditions.

In vitro skin permeation studies

In vitro skin permeation studies were performed through goat ear skin by using Franz diffusion cell. One end of the donor is covered with skin soaked in phosphate buffer of pH 7.4. The optimized ethosomal suspension or extract solution is kept on the dermal side of skin in the donor compartment. The reservoir was filled with 30 ml of the same buffer and was stirred using a magnetic stirrer at a speed of 300 rpm. The temperature of the medium was maintained at 37±0.5°C. Samples are withdrawn at predetermined intervals from the reservoir compartment. The reservoir is replenished with the same amount of buffer. Then the absorbance was measured at 209 nm. The findings were analyzed using Graph Pad software, by using paired T-test. The steady state flux (JSS) was calculated against a time (h) plot fromthe slope of the linear part of the cumulative amount of extract permeated per unit area (µg/cm^2). The permeability coefficient (KP) of extract through the goatskin was determined using the following equations:

$$\text{Permeability coefficient}\,(K_p) = \frac{J_{ss}}{C_0}$$

Where C_o is the initial concentration

Results and Discussions

Ethanolic extract of *Spondias pinnata* was a brownish color, and the percentage yield was found to be 17.93%, respectively. The preliminary phytochemical analysis showed the presence of flavonoids, alkaloids, saponins and phenols in the plant extract and results are shown in table 3.

FTIR Study

FTIR spectrum of crude extract was shown in Fig. 1a. The observed peak of the extract at 3353.51cm^{-1} indicates the presence of hydroxyl group of ethanol. Peak present at 1616.57cm^{-1} and 1335.55cm^{-1} show the presence of aromatic unsaturation which is due to the presence of flavonoids in the extract. The peak at 1457.90 cm^{-1} which is due to C=O and C=C aromatic stretching vibration in the structure of flavonoids. It was found that peaks observed for the extract is nearly retained in the peak of the optimized ethosomal suspension as shown in fig. 1b, which indicates that there is not much interaction of the extract with the components of the formulation.

Calibration of the crude extract

The crude ethanolic extract of *Spondias pinnata* was dissolved in little amount of ethanol and diluted with phosphate buffer of pH 7.4 and scanned in a UV-spectrophotometer have shown the maximum absorbance wavelength at 370 nm may be due to total flavonoids as said quercetin. The serially diluted samples of the extract exhibited the absorbance value in the linearity range and their regression was found to be 0.999 (Fig. 2).

Anti-bacterial screening of the extract

As shown in table 4, all concentrations of the extract showed some extent of the zone of inhibition. The concentrations, 8µg/ml showed similar zone of inhibition to the standard solution. Efficient anti-microbial activity was shown by the extract of *Spondias pinnata* due to presence of flavonoids (Da Silva *et al.,* 2012) and phenols content. Das *et al.,* 2011 was also performed the anti-bacterial activity and their results supported that the presence of total phenol and flavonoid content responsible for anti-bacterial activity.Further ethosomal suspension of extract also showed similar result; it concludes that antimicrobial activity of extract was retaining event after incorporating in ethosomal vesicles.

Characterization of ethosomes

Vesicle size, PDI and zeta potential

The increase in ethanol concentration from 20% to 40% v/v showed a small increase in the vesicle size at all SPC concentration due to the breakage of bilayer of film leading to aggregation of vesicles. Increased concentration SPC at a similar concentration of ethanol showed a decrease in the vesicle size due to the impact of sonication time on vesicle size as shown in table 5 and

Table 3: Phytochemical test.

Tests	Observation	Inference
Alkaloid	Presence of reddish brown precipitate	Presence of alkaloids confirmed
Saponin	Appearance of frothing	Presence of saponin confirmed
Flavonoids	Production of yellow colour in the organic layer	Presence of flavonoids confirmed
Phenol	Formation of bluish black colour	Presence of phenols confirmed

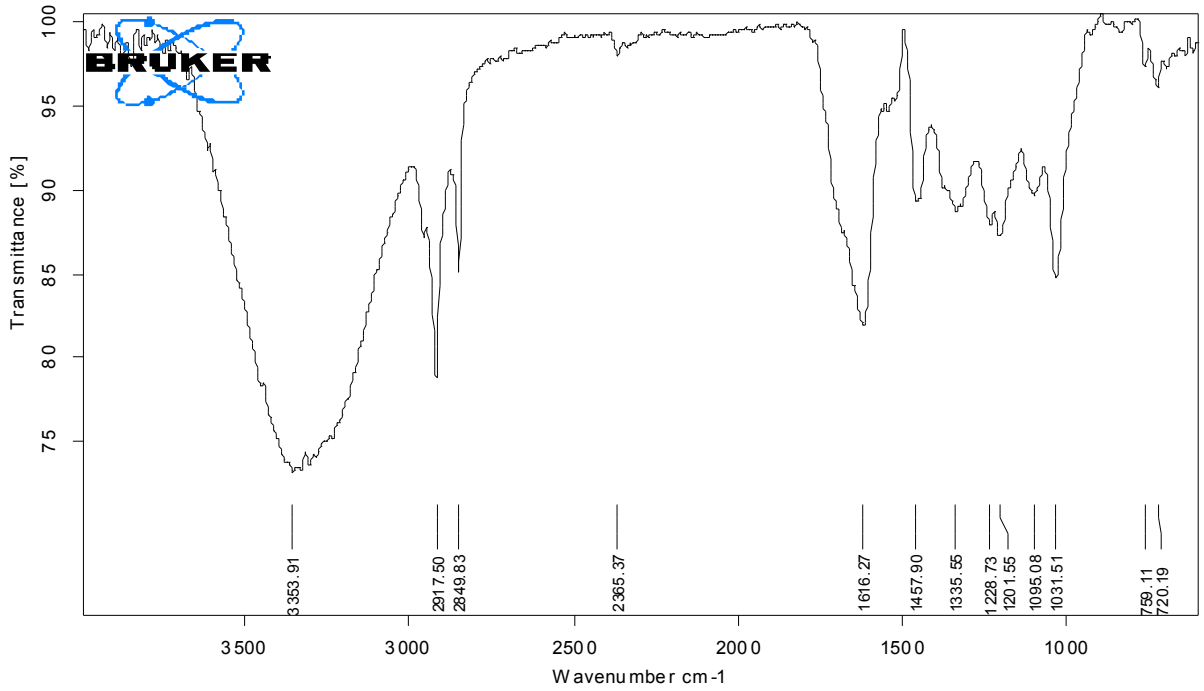


Fig. 1a: FTIR spectra of *Spondias pinnata* bark extract.

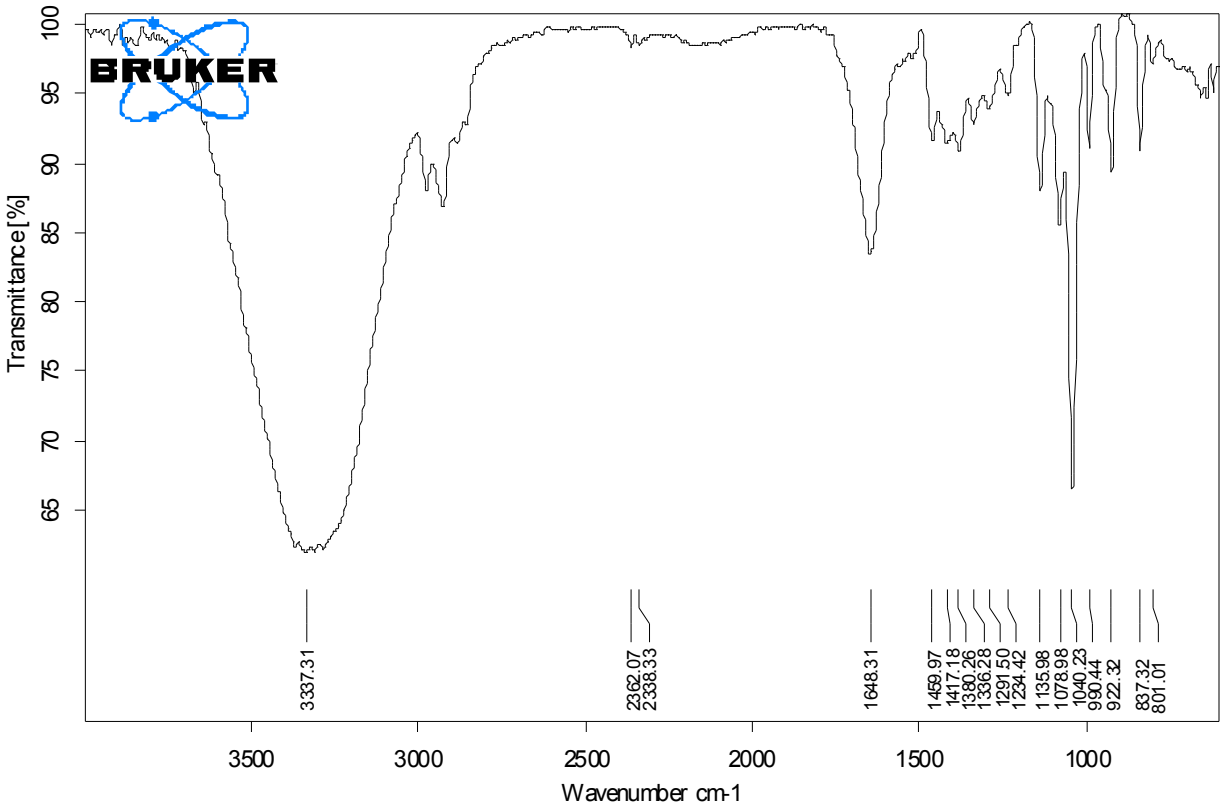


Fig. 1b: FTIR spectra of optimized ethosomal formulation.

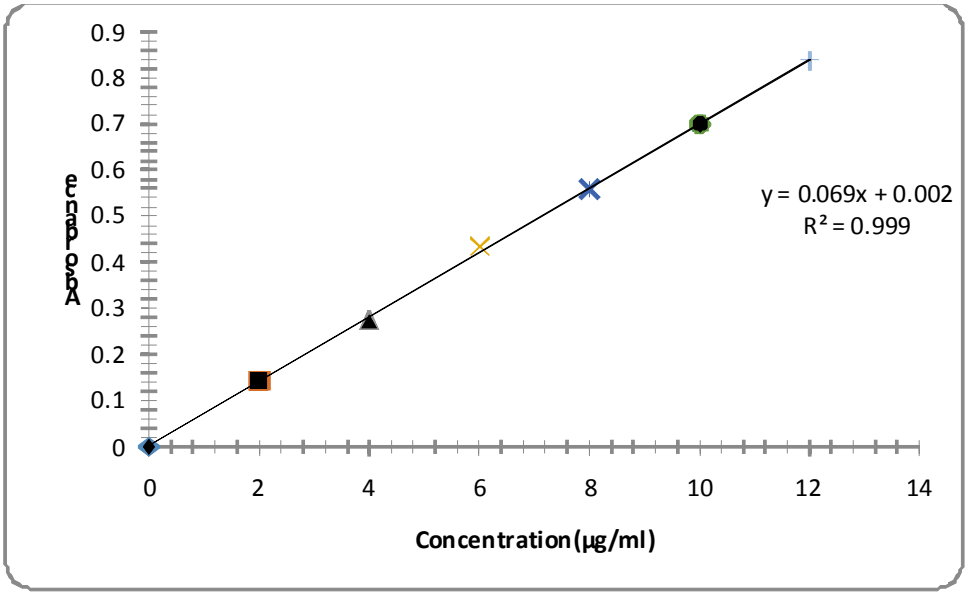


Fig. 2: Calibration curve of ethanolic extract of *Spondias pinnata in* phosphate buffer of pH 7.4

Table 4: Anti-microbial screening of extract.

S. No.	Substance used	Zone of inhibition (mm)
1.	Extract (2µg/ml)	7
2.	Extract (4µg/ml)	15
3.	Extract (6µg/ml)	19
4.	Extract (8µg/ml)	25
5.	Standard (Neomycin)	29
6.	Ethosomal suspension	27

fig. 3.

The Polynomial model implied significant with model f-value of 43.97. The Predicted R^2 of 0.8807 is in reasonable agreement with the Adjusted R^2 of 0.9609; *i.e.*, the difference is less than 0.2. The following polynomial equation was obtained from the results of the analysis:

Vesicle Size= +160.14 -29.42 (A)* +29.09 (B)* -7.61(AB) +11.43(BC)*

Where A is the concentration of soya phosphatidylcholine, B is the concentration of ethanol and C is the sonication time. The coefficient in this equation reflects the standardized beta coefficient and the asterisk symbol indicates the variable's importance. The regression model obtained was defined as statistically significant ($p<0.05$) with a high adjusted R2 value of 0.9609 as shown in table 6.

The zeta potential values for the prepared ethosomes were found within the range -30 mV to -70 mV which indicates that the formulations are stable. This is often because at higher zeta potential, the strong repulsion between the particles is present which avoids the tendency of vesicles to aggregate. PDI was found to be less than 0.5 which specifies that all the formulations were homogeneous in nature.

Percentage entrapment efficiency

The increase in ethanol concentration at all SPC concentration showed a significant decrease in entrapment efficiency due to partial fluidization lipid bilayers by ethanol, resulting in leakage of entrapped drug (Peram *et al.,* 2019) as given in table 5 and fig. 4.

As the concentration of SPC increases at all ethanol concentration, the entrapment efficiency decreases, because at higher concentration, chances of aggregation of vesicles increase resulting in reduced ability to form a stable film. As a result of this, drug leaching occurs and entrapment efficiency decreases accordingly. Further, as sonication time increases, there was decrease in entrapment efficiency as a result of breakage of bilayer of membrane.

As shown in table 6, Polynomial model implied significant with model f-value of 36.67. The Predicted R^2 of 0.8273 is in reasonable agreement with the Adjusted R^2 of 0.9622; *i.e.*, the difference is less than 0.2. The following polynomial equation was obtained from the results of the analysis:

Entrapment efficiency= +54.87-3.26 (A)-6.76 (B)*+4.89 (AB)*+6.82 (AC)*+10.46(ABC)*

Where A is the concentration of soya phosphatidylcholine, B is the concentration of ethanol and C is the sonication time. The coefficient in this equation reflects the standardized beta coefficient and the asterisk

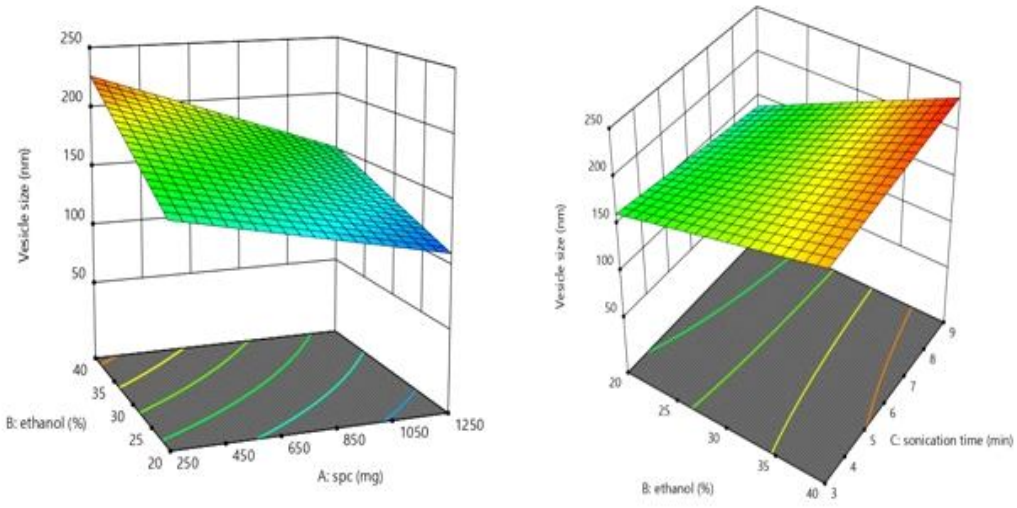


Fig. 3: Response surface curve depicting the effect of SPC, ethanol and sonication time on vesicle size.

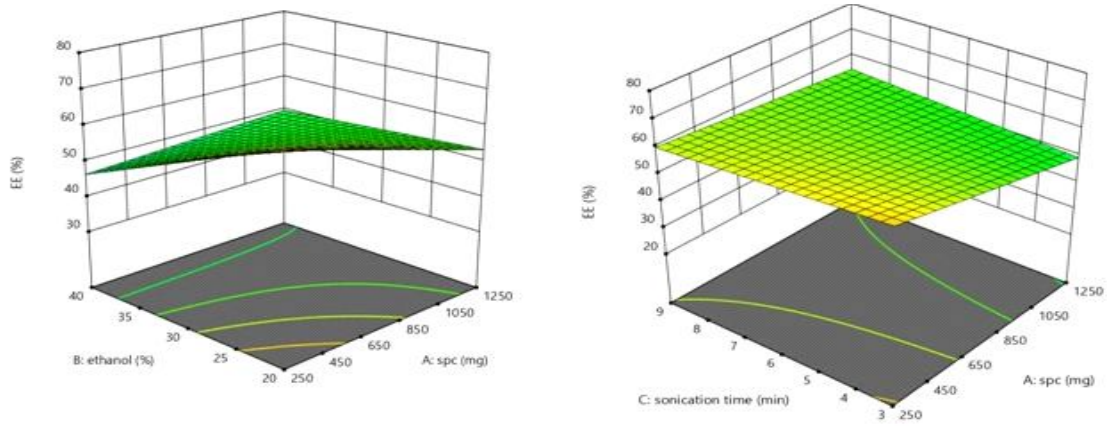


Fig. 4: Response surface curve depicting the effect of SPC, ethanol and sonication time on entrapment efficiency.

Table 5: Results of responses of ethosomes as per 2^3 full factoria.

Form.	Vesicle size (nm)(Y1)	Entrapment Efficiency (%)(Y2)	Zeta potential (mV)	PDI
F1	172.4±2.26	65.56±3.22	-44.2±1.97	0.403±1.01
F2	121.23±1.99	56.52±2.56	-56.52±1.88	0.497±1.14
F3	97.36±2.01	50.43±3.14	-50.76±2.12	0.441±1.40
F4	215.56±2.17	61.56±2.94	-54.23±2.01	0.486±1.23
F5	155.7±2.47	69.2±2.17	-61.53±1.57	0.417±1.09
F6	236.96±1.76	31.39±3.32	-55.16±2.64	0.336±1.16
F7	133.3±2.27	74.0±2.48	-41.1±1.94	0.457±1.31
F8	148.7±1.96	30.26±2.77	-30.26±2.32	0.438±1.34

symbol indicates the variable's importance. The regression model obtained was defined as statistically significant ($p<0.05$) with a high adjusted R2 value of 0.9622.

Optimization of ethosomes loaded with extract

The optimized formulation was selected based on the desirability value more than 0.8 and prepared according to the solution given by the software containing 250 mg SPC and 20% ethanol at a sonication time of 9 minutes. The observed value of vesicle size and percentage entrapment efficiency was found to be 141.41 nm and 73.41 % respectively, which is within 95% of CI of the predicted value which is acceptable as shown in Table 7. Zeta potential and PDI of optimized was found to be -54.1 mV and 0.321 respectively, which indicates that formulation is stable and vesicle is homogeneous in nature.

Scanning electron microscopy & Transmission electron microscopy

The particles were found to be uniform and having a spherical shape. The surface of the vesicles was found to be smooth as shown in fig. 5a. The TEM photographs showed the surface morphology of the vesicles with the presence of a unilamellar vesicular structure as shown in fig. 5b.

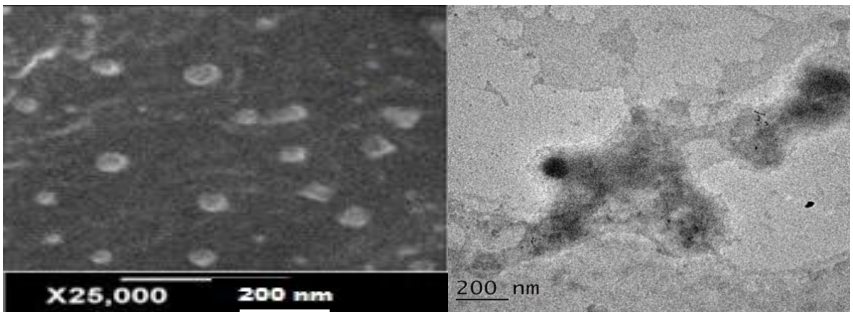
Table 6: Summary of regression analysis and ANOVA.

Factor	Vesicle size (Adjusted R^2 = 0.9609)		%Entrapment efficiency (Adjusted R^2=0.9622)	
	Estimated beta coefficient	p value	Estimated beta coefficient	p value
Intercept	+160.14	0.0054*	+54.87	0.0268*
A- SPC	-29.42	0.0029*	-3.26	0.1027
B- Ethanol	+29.09	0.0030*	-6.76	0.0270*
AB	-7.61	0.1034	+4.89	0.0499*
BC	+11.43	0.0401*	+6.82	0.0266*
ABC	-	-	+10.46	0.0116*

*Statistical significance of independent variables.

Table 7: Response of optimized formulation.

Response	Predicted Value	Observed Value	StdDev	95% CI low for Mean	95% CI high for Mean
%EE	73.412	71.24	3.21005	66.875	79.9488
Vesicle size	141.416	141.1	9.29732	132.151	150.68



a b

Fig. 5: (a) SEM and (b) TEM of optimized ethosomal vesicle.

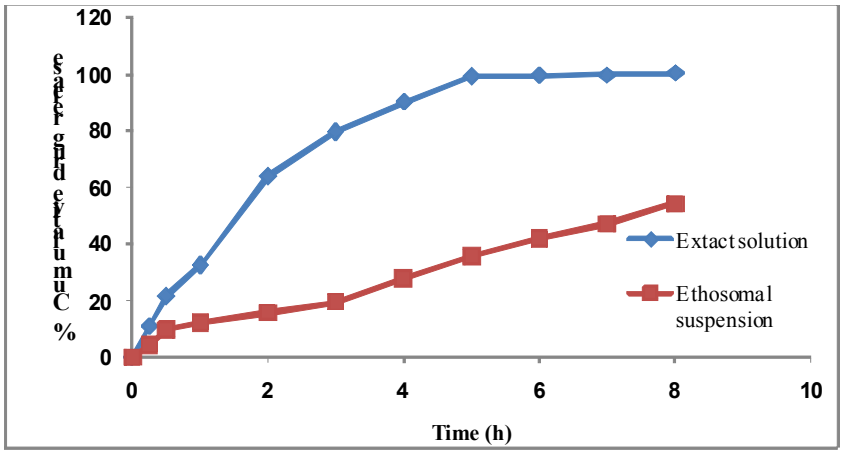


Fig. 6: Comparative *in vitro* drug release study of optimized ethosomal suspension with extract solution.

In vitro drug release study

The extract was release almost 100% from extract solution within 5 h, whereas from ethosomal suspension, it was released in a sustained manner for a prolonged period, as shown in Fig. 6. Sustained-release pattern was observed due to the compact wall present around the drug produced by the biodegradable lipid matrix. As shown in Table 8, the prepared optimized formulation of ethosomes followed first-order release kinetics with a regression coefficient (R^2) of 0.736. The drug release mechanism of ethosomes was studied by fitting the data to Higuchi model and Korsemeyer-peppas exponential model. Good linearity was found for the optimized formulation with a regression coefficient (R^2) of 0.851 based on the release plotted for Korsemeyer-peppas equation. The release component (n) was found to be 0.58 which is above 0.45. This indicated that the release was characterized by Non- Fickian diffusion which states that the release of the drug was regulated by two mechanisms *i.e.*, diffusion coupled with erosion mechanism.

In vitro permeability study

Fig. 7, demonstrated the permeation profile of extract from ethosomal suspension and extract solution. The total quantity of extract delivered from ethosomal suspension was found to be 1253.61 µg, which was substantially higher than the quantity found that the delivered by extract solution, which was 735.53 µg ($p < 0.0001$). The enhanced permeation of extract from ethosomes may be the presence of ethanol in the core of Ethosomes which helps to solubilize the lipid in the stratum corneum, allowing high vesicles penetration. Parameter of permeability, as shown in Table 9, in the case of ethosomal suspension the steady-state flux was greater than the extract solution. The steady-state flux and permeability coefficient of ethosomal suspension was found to be 200.99µg/cm^2.h & 0.2738 cm/h respectively whereas for extract solution was shown to be 80.39 µg/cm^2.h & 0.08039 cm/h

Table 8: Comparison of in vitro drug release kinetics of pure drug with optimized ethosomal suspension.

Formulation	Kinetic models								
	Zero order		First order		Higuchi		Korsmeyer-peppas		
	R^2	K	R^2	K	R^2	K	R^2	K	N
Extract solution	0.148	-0.376	0.436	0.0017	0.711	9.0076	0.2355	1.6181	0.2269
Ethosomal suspension	0.627	-2.492	0.736	-0.019	0.8173	15.324	0.8515	1.4176	0.5846

Table 9: Permeated amount of extract at 8 h, flux & permeability coefficient.

Form. Code	Permeated amount at 8 h (µg/cm²)	Flux (µg/cm².h)	Permeability coefficient (K_p)(cm/h)
Extract solution	735.45	80.39	0.08039
Ethosomal suspension	1253.12	200.99	0.273828

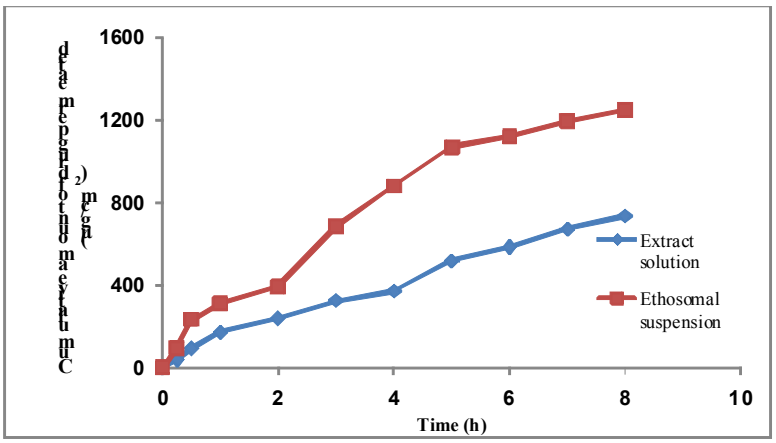


Fig. 7: Comparative *in vitro* permeability of extract through goat skin from ethosomal suspension and extract solution.

respectively after 8 hrs. Results indicated that the flux and permeability coefficient of ethosomes was 2.5 and 3.3 fold higher than extract solution respectively. It was found that there is a direct relationship between steady-state flux and permeability of coefficients, as shown in Table 9. The previous results could be attributed to ethanol content in the ethosomal core which dissolve the lipid of skin and overcome the skin barrier properties (Nimisha, *et al.,* 2017, Peram *et al.,* 2019).

Conclusions

From the above analysis, it was concluded that the factorial design (2^3) was capable of obtaining an optimized ethosomal formula, with high EE% and small vesicle size. In Addition, the preparation of ethosomal suspension of the extract can overcome the skin's barrier properties as opposed to extract solution. Antimicrobial activity of ethosomal extract suspension was found to be similar to that of an extract solution.

Conflict Of Interest

The author declares no conflict of interest.

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