

# Diosmin Phytosomes: Development, Optimization and Physicochemical Characterization

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## ABSTRACT

**Background:** Diosmin is a flavonoids glycoside that possesses different therapeutic activity include vascular-protecting agent used to help improving chronic venous insufficiency (CVI), haemorrhoids, etc. Diosmin is bioactive flavones constituent with wide range of biological activity. The poor solubility and dissolution rate limit its oral absorption and bioavailability. **Aim:** The aim of the present study is to develop diosmin - phospholipid complex (DN-PC) and characterized by physicochemical method. **Method:** DN-PC was prepared by refluxing followed by solvent evaporation technique in different ratios of Diosmin to Phosphatidylcholine. **Physicochemical Characterization:** DN-PC was characterised by various parameters like drug content, solubility studies, particle size determination, infrared absorption (FTIR), Differential scanning calorimetry (DSC), X-ray diffraction (XRD), Scanning electron microscopy (SEM), entrapment efficiency etc. **Results:** SEM and XRD revealed the reduction in crystallinity of diosmin in the phytosomes. FTIR and DSC confirm the formation of phyto-phospholipid complex. **Conclusion:** The results of the study revealed that the phospholipid complex may be considered as a promising drug delivery system that improves the absorption and bioavailability of plant constituents.

**Key words:** Phytosome, Diosmin, Phosphatidyl choline, Characterization.

## INTRODUCTION

The therapeutic uses of phytoconstituents are very popular for health maintenance by various means. Most of the bioactive constituents of plants are polar or water-soluble molecules (e.g. phenolics, glycosides, tannins and flavonoids). However, water soluble phytoconstituents are limited in their effectiveness because they are poorly absorbed due to large molecular size and poor lipid solubility when taken orally or when applied topically.<sup>1</sup>

Flavonoids are beneficially effective for antioxidant, anti-inflammatory, antiviral, anti-allergic, anticancer, etc.<sup>2</sup> The chemical structures and physicochemical properties of flavonoids verify their rate and extent of absorption. The biological activities of flavonoids depend on their bioavailability. The very limited information is available about bioavailability of flavonoids.<sup>3-4</sup>

Diosmin is flavonoid glycoside that can be isolated from various plant sources or derived from the flavonoid hesperidins.<sup>5</sup> Diosmin is considered to be a vascular-protecting agent used to help improving chronic venous insufficiency (CVI),<sup>6</sup> haemorrhoids, lymphedema, and varicose veins.<sup>7</sup> As a flavonoid, diosmin also exhibits anti-inflammatory,<sup>8</sup> free-radical scavenging,<sup>9</sup> antidiabetic<sup>10</sup> and antimutagenic properties.<sup>11</sup> The diosmin is practically insoluble in water.<sup>12</sup> Thus the dissolution rate of diosmin is limiting its absorption from the gastrointestinal tract. An attempt was made to increase the oral bioavailability of the drug chiefly centred on particle size reduction. The rate and extent of dissolution of diosmin was increase by nano sizing which led directly to an increase oral bioavailability which in turn enables dosage reduction.

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The therapeutic dose of diosmin is 500 mg twice daily which is very high, thus it is necessary to be administered frequently in order to maintain the normal therapeutic concentration of diosmin.<sup>13</sup>

The phytosome technique has emerged as one of the leading methods of improving bioavailability of phyto-pharmaceuticals having poor competency of solubilising and crossing the biological membranes.<sup>14</sup> Phytosome is a patented technology of Indena where plant polyphenolics are complexed with phospholipids to improve bioavailability.<sup>15-20</sup> Phospholipids are lipid molecules where glycerol is bonded to two fatty acids. Phospholipid mainly phosphatidylcholine, are lipophilic substances and readily form complex with polyphenolic compounds. Phosphatidylcholine is a major structural constituent of all biological membranes. Phosphatidylcholine is a major component of soybean lecithin which provides free choline in the blood for the manufacture of acetylcholine; regulates digestive, cardiovascular and liver functions.<sup>21</sup>

Diosmin shows low bioavailability because it is not soluble in water and is rapidly eliminated from the body. The aim of this study is to develop a diosmin loaded phospholipid complex that could have potential to increase the bioavailability. The key objective of the present study is to develop the phytosome of diosmin, to increase the solubility and bioavailability of drug, to prepare the diosmin phytosome by specific method. The complex (DN-PC) thus prepared was evaluated physico-chemically for drug loading, chemical interaction (FT-IR), thermal analysis (DSC), crystallinity (X-RPD) surface morphology (SEM), solubility and dissolution rate study. The developed complex may be suitable to reduce the dose and frequency and hence reduce toxic or side effect of diosmin.

## MATERIALS AND METHODS

### Materials

Diosmin was purchased from Sigma Aldrich, Mumbai (India). Phosphatidylcholine and dimethyl sulfoxide was purchased from Ozone international, Mumbai. All other chemicals and reagents were of analytical grade.

### Methods

#### Preparation of Diosmin Phospholipid complex (DN-PC)

DN-PC was prepared by refluxing followed by solvent evaporation technique.<sup>22</sup> DN-PC was prepared in different ratios, i.e., 0.5:1, 0.75:1, 1:1, 2.5:1 and 3:1 of Diosmin to Phosphatidylcholine. Diosmin and Phosphatidylcholine were dissolved in Dimethylsulphoxide (DMSO) and

dichloromethane respectively. Both the solutions were mixed and pour in a 200 ml round bottomed flask. The mixture was refluxed for 3-4 h and at various temperature 45-65°C. After 3 h the mixture was cooled and then poured to Petri dish. The dish was kept open overnight at room temperature for evaporation of solvent. Then the product was kept in hot air oven at 60°C for 2 h. The dried DN-PC were gathered and stored in desiccators for further use.

### Design of Experiment

A response surface design was used to study the influence of independent variables, viz., drug: phospholipids ratio ( $X_1$ , w: w), and temperature ( $X_2$ , °C) on the entrapment efficiency of diosmin. The two independent variables ( $X_1$  and  $X_2$ ) were selected at three levels resulting in thirteen possible combinations. The dependent variable is entrapment efficiency of diosmin. The experimental trials were performed using all thirteen possible combinations of the selected variables. The mathematical model containing coefficient effects, interactions, and polynomial terms was analyzed to assess the response using the following equation:

$$Y = b_0 + b_1X_1 + b_2X_2 - b_3X_1*X_1 - b_4X_2*X_2 - b_5X_1*X_2$$

Where Y is the dependent variable, b is the coefficient of the independent variable X. The main effects ( $X_1$  and  $X_2$ ) represent the possible aggregate effect of both factors as they change independently from their low to high level. The interaction term ( $X_1X_2$ ) shows how the response changes when two factors are simultaneously changed. The polynomial terms describes the non-linearity. The design levels and the real values of the independent variables are shown in Table 1. The composition of experimental trials along with obtained yield (%) values is shown in the Table 2.

### Characterization of DN-PC complex

#### Apparent Solubility

The apparent solubility was determined by adding excess of DN and DN-PC complexes to 5ml of water or n-octanol in sealed glass containers at room temperature (25-30° C). The liquids were agitated for 24 h then centrifuged for 20 min at 1,000 rpm to remove excessive DN or DN-PC complex. The supernatant was filtered through a membrane filter (0.45 m) then 1 ml filtrate was dilute with 9 ml of distilled water and absorbance were measured at 268 nm using UV spectrophotometer.<sup>23-24</sup>

#### Entrapment Efficiency

Entrapment efficiency (EE) was measured using UV-visible spectrophotometer (UV-1601, Shimadzu). A

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

Variables	Levels				
	-1.41	-1	0	1	1.41
X1	0.5	1.0	1.75	2.5	3.0
X2	45	50	55	60	65

**Table 2: Central Composite Design Formulation Batches.**

Formulation	X <sub>1</sub>	X <sub>2</sub>	Entrapment efficiency <sup>a</sup> (% w/w)
1	1	50	72.72±1.5
2	2.5	50	88.21±0.3
3	1	60	84.51±1.6
4	2.5	60	87.36±0.9
5	0.5	55	89.52±1.3
6	3	55	86.41±1.1
7	1.75	45	83.23±1.2
8	1.75	65	96.32±1.4
9-13	1.75	55	94.36±1.0

<sup>a</sup>All values are mean ± SD (n=3)

weighed quantity of phyto-phospholipid complex DN-PC equivalent to 10 mg of diosmin was added to 50 ml methanol in a 100 ml beaker. The contents were stirred on a magnetic stirrer for 4 h and then allowed to stand for one hour. Clear liquid was decanted and centrifuged at 5000 rpm for 15 min. After centrifugation the supernatant was filtered through 0.45 µ whatman filter paper and after suitable dilution absorbance was measured in UV at 268 nm; the concentration of drug was measured.<sup>25</sup> All measurements were performed in triplicate. The EE (%) was calculated using the following formula:

$$EE (\%) = T-S/T \times 100$$

Where, T- Total concentration of diosmin, S-is the diosmin contained in the filtrate.

### Particle size distribution

The particle size analysis of the prepared DN-PC sample was carried out using photon correlation spectroscopy, with dynamic light scattering on Zetasizer nano (Model: Nano series, S90 Zeta sizer, Malvern) The complex was dispersed in isopropyl alcohol by stirring on a magnetic stirrer for 10 min. The dispersion was analysed in size analyser.

### X-Ray diffraction (XRD) study

Diffractionmeter (Bruker, Germany) was used for measurements of the studied samples. The operating conditions were: voltage 45 kV; current 0.8 mA; scanning speed 1/min. The results were recorded over a range of 5–60° (2θ) using the Cu-Anode X-ray tube and scintillation detector.

### Differential scanning calorimetry (DSC)

DSC studies for pure DN and DN-PC were performed on a Perkin Elmer (USA) (Model JADE DSC) differential scanning calorimeter by heating samples over a temperature range of 50-300°C in closed metal pans at the rate of 10°C per min under the environment of nitrogen gas.

### Fourier Transform Infrared spectroscopy (FTIR) Study

FT-IR studies were performed on pure DN, PC, physical mixture (PM) and DN-PC was in an Alpha FTIR spectrophotometer IR Affinity-1 (Shimadzu Corporation). 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<sup>-1</sup>. The obtained IR spectra were interpreted for functional groups at their respective wave number (cm<sup>-1</sup>).

### Scanning electron microscopy (SEM)

DN and DN-PC were coated with gold in a Fine Coat Ion Sputter S-4800 TYPE II, Hitachi high technologies corporation, Japan. Analysis was done on the coated sample by placing a pinch of sample in the S-4800 TYPE II (Hitachi high technologies corporation, Japan) Scanning electron microscope and surface morphology was viewed and photographed.

### Dissolution Study (*in-vitro* Drug Release)

The *in vitro* dissolution profiles of DN, physical mixture (PM) and the prepared DN-PC were obtained and compared.<sup>26</sup> The dissolution studies were carried out in a USP XXIII, six station dissolution test apparatus, type II (VEEGO Model No. 6 DR, India) at 100 rpm and at 37°C. An accurately weighed amount of DN-PC of diosmin 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. Withdrawn samples were 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.

## RESULT AND DISCUSSION

### Preparation of DN-PC complex

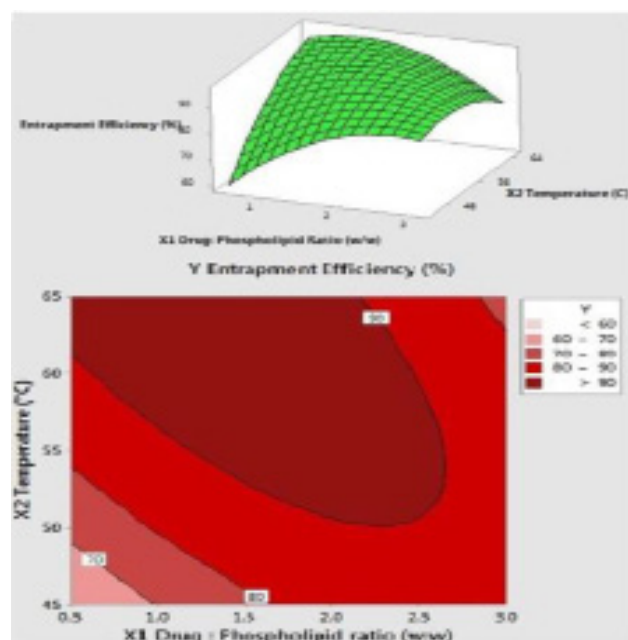
The initial investigation of the influence of factors revealed that all the studied factors, i.e., the drug to phospholipids ratio and temperature had a significant influence on the entrapment efficiency of the prepared phytosome. The results of the entrapment efficiency (%) are shown in Table 2. The measured values from the experimental trials revealed wide range (72.72–96.32, % w/w) entrapment efficiencies (Table 2). The fitted polynomial equations relating the response (entrapment efficiency, % w/w) to the transformed factors are shown in Figure 1. The polynomial equations could be used to draw conclusions after considering the magnitude of the coefficient, and its associated mathematical sign, i.e., positive or negative. The results from Figure 1 also indicated that all the coefficients were statistically significant ( $p < 0.05$ ). The value of correlation coefficient ( $R^2$ ) was found to be 0.9522 indicating a good fit to the quadratic model. The multiple regression analysis revealed that the coefficients were positive. This indicated that the entrapment efficiency increased with increasing  $X_1$  and  $X_2$ .

$$Y = -206 + 66.5 X_1 + 8.07 X_2 - 5.50 X_1 * X_1 - 0.0545 X_2 * X_2 - 0.825 X_1 * X_2$$

Based on the central composite design and response surface plots depicting the changes in the entrapment efficiency (%) as a function of  $X_1$  and  $X_2$ . The data from all 13 batches of the central composite design were used for generating interpolated values using Minitab software. The response surface plots and contour plot (Figure 1) indicated a strong influence of the studied factors on entrapment efficiency. Increasing levels of  $X_1$  and  $X_2$  were found to be favorable conditions for obtaining higher entrapment efficiency. Based on these observations, along with calculations from the developed quadratic model, the optimal values for the studied variables, i.e. drug: phospholipids ratio ( $X_1$ , w: w) and the reaction temperature ( $X_2$ , °C) were found to be 1:1 and 65°C respectively.

### Validation of model

An additional batch of DN-PC was prepared in order to validate the model using the optimized values of the variables, i.e.  $X_1$  and  $X_2$  1:1 and 65°C respectively. A comparison between the predicted (theoretical) value (%) of the DN-PC 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 diosmin in DN-PC was 95.38%, while the average observed value (%) from the prepared batches was found to be  $94.08 \pm 0.67$ ,



**Figure 1: The response surface plot and contour plots of entrapment efficiency (Y, %) as a function of the ratio of Diosmin to Phospholipid ( $X_1$ , w: w), and the reaction temperature ( $X_2$ , °C).**

**Table 3: Observed and predicted value of entrapment efficiency prepared under optimal protocol.**

Batches	Predicted value (%)	Observed value (%) <sup>a</sup>	Bias
1	95.38	94.23±0.14	1.48
2	95.38	94.87±1.53	0.53
3	95.38	95.19±0.34	2.8

<sup>a</sup>All values are mean ± SD (n=3)

**Table 4: Apparent solubility study of DN, PM and DN-PC in water and n-octanol.**

SN	Sample	Aqueous Solubility (µg/mL) <sup>a</sup>	n-Octanol solubility (µg/mL) <sup>a</sup>
1	DN	4.35 ± 0.34	305.65 ± 0.54
2	PM	8.12 ± 1.35	432.21 ± 0.04
3	DN-PC	80.32 ± 0.13	617.34 ± 0.58

<sup>a</sup>All values are mean ± SD (n=3)

(Table 3) indicating both applicability, and validity of the developed model. The bias (%), calculated using the equation, was also found to be less than 3% (1.35%), indicating the relative robustness of the model.

### Physico-chemical characterization of prepared CP

#### Apparent Solubility of CP

The results of the measured apparent solubility of the pure DN, the physical mixture of DN and PC, and the prepared DN-PC complex are shown in Table 4. It was

observed that the pure diosmin had poor aqueous solubility ( $4 \mu\text{g/mL}$ ), and a relatively higher solubility in n-Octanol ( $305 \mu\text{g/mL}$ ), indicating a rather lipophilic nature of the drug. The physical mixture (PM) revealed a non-significant change in the n-Octanol solubility and a modest increase (1.5 times) in the aqueous solubility. The prepared DN-PC, however, showed a dramatic, and a significant (over 12-fold) increase in the aqueous solubility. This increase in the solubility of the prepared complex may be explained by the partial amorphization (reduced molecular crystalline) of the drug, and the overall amphiphilic nature of the Phytosome.<sup>27-28</sup>

### Particle size distribution:

The mean particle size of the prepared DN-PC was carried out using dynamic light scattering technique. The mean particle size of DN-PC was distributed in a narrow range of  $233.4 \pm 20.0 \text{ nm}$ , and polydispersity index was  $0.642 \pm 0.03$ . The surface area/volume (SA/V) ratio of most particles is inversely proportional to the particle size. Thus, smaller particles of the DN-PC, having a higher SA/V, make it easier for the entrapped drug to be released from the phytosome via diffusion and surface erosion. They also have the added advantage for the drug entrapped phytosomes to penetrate into, and permeate through the physiological drug barriers. LeFevre *et al.* and Savic *et al.* have previously suggested that larger particles ( $\leq 5 \text{ mm}$ ) are taken up via the lymphatics, while the smaller particles ( $\leq 500 \text{ nm}$ ) can cross the epithelial cell membrane via endocytosis.<sup>29-31</sup>

### X-Ray diffraction (XRD) study

The x-ray diffraction (PXRD) patterns of (A) DN, (B) PC, (C) PM, and (D) DN-PC. The diffractogram of the DN (Figure 3) revealed sharp crystalline peaks at  $2\theta = 46.9^\circ, 43.5^\circ, 36.6^\circ,$  and  $28.0^\circ$ . A diffraction peak was observed for PC at  $35.5^\circ, 36.4^\circ,$  and  $25.2^\circ$ . The physical mixture (PM) showed most of the peaks associated with the DN and PC (Figure 3C). In comparison to the physical mixture, the diffractogram of the DN-PC revealed the disappearance of most of the crystalline peaks associated with the DN. These results were in agreement with the previously reported studies, where the disappearance of the active pharmaceutical ingredient (API) peaks was associated with the formation of drug phospholipid complex.<sup>32</sup> The disappearance of diosmin crystalline peak confirms the formation of diosmin-phospholipid complex.<sup>33</sup>

### Scanning electron microscopy (SEM)

SEM photographs give important insight into the solid state properties and surface morphology of DN and DN-PC. In the Figure 4 (A) the shows crystalline

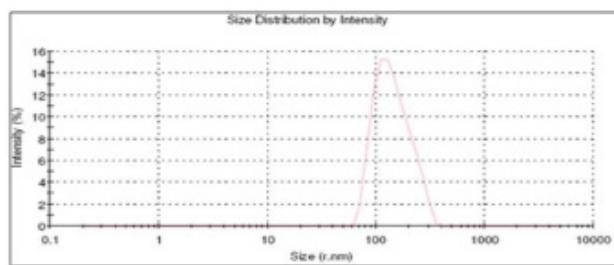


Figure 2: Particle size analysis of DN-PC.

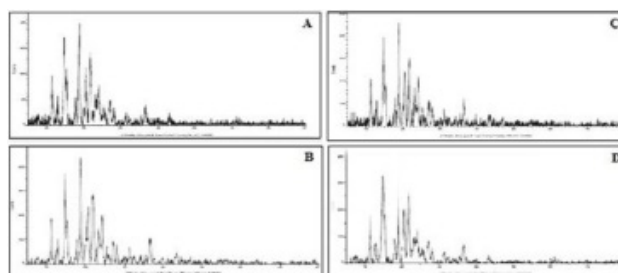


Figure 3: XRD spectra of (A) DN, (B) PC, (C) PM and (D) DN-PC.

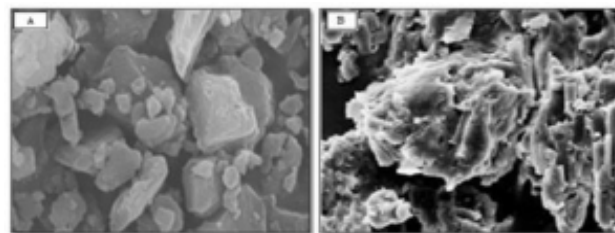


Figure 4: SEM of DN (A) and DN-PC (B).

state of DN was visualized in the SEM photograph as numerous crystals. In Figure 4 (B) drug was completely converted in to phyto-phospholipid (DN-PC) complex where DN was physically enwrapped by PC imparting amorphous nature to the complex due to which crystals disappeared.

### FT-IR study

The results from the Fourier transform infrared spectroscopy (FTIR) analyses of the DN, PC, the physical mixture of DN with PC (PM), and the prepared DN-PC were studied in order to get insight into occurrence of interaction between DN and PC. The FTIR spectrum of DN (Figure 5A) exhibited a broad peak at  $3556 \text{ cm}^{-1}$ , representing the aliphatic alcoholic ( $-\text{OH}$ ) group,  $2900 \text{ cm}^{-1}$  (CH stretching),  $1660 \text{ cm}^{-1}$  ( $\text{C}=\text{O}$  stretching),  $1559 \text{ cm}^{-1}$  ( $\text{C}=\text{C}$  stretching). Prominent peak observed at  $1160 \text{ cm}^{-1}$  and  $1050 \text{ cm}^{-1}$  typically relates to presence of acidic functional group. FTIR spectrum of PC (Figure 5B) revealed the characteristic absorption at  $2921$  and  $2850 \text{ cm}^{-1}$  (CH stretching),  $1775 \text{ cm}^{-1}$  ( $\text{C}=\text{O}$  stretch-

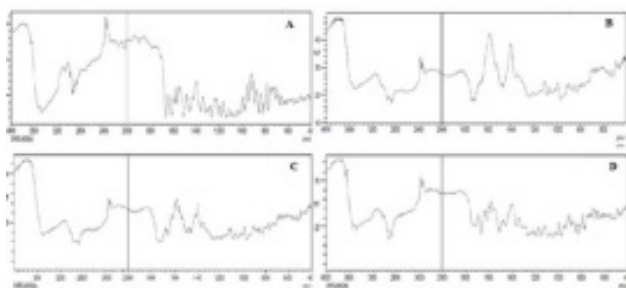


Figure 5: FTIR spectra of (A) DN, (B) PC, (C) Physical mixture and (D) DN-PC.

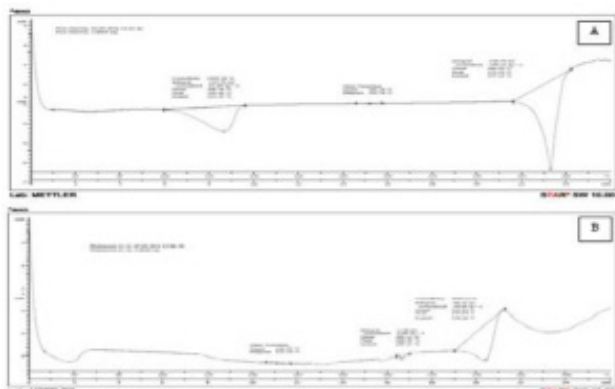


Figure 6: Differential scanning Calorimetry of (A) DN and (B) DN-PC.

ing),  $1235\text{ cm}^{-1}$  (P=O stretching),  $1081\text{ cm}^{-1}$  (P–O–C stretching) and  $975\text{ cm}^{-1}$  (C–C–N stretching). The FTIR spectrum of the prepared DN-PC (Figure 2D) is quite different from that of DN and PC. The peaks at  $1660\text{ cm}^{-1}$ ,  $1559\text{ cm}^{-1}$  and  $3556\text{ cm}^{-1}$  were shielded by phospholipids. The absorption at  $1660\text{ cm}^{-1}$  shifted to lower field in the spectrum of complex, indicating the formation of hydrogen bond and existence of electrostatic interaction between drug and phospholipids.

#### Differential scanning calorimetry (DSC)

DSC is a fast, reliable method to investigate the interaction between multiple component and drug excipient compatibility. These interactions are observed as the elimination of endothermic peak, the appearance of new peak, the change in peak shape, onset temperature/melting point, relative peak area or enthalpy.<sup>34,35</sup> The DN revealed broad endothermic peaks at  $126.48^\circ\text{C}$  and  $272.79^\circ\text{C}$ . The DSC thermo gram of DN-PC showed complex Figure 6 (B) gives two endothermic peaks at  $206.40^\circ\text{C}$  and  $244.54^\circ\text{C}$ . Therefore from (Figure 6) it was revealed that the shift of endothermic peak at the difference of around  $25\text{--}30^\circ\text{C}$  suggest possible interaction of DN with PC and can account for enhanced entrapment.

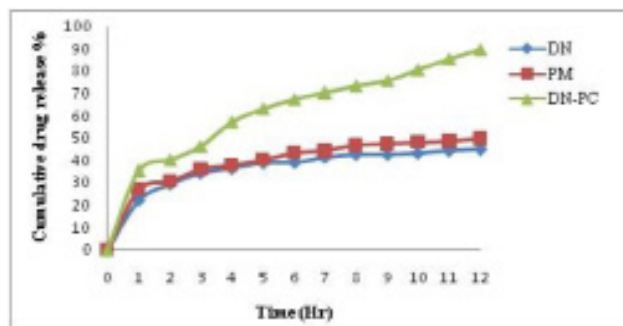


Figure 7: *In vitro* dissolution study of DN, PM and DN-PC.

#### Percentage drug release

The results of *in vitro* drug release studies are shown in the Figure 7. The 12-h dissolution in the phosphate buffer (pH 6.8) revealed that, the pure diosmin showed the slowest rate of dissolution, i.e., at the end of the dissolution period only about 44% w/w of diosmin was dissolved. The dissolution rate of the physical mixture was found not to be significantly different ( $\sim 49\%$  w/w dissolved in 12 h) compared to the pure diosmin. The prepared DN-PC, revealed a significantly faster release of diosmin at the end of dissolution period. The dissolution profile of the DN-PC at the end of 12 h, over 89% w/w diosmin was observed to be released from the DN-PC. The dissolution rate is largely influenced by the crystal morphology and the wettability of the solids,<sup>36</sup> and the improved dissolution rate of diosmin from the DN-PC may be explained by the improved solubility, and the partially disrupted crystalline phase (amorphous form) in the prepared complex. The relatively higher amorphous state of the phytosome and their increased water-solubility may have a positive impact on the cumulative release of the drug.<sup>37</sup>

#### CONCLUSION

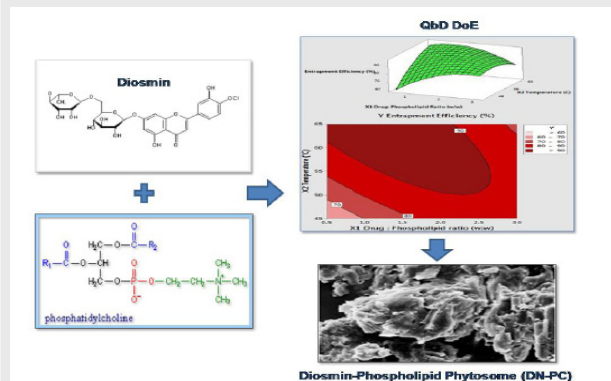
In the present study, an attempt was made to enhance the aqueous solubility of diosmin via its complexation with phospholipids. A central composite design was used to optimize the formulation and process variables. The prepared DN-PC was evaluated for physicochemical and functional attributes. The FTIR, DSC, PXRD, photo microscopy, and the SEM studies indicated the successful formation of vesicular drug-phospholipids complex. The apparent solubility, the *in vitro* dissolution, studies indicated a significant improvement in the aqueous solubility, the drug release, and the membrane permeation of the diosmin from DN-PC respectively. Additional studies analyzing the pharmacokinetic parameters are required to substantiate

the increased absorption, and the enhanced bioavailability hypothesis.

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## PICTORIAL ABSTRACT



## SUMMARY

- Diosmin is flavonoid glycoside that can be isolated from various plant sources or derived from the flavonoid hesperidins. Diosmin is considered to be a vascular-protecting agent used to help improving chronic venous insufficiency (CVI), haemorrhoids, lymphedema, and varicose veins.
- Diosmin shows low bioavailability because it is insoluble in water and is rapidly eliminated from the body. The phytophospholipid complex of diosmin (DN-PC) was developed to increase the solubility and bioavailability of drug.
- DN-PC was prepared by refluxing followed by solvent evaporation technique. The formulation and the process variables for the preparation of the DN-PC were optimized using a Quality by design (QbD) approach. Response surface design was employed for the optimization of the critical process parameters (CPP) on the diosmin entrapment rate of DN-PC.
- The model-predicted value for the entrapment efficiency of diosmin in DN-PC was 95.38%, while the average observed value (%) from the prepared batches was found to be  $94.08 \pm 0.67$ , indicating both applicability, and validity of the developed model.
- The mean particle size of DN-PC was distributed in a narrow range of  $233.4 \pm 20.0$  nm, and polydispersity index was  $0.642 \pm 0.03$ . SEM and XRD studies of the diosmin and its phytosome revealed the reduction in crystallinity of diosmin in the phytosomes. FTIR and DSC confirm the formation of phyto-phospholipid complex.
- The prepared DN-PC, revealed a significantly faster release of diosmin at the end of dissolution period. The dissolution profile of the DN-PC at the end of 12 h, over 89% w/w diosmin was observed to be released from the DN-PC.
- The apparent solubility and in vitro dissolution studies indicated a significant improvement in the solubility and the drug release of diosmin (DN) from DN-PC respectively.

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