QbD Approach for the Design, Optimization and Characterization of Piperine Phytosomes to Enhance Bioavailability

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ABSTRACT

Background and Purpose:

Diabetes type 2 (adult-onset diabetes) is a chronic metabolic disorder characterized by high blood sugar (glucose) levels. Piperine is an alkaloid compound found naturally in black pepper has antioxidant properties, which means it can aid in the fight against oxidative stress and Inflammation. Some preliminary in vitro and animal studies indicate that may have insulin-mimicking properties. Insulin resistance and type 2 diabetes are both linked to chronic inflammation. The aim of this study was to examine the anti-diabetic activity of formulated piperine phytosomes in an in vitro glucose uptake assay.

Experimental Approach:

Phosphatidylcholine was used to prepare the phytosomes, and Quality by design was used to determine the model's robustness. An additional batch of PP was prepared in order to evaluate the optimization capability of the models using the optimized values of the variables. The prepared phytosomes were physio-chemically characterized. To determine the acute oral toxicity and effect of PP on cellular glucose uptake behavior, the glucose uptake assay was performed on L6 cell lines. Furthermore, the phytosomes anti-diabetic activity was compared to that of commercially available standard anti-diabetic drugs such as Insulin (1 IU/mL) and Metformin (100 µg/mL).

Findings:

The comparison between the predicted (theoretical) value (%) of the PP obtained from the developed model and the observed value (%) achieved from the prepared formulation was carried out using QbD approach. L6 cell lines were used to measure the *in vitro* glucose uptake assay to ascertain the impact of phytosomes on cellular glucose uptake behavior.

Conclusion:

The current study achieved an optimized formulation by QbD approach and in vitro antidiabetic activity, the prepared test samples PPN, PP exhibited significant glucose uptake activity on L6 cell lines.

Keywords: Formulation, Optimization, Piperine, Phytosomes, Quality.

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1. INTRODUCTION

Piperine (PPN) is a natural alkaloid compound found in Piper nigrum and other related spices, such as white pepper and long pepper [1]. It is responsible for the pungent and spicy flavor of these peppers and has been used for centuries to enhance the taste of various dishes. Piperine is not only known for its culinary significance but also for its potential health benefits and medicinal properties [2]. One of the most notable properties of piperine is its ability to enhance the bioavailability of certain nutrients and compounds in the body. It does this by inhibiting enzymes that break down these substances in the digestive system, allowing them to be absorbed more effectively [3]. Piperine has been studied for its potential health-promoting effects. Some research suggests that it may have antioxidant, anti-inflammatory, and even anti-cancer properties [4]. Additionally, it may help improve digestive health and aid in weight management. It contains alkaloids, flavones, terpenes, and steroids. Piperine and piperidine are the two main alkaloids extracted from black pepper and piperine in recent studies proved to be PPAR-agonist and insulin sensitivity [5]. As a result, piperine could be used as anti-diabetic medication. Thus, piperine has shown promise in various studies, it's important to note that more research is needed to fully understand its effects on health and to determine safe and effective dosages.

2. EXPERIMENTAL METHODS

2.1. Preliminary phytochemical screening: The pure piperine (1 g) was thoroughly dissolved in 100 mL of its mother solvents before being tested for the presence of various phytochemical components like polyphenols, flavonoids, terpenoids, steroids, saponins, tannins, alkaloids, and glycosides. The prepared solution was used. The obtained stock solution was put to use for phytochemical screening in accordance with Harborne and Kokate's [6-8] methodology.

2.2. Preparation of Phytosomes: The piperine phytosomes (PP) were prepared by refluxing followed by solvent evaporation technique. Phytosomes were prepared in different ratios, i.e., 0.5:1, 0.75:1, 1:1, 2.5:1 and 3:1 of Phosphatidylcholine to pure piperine. Phosphatidylcholine and pure piperine were dissolved in dichloromethane and Methanol respectively. Both the solutions were mixed and pour in a 200 ml round bottomed flask. The mixture was refluxed for different duration i.e. 1-4 hr and at various temperature 45-65°C. The resulting clear solution was evaporated, dried under vacuum (40°C);

the residues were gathered and stored in desiccators for further use. The mixture was refluxed for different duration i.e. 1-4 hr and at various temperature 45-65°C. The resulting clear solution was evaporated, dried under vacuum (40°C); the residues were gathered and stored in desiccators for further use.

2.3. Quality by Design-based Design of Experiment[9]

A QbD-based approach using a central composite design to obtain a response surface design was employed to systematically study the combined influence of the formulation and process variables such as the phospholipids-drug ratio (X_1, w/w), the reaction temperature (X_2, °C), and the reaction time (X_3, h) on the critical quality attributes (CQAs) of the product i.e., the entrapment efficiency and drug content (%) [16]. Using this design, the influence of three factors was evaluated, and the experimental trials were carried out at all 20 possible combinations. A statistical model incorporating interactive and polynomial terms was used to evaluate the response employing the equation:

$$Y= b_0+b_1X_1+b_2X_2+b_3X_3+b_4X_1^2+b_5X^2_2 +b_6X^2_3 +b_7X_1X_2+b_8X_1X_3+b_9X_2X_3$$

Where Y is the dependent variable, b_0 is the intercept representing arithmetic mean response of the 20 runs, and b_1 to b_9 is the estimated coefficient for the factor (Xi, i=1,2,3). X_1, X_2, and X_3) are the coded levels of independent variables. The interaction terms (X_1X_2, X_2X_3, and X_1X_3) showed how the response changes when all three factors were simultaneously changed. The polynomial terms (X_1^2, X_2^2 and X_3^2) were included to investigate nonlinearity. The level values of the three factors, the real values of the central composite batches, are shown in table 1.

Table 1: Coded level and real values for each factor under study

Variables	Levels				
	-1.68	**-1**	**0**	**1**	**1.68**
X1	0.5	1.0	1.75	2.5	3.0
X2	45	50	55	60	65
X3	1	1.5	2	2.5	3.0

2.4. Validation of optimized model [10]

An additional batch of phytosomes was prepared in order to evaluate the optimization capability of the models generated according to the result of central composite design- response surface methodology. A comparison between the predicted (theoretical) value (%) of the phytosomes obtained

from the developed model and the observed value (%) achieved from the prepared formulation was carried out. The model-predicted value for the entrapment efficiency of piperine in phytosomes and average observed value (%) from the prepared batches was compared. The bias (%) was calculated using following equation which should be less than 3%.

2.5. Physico-chemical characterization of prepared phytosomes [11, 12]

2.5.1. Apparent solubility: The apparent solubility of piperine and phytosomes was determined by adding excess of piperine and phytosome to 5ml of water or n-octanol in sealed glass containers at room temperature (25-30ºC). The liquids was agitated for 24 hours then centrifuged for 20 min at 1,000 rpm to remove excess of piperine. The supernatant was filtered through a membrane filter (0.45 m) then 1 ml filtrate was diluted with 9 ml of distilled water or n- octanol and these samples were measured spectrometrically at 268 nm using UV spectrophotometer.

2.5.2. Entrapment efficiency: Entrapment efficiency (EE) was measured using UV visible spectrophotometer (UV-3000+, Lab India). Weighed quantities of Phytosomes equivalent to 10 mg of piperine were added to 50 ml methanol in a 100 ml beaker. The contents were stirred on a magnetic stirrer for 4 hours and then allowed to stand for one hour. Clear liquid was decanted and centrifuged at 5000 rpm for 15 minutes. After centrifugation the supernatant was filtered through 0.45µm Whatman filter paper and after suitable dilution absorbance was measured in UV at 268 nm; the concentration of drug was measured. All measurements were performed in triplicate.

2.5.3. Particle size distribution: The particle size analysis of the prepared samples was carried out using photon correlation spectroscopy, with dynamic light scattering on Zetasizer nano (Model: Nano series, S90 Zeta sizer, Malvern). The phytosomes were dispersed in isopropyl alcohol by stirring on a magnetic stirrer for 10 minutes. The dispersion was analyzed in size analyzer.

2.5.4. X-Ray diffraction (XRD) study: The polymorphic states of samples were evaluated using X ray diffractometer (D- 8 Advance Bruker, USA). The operating conditions were: voltage 45 kV; current 0.8 mA and scanning speed 1/min. The samples were scanned with the diffraction angle over a range of 5–60° (2θ angle), using the Cu-Anode X-ray tube and scintillation detector.

2.5.5. Differential scanning calorimetry (DSC): The thermal analyses of samples were carried out using differential scanning calorimeter Perkin Elmer (USA) (Model JADE DSC). The analysis was performed under a purge of dry nitrogen gas with high-purity medium was used to calibrate the heat

flow and the heat capacity of the instrument. The samples (~5 mg) were held in closed metal pans. Each sample was subjected to a single heating cycle from 0°C to 300°C at a heating rate of 10°C/min.

2.5.6. Fourier Transform Infrared spectroscopy (FTIR) Study: Fourier transform infrared spectrophotometer (Model: IR Prestige-21, Shimadzu, Japan) was employed to study the interaction between piperine and PC and to establish the structure and chemical stability of prepared phytosomes. The IR spectra of samples were obtained by the potassium bromide (KBr) method. KBr pellets were prepared by gently mixing small quantity of sample (1 mg) with 100 mg KBr. A small quantity of sample was placed just below the probe on to which the probe was tightly fixed and scanned in the wave number region 4000-500 cm-1. The obtained IR spectra were interpreted for functional groups at their respective wave number (cm^{-1}).

2.5.7. Scanning electron microscopy (SEM): Samples were coated with gold in a Fine Coat Ion Sputter (S-4800 TYPE II, Hitachi, Japan). Analysis was done on the coated sample by placing a pinch of sample in the Scanning electron microscope and surface morphology was viewed and photographed to observe their particle shape and surface morphology.

2.5.8. Dissolution Study *(In-vitro* drug release): The *In vitro* dissolution profiles of prepared phytosomes were obtained. The dissolution studies were carried out in a Lab India DS 8000, eight station dissolution test apparatus, type II at 100 rpm and 37°C. An accurately weighed amount of phytosomes 50 mg was put in to 900 ml of pH 6.8 phosphate buffer. Samples (3 ml each) of dissolution fluid were withdrawn at different time intervals and replaced with an equal volume of fresh medium to maintain sink conditions. Samples were withdrawn and filtered through a 0.45 µm membrane filter, diluted suitably and then analyzed spectrophotometrically at 268 nm to determine drug release from the complex and the drug.

2.6. Acute Oral Toxicity Studies

The AOT study was performed as per the OECD-425 guidelines and the protocol for AOT activity was approved by Institutional Animal Ethics Committee (IAEC) of Sri Padmavati Mahila Visvavidyalayam (Women's University), Tirupati (CPCSEA Reg. No.1677/PO/Re/S/2012/CPCSEA)[13]. The test was carried out for the piperine of phytosomes (PP) using overnight fasted wistar rats (3F+3M). Each piperine with the limit test dose in sequence at a 24 h interval was administered using a stainless curved oral feeding needle. In brief, the first female rat received a limit test dose of 2000 mg/kg. Then the remaining two female rats received the same dose and then further, three male rats received the same

dose and were observed for toxicity symptoms for 30 minutes carefully for individual, 2 h occasionally, 4 h special care, and each day14 days. General behavior, including writhing, gasping, palpitation, decreased respiratory rate, and mortality were observed.

2.7. *In vitro* antidiabetic activity[14, 15]

***2.7.1. In vitro* antidiabetic activity by glucose uptake method:** The effect of piperine on glucose uptake was examined using differentiated rat skeletal muscle cells (L-6 cells). The cell cultures (70–80% confluence) were allowed, for 4–6 days, to differentiate in Dulbecco's modified eagle growth medium (DMEM) comprising 2% FBS. Then, the differentiated cells were serum-starved overnight, washed once with HEPES buffered Krebs Ringer Phosphate solution (KRP buffer), and incubated at 37°C for 30 min in KRP buffer containing 0.1% BSA. The cells were further incubated with test and standard drugs (non-toxic concentrations) and negative controls for 30 min at 37°C. The D-glucose solution (1 M, 20 µL) was added at the same time to all wells before incubation. Post incubation, the supernatant solutions aspired from wells and the cells were washed three times using the KRP buffer solution (ice-cold). Aliquots of cell lysates (prepared in 0.1 M NaOH solution) were analyzed for cell-associated glucose using a glucose assay kit (ERBA).

3. RESULTS AND DISCUSSION

3.1 Preliminary phytochemical screening

The pure piperine (PPN) were screened for the presence of various phytochemicals such as polyphenols, flavonoids, terpenoids, steroids, saponins, tannins, alkaloids, and glycosides (Table 2a). These results revealed the solubility pattern of the above-screened phytoconstituents.

Table 2a: The phytochemicals of the selected plant extracts

Test Compound	P	F	T	St	S	Ta	Al	Gl
Piperine	+	+	-	+	-	-	+	-

The sign (+) indicates the presence and (-) indicates the absence of phytochemicals: Polyphenols; F: Flavonoids; T: Terpenoids; St: Steroids; S: Saponins; Ta: Tannins; Al: Alkaloids; Gl: Glycosides.

Total phenol and flavonoid content of the extracts

The total phenol and flavonoid concentrations of the extracts are presented (Table 2b). The pure piperine showed a considerably elevated amount of flavonoids and phenolic compounds. In addition, the results revealed that the total phenolic content are 6.51 mg/gm whereas the total flavonoid content present the piperine extract was 53.11 mg/gm equivalent.

Table 2b: **Total phenol and flavonoid content of the selected plant extracts (PPN)**

Extract	TPC*	TFC*
Piperine (PPN)	6.51±0.31	53.11±2.15

values are mean±SD. TPC: Total phenolic content; TFC*: Total-flavonoid-content of extracts (mg/gm equivalent).*

3.2. Preparation of piperine phytosomes (PP): Piperine phytosomes were prepared by solvent evaporation technique. The influence of three factors was evaluated, and the experimental trials were carried out at all 20 possible combinations. Influence of factors revealed that all the tested variables, i.e., the drug to phospholipids ratio, the reaction temperature, and the reaction time had a significant influence on the entrapment efficiency of the prepared PP and also on the drug content present in the PP. The measured values from the experimental trials revealed wide range of (65.34–95.24 %w/w) entrapment efficiencies. The results of the entrapment efficiency (%) and Drug content are shown in Table. A mathematical relationship between factors and parameters was generated by response surface regression analysis in the software Minitab 17. The three-dimensional response surface plots for the most statistically significant variables on the evaluated responses are shown in Figure. The equations represented the quantitative effect of process variables (X_1, X_2, and X_3), and their interactions on response Y are as follows:

$$Y = 89.442 + 3.786X_1 + 1.925X_2 + 3.344X_3 - 0.947X_1^2 - 0.685X_2^2 - 0.727X_3^2 + 0.624 X_1X_2 - 0.970 X_1X_3 - 0.606 X_2X_3$$

The polynomial model for Y were found to be significant, with F values of 11.47 ($P<0.05$). The value of correlation coefficient (R^2) was found to be 0.9117, indicating a good fit to the quadratic model. Based on the central composite design, the response surface plots show the changes in the entrapment efficiency (%) and drug content as a function of X_1, X_2, and X_3. The data from all 20 batches of the central composite design were used for generating interpolated values using software. The response surface plots indicated a strong influence of the studied factors X_1, X_2, and X_3 on the entrapment efficiency. Based on these observations, along with the multiple regression models, the optimal values

of the studied factors, i.e., the drug -to-phospholipids ratio, the reaction temperature, and the reaction time were 1:3, 65°C, and 2 h, respectively.

Table 3: Independent variables, along with coded and actual values and respective responses for different batches of PPs

Std	Run	Coded Value Variables			Actual Value Variables			Response Value	Response Value
		X_1	X_2	X_3	X_1	X_2	X_3	% EE (Y)[a]	Drug content [b]
1	1	-1	-1	-1	1:0.75	50	1.5	65.34 ± 0.23	88.52± 0.12
2	2	1	-1	-1	1:2.5	50	1.5	80.45 ± 0.54	79.24± 0.25
3	3	-1	1	-1	1:0.75	60	1.5	72.34 ± 0.87	86.23± 0.18
4	4	1	1	-1	1:2.5	60	1.5	89.56 ± 0.74	91.25± 0.16
5	5	-1	-1	1	1:0.75	50	2.5	88.12 ± 0.89	94.25± 0.15
6	6	1	-1	1	1:2.5	50	2.5	87.31 ± 0.52	88.17± 0.19
7	7	-1	1	1	1:0.75	60	2.5	83.32 ± 1.05	92.36± 0.10
8	8	1	1	1	1:2.5	60	2.5	94.51 ± 0.32	75.26± 0.59
9	9	-1.68	0	0	1:0.5	55	2	68.93 ± 0.35	94.88± 0.08
10	10	1.68	0	0	1:3	55	2	95.24 ± 0.65	90.25± 0.15
11	11	0	-1.68	0	1:1	45	2	76.54 ± 0.68	91.57± 0.15
12	12	0	1.68	0	1:1	65	2	91.82 ± 1.31	87.65± 0.18
13	13	0	0	-1.68	1:1	55	1	74.56 ± 0.36	75.28± 0.23
14	14	0	0	1.68	1:1	55	3	93.13 ± 0.24	94.62± 0.02
15-20	15-20	0	0	0	1:1	55	2	89.43 ± 0.54	90.17± 0.10

[a]*Values represent mean ± standard deviation (n=3)*

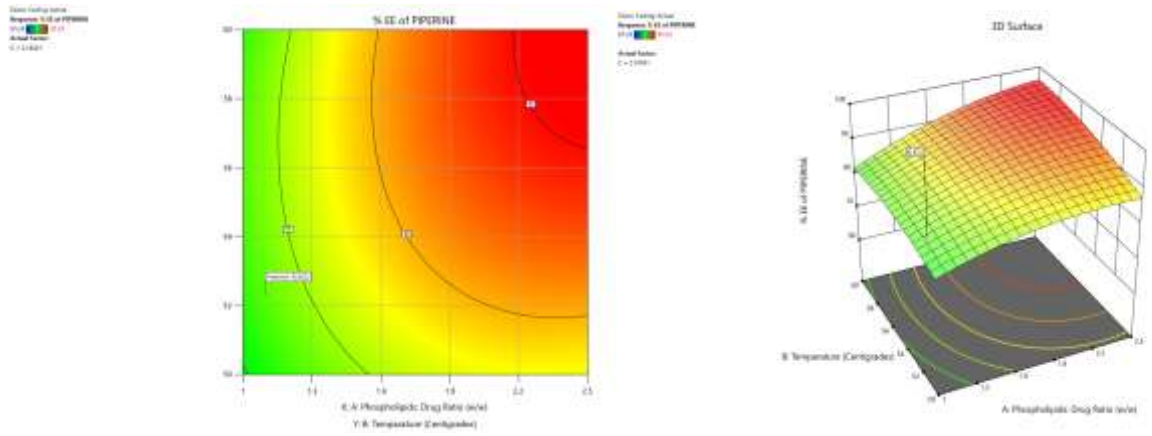


Figure 1a: Response surface plot showing the influence of phospholipids-drug ratio (X_1, w/w), the reaction temperature (X_2, °C), and the reaction time (X_3, h) on entrapment efficiency of PP.

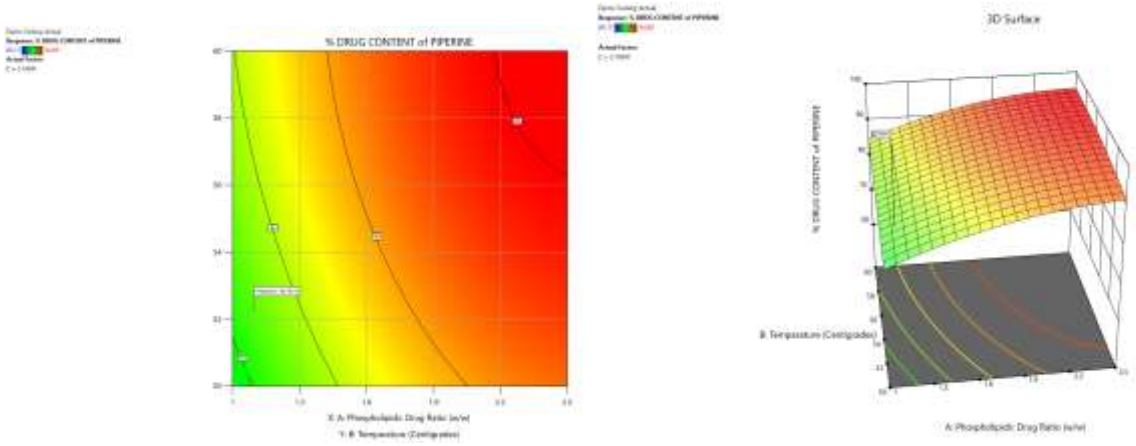


Figure 1b: Surface response plot showing the influence of phospholipids-drug ratio (X1, w/w), the reaction temperature (X2, °C), and the reaction time (X3, h) on Drug Content of PP

3.3. Validation of optimized model: An additional batch of PP was prepared in order to evaluate the optimization capability of the models using the optimized values of the variables. A comparison between the predicted (theoretical) value (%) of the PP obtained from the developed model and the observed value (%) achieved from the prepared formulation was carried out and it is shown in Table. The model-predicted value for the entrapment efficiency of PIPERINE in PP was 82.82% and the drug content was 82.18, while the average observed value (%) from the prepared batches was found to be 84.50% and the

drug content was 83.16 %, indicating both applicability, and validity of the developed model. The bias (%) was found to be less than 3% (1.16%), indicating the relative robustness of the model. The results are illustrated in table 2.

Table 4: Observed and Predicted value of entrapment efficiency and Drug Content of optimized Phytosomes.

Solution 1 of 100 Response	Predicted Mean	Predicted Median	Observed	Std Dev	SE Mean	95% CI low for Mean	95% CI high for Mean	95% TI low for 99% Pop	95% TI high for 99% Pop
% EE of PIPERINE	82.8222	82.8222	84.50	2.22931	1.01954	80.5506	85.0939	71.8264	93.8181
% DRUG CONTENT of PIPERINE	82.1811	82.1811	83.16	2.72808	1.24764	79.4012	84.961	68.7251	95.637

3.4. Physico-chemical characterization of prepared phytosomes

3.4.1. Solubility of PP: The average apparent solubility of the Piperine, the physical mixture of Piperine and PC, and the prepared complex PP. The data indicated that PP significantly increases the solubility of Piperine. The physical mixture (PM) enhanced the solubility of Piperine but this effect was weaker. The prepared PP showed a significant increase in the aqueous solubility and the results were illustrated in table 3.4.1.

Table 5: Apparent solubility study of Piperine, PM and PP in water and n-octanol

S. No.	Sample	Aqueous Solubility	n-Octanol solubility (µg/mL) [a]
1	Piperine	5.76 ± 0.53	275.13 ± 0.78
2	Physical mixture	9.35 ± 1.05	417.21± 0.56
3	PP	81.48 ± 0.63	689.43 ±1.03

All Values are mean ± SD(n=3)

3.4.2. Particle size distribution: The particle size of the prepared PP and Piperine was showed in table 6. The particle size of PP was distributed in a narrow range of 250±16.0 nm, and polydispersity index was 0.253±0.03. The zeta potential of PP was found to be 35.2 mV. Zeta potential is a key factor

that influences some properties of particle, such as stability of particles in liquid medium and the possible interactions with other materials.

Table 6: Particle size analysis of Piperine and Phytosomes

S.No	Material	Avg Size (r.nm)	% Intensity	Width (r.nm)	PDI
1	PIPERINE	611.4±16.0	100	135.6	0.28±0.03
2	PP	250±16.0	100	86.26	0.253±0.03

3.4.3. X-Ray diffraction (XRD) study: Figures showed the powder X-ray diffraction (PXRD) patterns of (A) Piperine, (B) PC, (C) PM, and (D) PP. The powder of X-ray diffraction patterns of Piperine displayed partial sharp crystalline peaks, which is characteristic of a molecule with some crystallinity. The physical mixture exhibited both crystalline peaks and a wide peak due to the phospholipids. Compared with the above two, the crystalline peaks disappeared in the complex. The diffractogram of the PP revealed the disappearance of most of the crystalline peaks associated with the Piperine and when compared with physical mixture (PM). The disappearance of Piperine crystalline peak confirms the formation of Piperine- phospholipid complex.

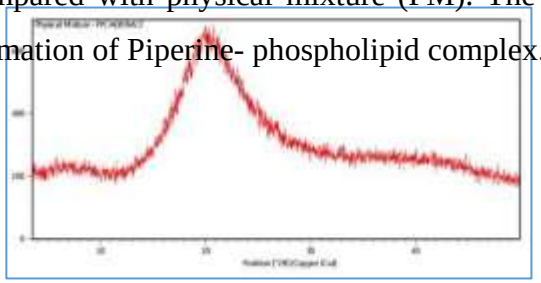


Figure 2a: X Ray Diffraction pattern of Piperine

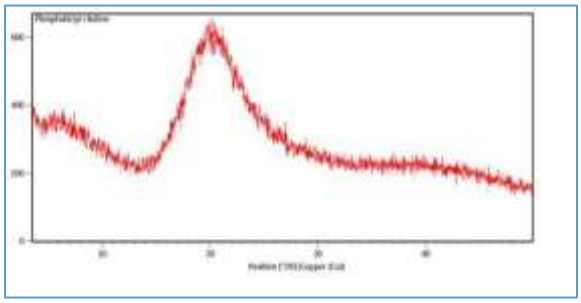
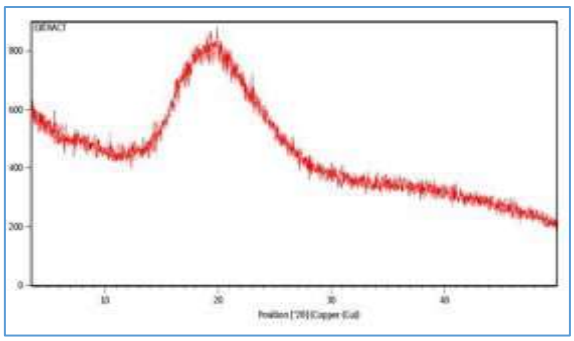


Figure 2b: X Ray Diffraction pattern of PC



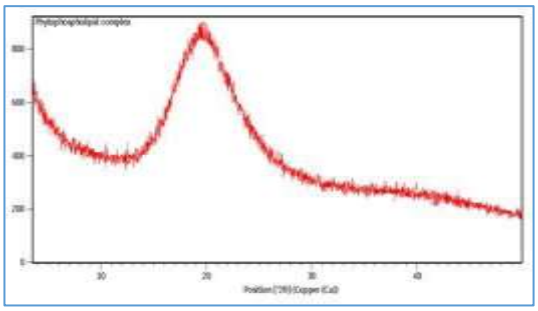


Figure 2c: XRD spectra of PM of Piperine and PC

Figure 2d: XRD spectra of PP

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3.4.4. Differential scanning calorimetry (DSC): Figures showed DSC thermograms of P Piperine, PC, the physical mixture of Piperine with PC (PM), and the prepared PP. Thermogram of Piperine revealed broad endothermic peak at 197.30C. PC shows endothermic peak at 167.40C. PM showed a major peak at 143.50c. The thermogram of complex showed potentially broad endothermic peak at 210.50c. This peak differs from extract and phospholipid. The original peaks of Piperine and PC disappeared from thermogram of complex, thus indicating the successful formation of complex.

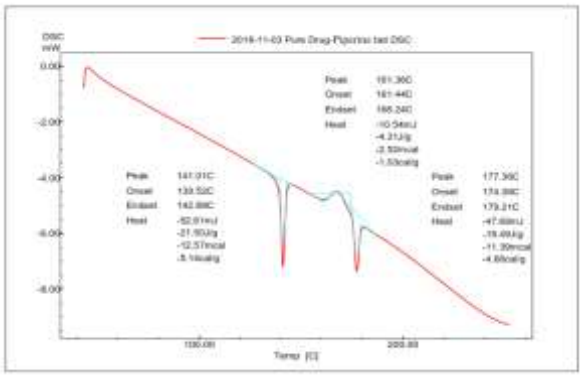

Figure 3a: DSC of piperine

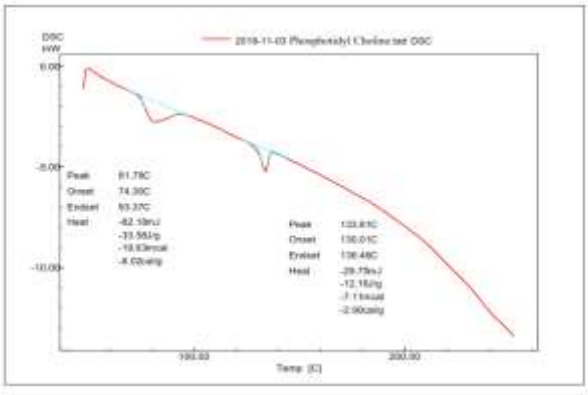

Figure 3b: DSC of PC

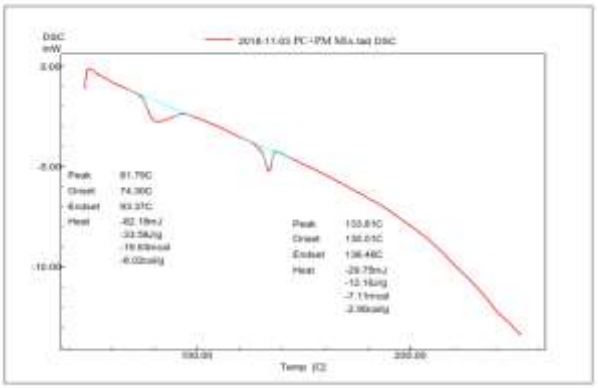

Figure 3c: DSC of PM of Piperine and PC

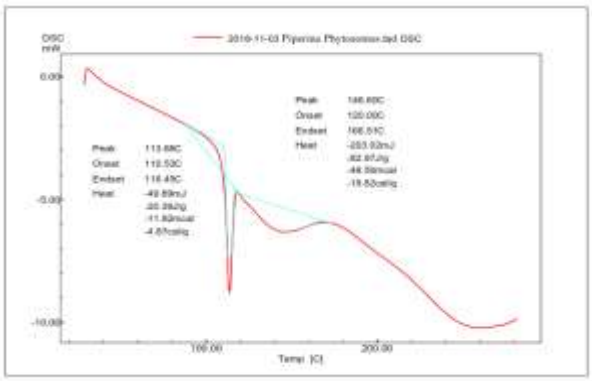

Figure 3d: DSC of PP

3.4.5. FT-IR study: Table No. 7 shows Fourier transform infrared Spectroscopy (FTIR) of the piperine (PPN), PC, the physical mixture of piperine with PC (PM), and the prepared PP. These Spectrums were

studied interaction between piperine and PC. The FTIR Spectrum of piperine showed broad peak at 3491 cm−1 representing alcoholic (−OH) group, 3205 cm−1 (Ar CH stretching), 1712 cm−1 (C=O stretching), 1064 cm−1 (C-O-C stretching). FTIR Spectrum of PC (Figure) revealed the characteristic absorption at 3483 cm−1 (NH stretching), 2920 cm−1 (CH stretching), 1739cm−1 (C=O stretching), 1234 cm−1 (P=O stretching), 1087cm−1 (P–O–C stretching) and 972 cm−1 (C-C-N stretching). The FTIR Spectrum of the prepared PP (Figure 4) is quite different from that of piperine and PC. Some absorption peaks were shielded by phospholipids. The absorption peak of hydroxyl stretching of PP showed remarkable broadening from 3278 -3464 cm−1.

Table 7: Interpretation of IR spectra for PPN, Phosphatidylcholine (PC), PPN+PC, and PP

Sample	Stretcting	Frequency (cm^{-1})
PPN	O-H stretching	3491
	Aro C-H stretching	3205
	Ali C-H stretching	2994
	C=O stretching	1712
	C-O-C stretching	1037
PC	N-H stretching	3280
	Ali C-H stretching	2927
	C=O stretching	1590
	P-O-C stretching	1014
	P=O stretching	1285
PPN+PC	O-H stretching	3456
	Aro C-H stretching	2996
	Ali C-H stretching	2587
	C=O stretching	1710
	O-H bonding	3071
	C-O-C stretching	1472
	P=O stretching	1248
PP	O-H stretching	3345
	Aro C-H stretching	2976
	Ali C-H stretching	2934
	C=O stretching	1643
	C-O-C stretching	1458,1379
	P=O stretching	1251

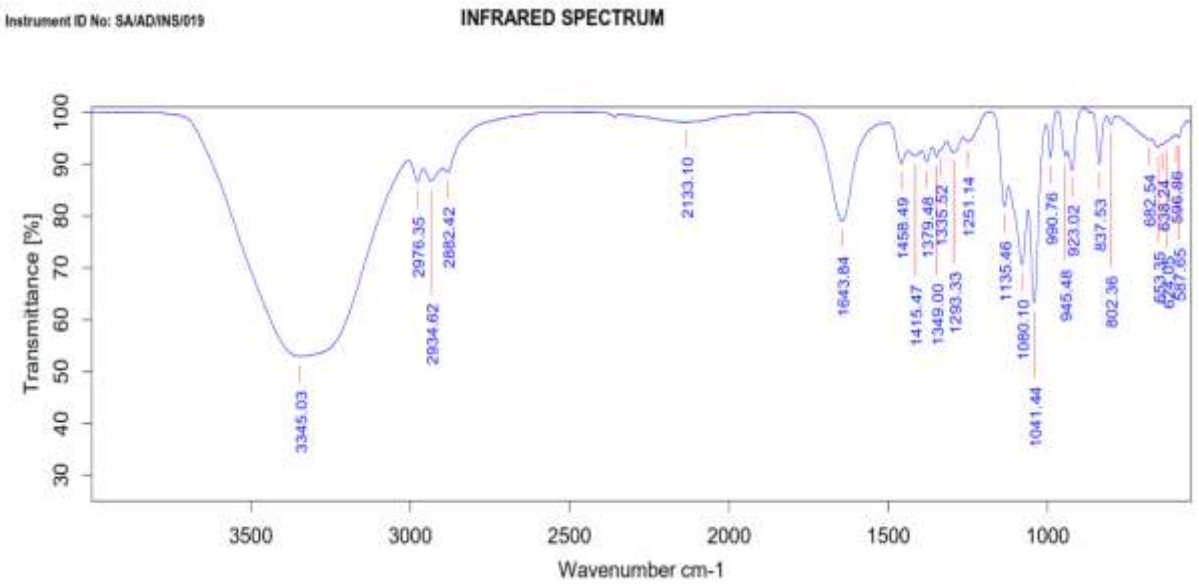

Figure 4: FTIR Spectra of PP

3.4.6. Scanning electron microscopy (SEM): SEM photographs of PPN and PP are shown in figures. Crystalline state of PPN was visualized in the SEM photograph as numerous crystals in figure. In figure the drug was completely converted into phyto-phospholipid complex where piperine was physically enwrapped by phospholipids imparting amorphous nature to the complex due to which crystals disappeared.

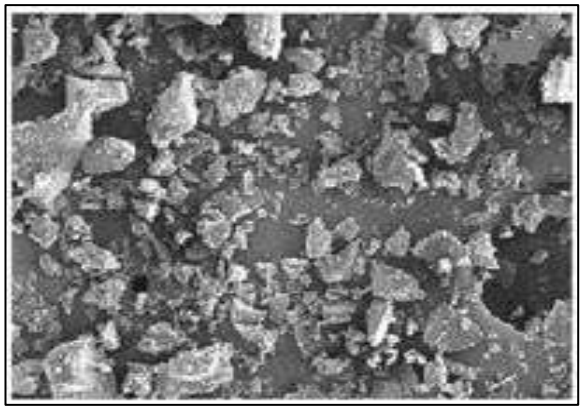

Figure 5a: SEM of Piperine (PPN)

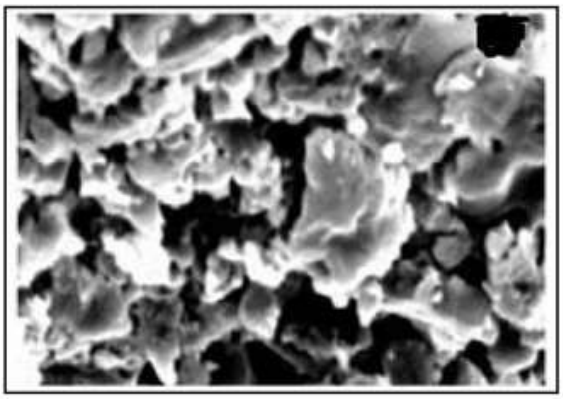

Figure 5b: SEM of PP

3.4.7. *In vitro* drug release: The results of in vitro drug release studies are shown in figure. The 12-h dissolution in the phosphate buffer (pH6.8) revealed that, the pure piperine (PPN) showed the slowest rate of dissolution, i.e., at the end of the dissolution period only about 43.35 % w/w of piperine was dissolved. The prepared PP revealed a significantly faster release of PPN at the end of dissolution period. At the end of 12 h, over 88.34% w/w piperine was observed to be released from PP (figure 6). The improved dissolution rate of PPN from PP may be explained by the improved solubility, and the amorphous form in the prepared complex. The results are summarized in table no. 10.

Table 10: *In vitro* dissolution study of PPN, and PP

S. No	Time (h)	Piperine (PPN)	PP
01	1	22.0911	32.7742
02	2	24.4434	38.7511
03	3	26.3253	42.0225
04	4	29.4164	48.0517
05	5	32.6487	53.1476
06	6	36.1892	57.1679
07	7	38.1962	64.1872
08	8	39.3211	71.1913
09	9	40.1245	74.3421
10	10	41.3213	78.0754
11	11	42.3214	84.3421
12	12	43.3543	88.3421

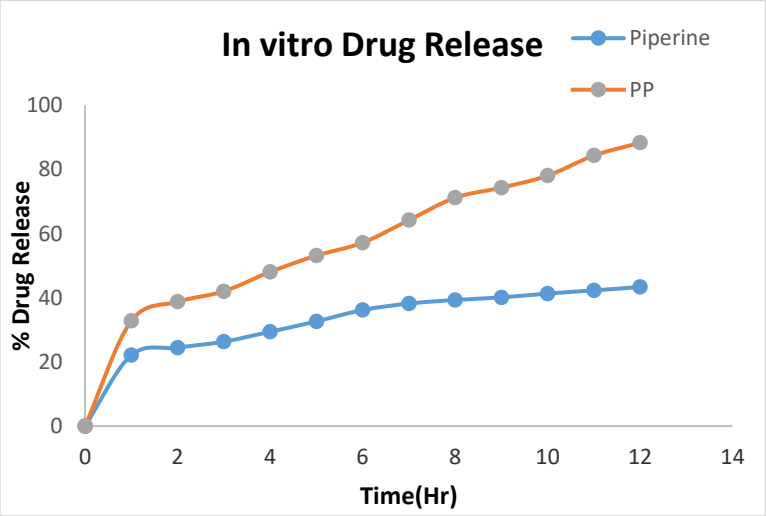

Figure 6: % Drug release profile of Piperine and PP

3.4.8 Pharmacokinetic Study of Piperine Phytosomes:

Pharmacokinetic of drugs following their administration from dosage forms is an integral part of part of research investigations in order to obtain vital information with respect to bioavailability of the newly developed dosage forms.

Analysis of Pharmacokinetic Parameters

Non compartmental method was used for the estimation of pharmacokinetic parameters of Pure Extracts and Prepared Phytosomes and the concentration vs. time data.PK parameters were estimated by using Thermo Scientific KINETICA 5.2 software. The results were summarized in table 11.

Statistical Analysis

With the help of Graph Pad Prism 6 software data was statistically analyzed. For comparison of PK parameters of test and control samples paired t-test was used and a value of $p<0.05$ was considered to be significant. ANOVA was used to determine any differences PK parameters obtained in a group (in six animals).

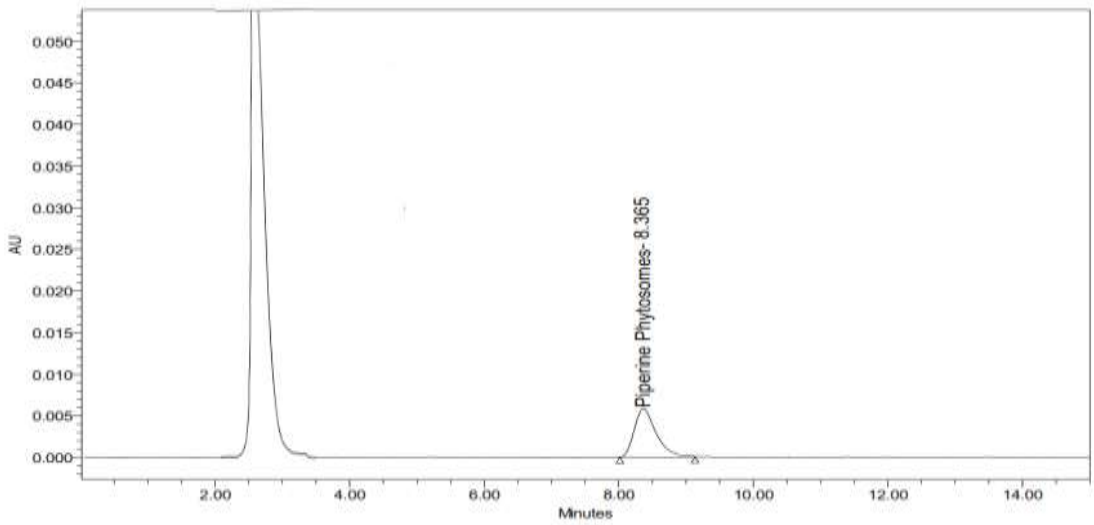

Figure 7: Chromatogram of Piperine Phytosomes group at 4th Hour

Table 11: Comparative bioavailability parameters of PPN and PP

PK parameter	**PPN**	**PP**	**'t' test at 0.05**
C_{max} (µg/mL)	207.50	523.63	significant
T_{max} (Hours)	1.85	4.25	Significant
$t_{1/2}$ (hrs)	3.60	4.12	Significant
MRT (h)	0.12	0.15	Significant
Total AUC (µg-hr/mL)	2354.58	5234.46	Significant
Total AUMC (µg-hr/mL)	4896.24	6428.76	Significant
Cl (mL/min)	1.3	1.4	Significant
K_{el} (hrs^{-1})	0.05	0.06	Significant

3.5. Acute Oral Toxicity: In acute oral toxicity of piperine phytosomes (PP) on selected Wistar rats (both sex) for 14 days, no signs and symptoms of toxicity were noticed at the limit test dose of 2000mg/kg in Wistar rats.

3.6. In Vitro Antidiabetic activity

3.6.1. Glucose Uptake Assay (GUA): The GUA was measured using L6 cell lines to determine the effect of piperine phytosomes (PP) on cellular glucose uptake behavior. Further, the anti-diabetic activity of the phytosome was compared with marketed standard anti-diabetic drugs such as Insulin (1 IU/mL) and metformin (100 µg/mL). Insulin and metformin treatments produced significantly augmented glucose uptake activity (about 120% and 92% respectively) over the untreated control group. The treatment of L6 cells with PP produced significantly higher glucose uptake activity when compared to pure piperine (PPN). Between PPN, and PP caused about 2.26-fold superior glucose uptake activity when compared to the PPN. However, when compared with the standard (Insulin, 1 IU/mL) the PP (200 µg/mL) produced less activity (about 47%) glucose uptake. Further, the glucose uptake activity by PP was found about 22% less when compared to standard metformin (100 µg/mL). Although PP (200 µg/mL) produced significantly less L6 glucose uptake activity as compared with standard drugs could be the cause for comparison of glucose uptake activity with standard drugs at higher concentrations. However, further evaluations are required to authenticate this fact. Lastly, all the prepared test samples – PPN, PP exhibited significant glucose uptake activity on L6 cell lines. All the obtained results are presented (Table nos. 12).

Table 12: In vitro glucose uptake assay (GUA) in L6 cells

Test Sample	**% Glucose uptake over control**
Control	0.021±0.025
Insulin (1 IU/mL)	115.20±2.085***
Metformin (100 µg/mL)	92.54±1.202***
PPN (400 µg/mL)	44.60±1.021***
PP (200 µg/mL)	78.15±1.235***

*Values presented are mean ± SD, (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 over control*

3.7. Statistical Analysis: The results are presented as mean ± standard deviation. The statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Student's t-test. P values < 0.05 were assumed as statistically significant.

4. CONCLUSION

The phytochemical analysis of PPN reveals the presence of polyphenols, flavonoids, steroids, and alkaloids. The total flavonoid and phenolic content of the piperine were 53.11 and 6.51 mg/gm equivalent, respectively. The preliminary investigation of factor influence revealed that all of the tested variables, namely the phospholipids to drug ratio, reaction temperature, and reaction time, had a significant influence on the entrapment efficiency of the prepared phytosomes. Based on the observations and multiple regression models, the optimal values for the investigated factors, namely the phospholipids-to-drug ratio, reaction temperature, and reaction time, were 1:3, 65°C, and 2 h, respectively. The model-predicted value for the entrapment efficiency of PP and drug content was found to be 82.18% and 84.50% respectively, while the average observed values from the prepared batches was found to be 84.50% and 83.16%, which indicates both applicability, and validity of the developed model. The bias (%) was found to be less than 3% (1.16%) indicating the relative robustness of the model. The physio-chemical characterization of PP in comparison to pure piperine (PPN) revealed that the apparent solubility of PP was amphiphilic, and the mean particle size of PP was found to be in a narrow range. The prepared PP demonstrated significantly faster PP release at the end of the dissolution period. The crystal morphology and wettability of the solids have a large influence on the dissolution rate, and the improved dissolution rate of PP from phytosomes may be explained by the improved solubility and amorphous form in the prepared complex. The values of C_{max}, T_{max}, and $t_{1/2}$ for PPN and PP were found to be 207.50 and 523.63 µg/ml, 1.85 and 4.25 hours, and 3.60 and 4.12 hours, respectively, after the pharmacokinetic parameters for PPN and PP were performed. Thus, when compared to pure piperine, PP had a higher bioavailability, according to the comparative bioavailability parameters. The phytosome's relatively higher amorphous state and increased aqueous solubility may have a positive impact on the drug's cumulative release. L6 cell lines were used in an *in vitro* glucose uptake assay to determine the effect of PP on cellular glucose uptake behaviour. Furthermore, the phytosome's anti-diabetic activity was compared to that of commercially available standard anti-diabetic drugs such as Insulin (1 IU/mL) and Metformin (100 µg/mL). L6 cells treated with PP had significantly higher glucose uptake activity than PPN and about 1.55-fold superior glucose uptake activity. The increased bioavailability of the nanosized piperine-loaded phytosome formulation was most likely responsible for the antidiabetic effect.

5. ACKNOWLEDGMENT

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6. CONFLICT OF INTEREST: None

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