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DEVELOPMENT, CHARACTERIZATION AND OPTIMIZATION OF KUTKI PHOSPHOLIPID COMPLEX USING CENTRAL COMPOSITE DESIGN AND RESPONSE SURFACE METHODOLOGY

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Keywords:

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ABSTRACT: Kutki (*Picrorrhiza kurroa*) is a medicinal herb with remarkable pharmacological properties. However, poor solubility of the active principles limits its medicinal value. This study sought to prepare kutki phospholipid complex in phospholipids such as phosphatidyl choline in order to improve its solubility and permeability. Kutki phospholipid complex (phytosome) was prepared by solvent evaporation method. The study was conducted using central composite design (CCD) with a 3^3 designs and total of 20 experimental runs. The complex was characterized for solubility, entrapment efficiency, Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), particle size and zeta potential, Differential scanning calorimetry (DSC), X-ray diffraction (XRD), drug release, and *in-vitro* antioxidant activity. The optimized batch showed substantial enhancement of solubility and entrapment efficiency. FTIR study indicates the interaction between phosphatidylcholine and Kutki extract. SEM showed the surface morphology of complex depicting the conversion of crystalline drug into amorphous complex. The *in-vitro* release was significantly higher for the complex (93.43%) than the drug (19.65%) after 7 h. Enhanced solubility, absorption and antioxidant effect may improve the overall pharmacological effects and medicinal value of *Picrorrhiza kurroa* extract.

INTRODUCTION: Herbal medicines stand out as a recent promising tool for the treatment of various diseases. The popularity of Herbal medicine in recent time is based on plant constituents that can promote health and would help to alleviate illness [1]. Herbal drugs, medicinal plants, their extracts, and isolated compounds have shown a wide spectrum of biological activities.

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The medicinal plants are rich in sources of secondary metabolites like alkaloids, glycosides, steroids, and flavonoids, which are a potential source of drugs. These are important molecules but limited in their effectiveness because they are poorly absorbed when taken orally or when applied topically, either due to their large molecular size which cannot absorb by passive diffusion, or due to their poor lipid solubility; severely limiting their ability to pass across the lipid-rich outer membranes of the enterocytes, the cells that line the small intestine, resulting poor bioavailability [2].

Moreover, the standardized extracts, when administered orally, lose some of their constituents in the presence of gastric fluids.

This has restricted the use of pharmacologically effective polyphenolic plant actives for treating different disorders [3, 4]. To counter these problems and to make herbal therapy more effective, these drugs have been incorporated into several novel delivery systems in recent times. The technique of complexation of plant drugs with phospholipids has emerged as a challenging and one of the most successful methods for improving bioavailability and therapeutic efficacy of a number of poorly absorbed plant constituents [5].

Several approaches explored in recent years for the improvement of bioavailability of drugs, drug-phospholipid complexes appear to be among the promising ones. Incorporating bioactive phytoconstituents into phospholipid molecules is reported to improve the solubility, the membrane permeability, and hence the systemic absorption and bioavailability of the active phytoconstituents. Such complexes are appropriately called as phytosomes [6]. Phytosome is a novel approach to drug delivery system which addresses the limitations of the conventional drug delivery systems. Phytosomes are developed by incorporating standardized plant extract or water-soluble bioactive plant constituents into phospholipids to produce lipid compatible molecular complexes and so improve their absorption and bioavailability [7-10].

Kutki, *Picrorrhiza kurroa* is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers, and to treat dyspepsia, chronic diarrhea, and scorpion sting [11-13]. *Picrorrhiza kurroa* (Pk), is a well-known herb native of the Himalayan high altitude (3000 m) region. This is used as a dietary supplement to maintain healthy bile production and liver function by offering multiple pharmacological effects [14]. *P. kurroa* is effectively recommended for the restoration of various liver-related complications such as jaundice, nausea anorexia, dyspepsia, viral hepatitis, periodic fevers [15], antimicrobial [16], antibacterial [17] antineoplastic [18] and liver chemo-preventive activities [19]. Kutkin is the active principal of *Picrorhiza kurroa* and is comprised of kutkoside and the iridoid glycoside picrosides I, II, and III. Other identified active constituents are apocynin, drosin, and nine cucurbitacin glycosides [20]. Apocynin is a catechol that has been shown to inhibit neutrophil oxidative burst in addition to being a powerful anti-inflammatory agent, [21] while the curcubitacins have been shownto be highly cytotoxic and possess antitumor effects. Picrorhiza is not readily water-soluble therefore limit its absorption. The hepatoprotective function of Kutki is mainly due to the inhibition of oxygen anions generation and scavenging free radicals similar to superoxide dismutase, metal ion chelators, xanthine oxidase inhibitors and anti-lipid peroxidative effect [22].

The usefulness of pharmaceutical products depends on the safety, efficacy, and quality of that product. The effect of process parameters and the physicochemical properties of phytosome, like particle size, surface morphology, drug release, *etc*., can be improved by an optimization technique. The procedure of optimization involves the systematic formulation of designs to minimize the number of trials and analyzes the response surfaces in order to realize the effects of factors and to obtain the appropriate formulations with the target goals. Statistical models are extensively used in diversified areas, especially in pharmaceuticals, to strengthen the art of drug formulation. The objective of the present study was to develop a mathematical model using a Central Composite Design (CCD), combined with Response Surface Methodology (RSM), in order to deduce the adequate conditions to prepare SLNs with the desired optimized characteristics. This will help to determine the relationship between the selected independent variables and their responses. The variables selected are fitted in a three-factor, three-level CCD, with three replicates. A quality by design (QbD) approach is now considered to be an ideal pathway to ensure the development of a quality product, and the minimization of product variabilities. Quality by Design (QbD) is defined in the ICH Q8 guideline as ‘a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management’ [23].

Thus, the objective of the current study was to evaluate the feasibility of enhancing the solubility of Standardized *Picrorrhiza kurroa* extract (SPE) by preparing its vesicular complex with

phosphatidyl-choline. This complex is hereby referred to as Kutki Phytosome. KP was prepared using a solvent evaporation method. The formulation and the process variables for the preparation of the KP were optimized using a QbD approach. Response surface analysis by means of the central composite design was employed for the optimization of the critical process parameters (CPP) on the SPE entrapment rate of KP. The prepared KP was evaluated for their physicochemical, functional, and preliminary pharmacological properties.

MATERIALS AND METHODS:

Materials: Standardized extract of *Picrorrhiza kurroa* was obtained as a gift sample from Kisalaya herbals Pvt. Ltd., Indore. Phosphatidylcholine was purchased from Ozone International, Mumbai. All other chemicals and reagents were of analytical grade.

Methods:

Preformulation Studies: The presence of a phenolic compound was studied by a specific identification test by taking 0.5 g of moistened drug in a test tube. 2 ml of water was added. 1 to 2 drops of diluted ferric chloride solution was added. A dark green or blue-green coloration indicates the presence of phenolic compound [24]. Melting points were determined using Thiele tube method [25].

The analytical method development was done using UV spectroscopic analysis. Calibration of standardized *Picrorrhiza kurroa* extract (SPE) was performed in methanol and phosphate buffer pH 7.4. A standard stock solution of 1000 µg/ml was prepared, and from this, a 10 µg/ml solution was prepared and used to determine the λ_{max} and calibration curves were obtained [26].

Characterization of SPE was done by obtaining FTIR spectra using the FTIR spectrophotometer. The thermal behavior of the drug was evaluated using differential scanning calorimeter, and the enthalpy and melting point range was determined.

Preparation of Kutki Phytosome (KP): The Kutki Phytosomes (KP) were prepared by refluxing followed by the solvent evaporation technique. [27]KP was prepared in different ratios, *i.e.*, 1:0.5, 1:0.75, 1:1, 1:2.5 and 1:3 of SPE to Phosphatidylcholine. SPE and Phosphatidylcholine were dissolved in ethanol and dichloromethane, respectively. Both the solutions were mixed and pour in a 200 ml round-bottomed flask. The mixture was refluxed for different duration *i.e.* 1-3 h and at various temperatures 45-65 °C. The resulting clear solution was evaporated and dried under vacuum to remove any traces of solvents. The resulting residues were then gathered and stored at room temperature for further use.

QbD based Design of Experiment: Central Composite Design: 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) and reaction time (X_3, hr) on the entrapment efficiency of SPE. Three independent variables (X_1, X_2 and X_3) were selected at three levels resulting in twenty possible combinations. The dependent variable is the entrapment efficiency of SPE. The experimental trials were performed using all twenty 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:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3$$

Where Y is the dependent variable, b_0 is the intercept representing arithmetic mean response of the 20 runs, b_i 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 describe nonlinearity. The coded levels and the real values of the independent variables are shown in **Table 1**. The compositions of experimental trials along with entrapment efficiency are shown in **Table 2**.

TABLE 1: CODED LEVEL AND REAL VALUES FOR EACH FACTOR UNDER STUDY

Variables	Levels				
	-1.68	**-1**	**0**	**1**	**1.68**
X1	1:0.5	1:0.75	1:1	1:2.5	1:3
X2	45	50	55	60	65
X3	1	1.5	2	2.5	3.0

Characterization of Kutki Phytosome (KP):

Apparent Solubility: The apparent solubility was determined by adding excess of SPE and KP to 5ml

of water or n-octanol in sealed glass containers at room temperature (25-30 ºC). The liquids were agitated for 24 h then centrifuged for 20 min at 1,000 rpm to remove excessive SPE and KP. 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 270 nm using UV spectrophotometer [28].

Entrapment Efficiency: Entrapment efficiency (EE) was measured using UV visible spectro-photometer (UV-1601, Shimadzu). A weighed quantity of Kutki Phytosome (KP) equivalent to 10 mg of SPE was 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 min. After centrifugation, the supernatant was filtered through 0.45 µ Whatman filter paper, and after suitable dilution absorbance was measured in UV at 270 nm; the concentration of drug was measured [29]. All measurements were performed in triplicate. The EE (%) was calculated using the following formula:

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

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

Particle Size Distribution: The particle size analysis of the prepared KP 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 analyzed in size analyzer.

Zeta Potential Measurements: The zeta potential was measured by analyzing 0.1 g of ZnO in 10 ml of water (or additives solutions) using the Zetasizer 7.12 (Malvern Instruments Ltd., GB). Before zeta potential measurements all samples were sonicated for 5 min. Zetasizer 7.12 uses Laser Doppler Velocimetry is used to determine electrophoretic mobility. The dynamic method consisted of using ZS Malvern Zetasizer device coupled with an automatic titrator (Malvern MPT-2).

X-Ray Diffraction (XRD) Study: Diffractometer (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 Calorimeter (DSC): DSC studies for pure SPE, PC, PM and KP 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 SPE, PC, PM, and KP 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 wavenumber region 4000-500 cm^{-1}. The obtained IR spectra were interpreted for functional groups at their respective wave number (cm^{-1}).

Scanning Electron Microscopy (SEM): SPE and KP were coated with gold in a Fine Coat Ion Sputter S-4800 TYPE II, Hitachi high technologies corporation, Japan. The analysis was done on the coated sample by placing a pinch of the sample in the S-4800 TYPE II (Hitachi high technologies corporation, Japan) Scanning electron microscope and surface morphology were viewed and photographed.

Dissolution Study (*in-vitro* Drug Release): The *in-vitro* dissolution profiles of SPE, physical mixture (PM), and the prepared KP were obtained and compared [30]. 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 KP equivalent to 50 mg of SPE, PM, and SPE 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 270 nm to

determine drug release from the complex and the drug.

Stability Studies of KP: The optimized KP was subjected to stability studies to evaluate any physical or chemical changes in storage. The storage stability studies of the optimized KP were performed as per the ICH guidelines. The optimized KP was stored in sealed amber-colored vials. For this, 10 ml of KP dispersion with the drug of concentration 2 mg/ml was taken into glass vials and stored at two different temperature conditions, 4 °C and 25 °C, for a period of 3 months. The effect of storage on color and entrapment efficiency was determined. The sampling was done with a frequency of 1 month.

***In-vitro* Antioxidant Activity:**

DPPH Assay: A methanolic solution of test sample was prepared at various concentrations (100-1000 µg/ml). To a set of test tubes, 2.9 ml of DPPH solution (100µg/ml in methanol) and 0.1 ml of varying concentrations of test sample were added. The mixture was then shaken vigorously and allowed to stand in dark for 30 min; absorbance at 517 nm was measured using a spectrophotometer. A control solution was consisting of 0.1 ml of methanol and 2.9 ml of DPPH radical solution. Percentage scavenging of DPPH radical was calculated by comparing the absorbance between the test, mixture and control. % scavenging of DPPH radical was calculated by the formula, [31]

% Scavenging of DPPH = Absorbance(control) - Absorbance (sample) ×100 / Absorbance(control)

Reducing Power Assay: The reducing power of the SPE, KP, and vit C were determined according to the conventional method. Various concentrations of SPE, KP, and vit C(100- 1000 µg/ ml) in 1.0 ml of deionized water were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50 °C for 20 min. Aliquots of trichloroacetic acid (2.5 ml, 10%) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml, 1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power [32].

Hydrogen Peroxide Scavenging Activity: The ability of the extracts to scavenge hydrogen peroxide (H_2O_2) was determined. A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer pH 7.4. Various concentrations of SPE, KP and vit C (10- 100 µg/ ml) in distilled water was added to hydrogen peroxide solution (0.6 ml, 40 mM). Hydrogen peroxide concentration was determined after 10 min spectrophotometrically from absorption at 230 nm against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as the standard.

The experiment was performed in triplicate [33]. Hydrogen peroxide scavenging activity was expressed as the percentage inhibition, calculated using the following formula,

% Inhibition = Absorbance (control) – Absorbance (sample) ×100 / Absorbance (control)

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.

RESULTS AND DISCUSSION:

Pre-formulation Studies: The melting point range by the Thiele tube method was found to be 195-220°C. The λ_{max} of SPE in methanol was found to be 270 nm. The regression equation and correlation coefficient for calibration in methanol was found to be 0.026x + 0.010 and 0.999 respectively. The FT-IR spectrum of SPE showed characteristic peaks. The DSC graph of SPE showed an exothermic peak and phase transition from solid at 235.34 °C to liquid at 243.45 °C.

Preparation of KP: In the present study, the 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 phytosome. The variables used in the study were selected on the basis of preliminary experiments. The results of the entrapment efficiency (%) are shown in **Table 2**. The measured values from the experimental trials revealed a wide range (65.34-95.24% w/w) entrapment efficiencies **Table 2**. 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 **Fig. 1**. 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:

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

The polynomial model for Y was 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. The multiple regression analysis revealed that the coefficients b1, b2, and b3 were positive.

Based on the central composite design, the response surface plots show the changes in the entrapment efficiency (%) 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 **Fig. 1**. Increasing levels of X_1, X_2, and X_3 were found to be favorable conditions for obtaining higher 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 2: INDEPENDENT VARIABLES, ALONG WITH CODED AND ACTUAL VALUES AND RESPECTIVE RESPONSES FOR DIFFERENT BATCHES OF KPs

Std	Run	Coded Value Variables			Actual Value Variables			Response Value
		X_1	X_2	X_3	X_1	X_2	X_3	% EE (Y)
1	1	-1	-1	-1	1:0.75	50	1.5	65.34 ± 0.23
2	2	1	-1	-1	1:2.5	50	1.5	80.45 ± 0.54
3	3	-1	1	-1	1:0.75	60	1.5	72.34 ± 0.87
4	4	1	1	-1	1:2.5	60	1.5	89.56 ± 0.74
5	5	-1	-1	1	1:0.75	50	2.5	88.12 ± 0.89
6	6	1	-1	1	1:2.5	50	2.5	87.31 ± 0.52
7	7	-1	1	1	1:0.75	60	2.5	83.32 ± 1.05
8	8	1	1	1	1:2.5	60	2.5	94.51 ± 0.32
9	9	-1.68	0	0	1:0.5	55	2	68.93 ± 0.35
10	10	1.68	0	0	1:3	55	2	95.24 ± 0.65
11	11	0	-1.68	0	1:1	45	2	76.54 ± 0.68
12	12	0	1.68	0	1:1	65	2	91.82 ± 1.31
13	13	0	0	-1.68	1:1	55	1	74.56 ± 0.36
14	14	0	0	1.68	1:1	55	3	93.13 ± 0.24
15-20	15-20	0	0	0	1:1	55	2	89.43 ± 0.54

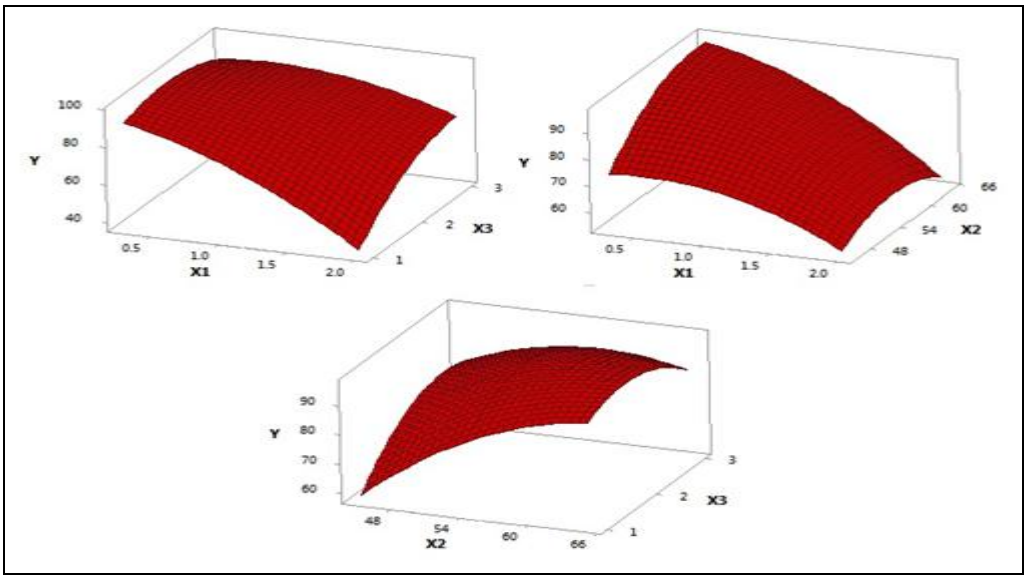

FIG. 1: SURFACE RESPONSE PLOT SHOWING THE INFLUENCE OF PHOSPHOLIPIDS-DRUG RATIO (X_1, w/w), THE REACTION TEMPERATURE (X_2, °C), AND THE REACTION TIME (X_3, h) ON ENTRAPMENT EFFICIENCY

Validation of Optimized Model: An additional batch of KP was prepared in order to evaluate the optimization capability of the models generated according to the result of central composite design-response surface methodology using the optimized values of the variables, *i.e.*, X_1, X_2, and X_3 1:3, 65 °C, and 2 h, respectively. A comparison between the predicted (theoretical) value (%) of the KP 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 SPE in KP was 96.81%, while the average observed value (%) from the prepared batches was found to be 95.67%, indicating both applicability, and validity of the developed model. The bias (%), calculated using the equation, was also found to be less than 3% (1.16%), indicating the relative robustness of the model.

Bias% = Predicted value – observed value × 100 / Predicted value

TABLE 3: OBSERVED AND PREDICTED VALUE OF ENTRAPMENT EFFICIENCY

Batches	Predicted value (%)	Observed value (%)[a]	Bias
1	96.81	96.01 ± 1.32	0.82
2	96.81	95.14 ± 0.54	1.72
3	96.81	95.89 ± 0.78	0.95

[a]All values are mean ± SD (n=3)

Physico-chemical Characterization of Prepared KP:

Apparent Solubility of KP: Table 4 showed an average apparent solubility of the SPE, the physical mixture of SPE and PC, and the prepared complex (KP). The data indicated that KP significantly increases the solubility of SPE. The physical mixture (PM) enhanced the solubility of SPE but this effect was weaker. The prepared KP showed a significant increase in the aqueous solubility. This increase in the solubility of the prepared complex may be explained by reduced molecular crystallinity of the drug and the overall amphiphilic nature of the phytosome [34].

TABLE 4: APPARENT SOLUBILITY STUDY OF SPE, PM AND KP IN WATER AND N-OCTANOL

S. no.	Sample	Aqueous solubility (µg/mL)[a]	n-Octanol solubility (µg/mL)[a]
1	SPE	5.76 ± 0.53	275.13 ± 0.78
2	PM	9.35 ± 1.05	417.21 ± 0.56
3	KP	81.48 ± 0.63	689.43 ±1.03

[a]All values are mean ± SD (n=3)

Particle Size Distribution: The particle size of the prepared KP was carried out using dynamic light scattering technique. The mean particle size of KP was shown in **Fig. 2**. The particle size was distributed in a narrow range of 611.4 ± 16.0 nm, and the polydispersity index was 0.323 ± 0.03 **Fig. 2**. The surface area to volume (SA/V) ratio of most particles is inversely proportional to the particle size. Thus, smaller particles of the KP, 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 [35-36].

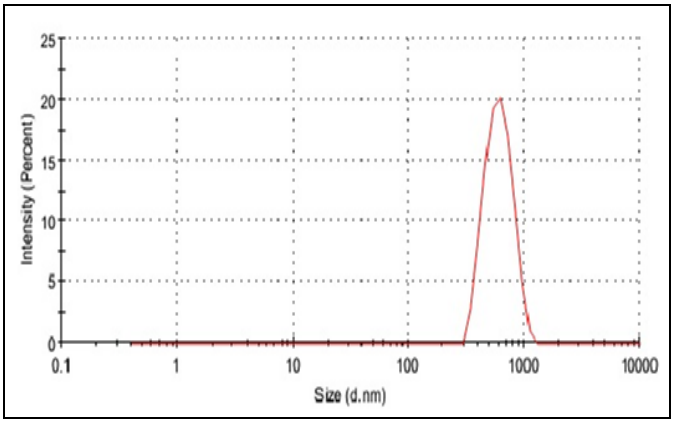

FIG. 2: PARTICLE SIZE ANALYSIS OF KP

Zeta Potential Measurement: The zeta potential of KP was found to be 10.7 mV, as shown in **Fig. 3**. Zeta potential is a key factor that influences some properties of the particle, such as stability of particles in a liquid medium and the possible interactions with other materials [37].

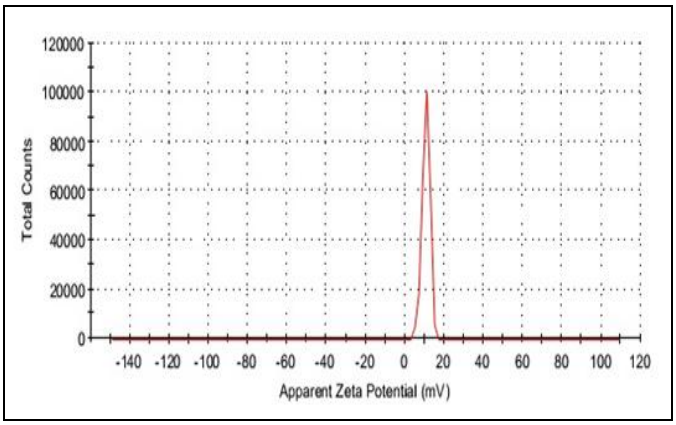

FIG. 3: ZETA POTENTIAL OF KP

X-Ray Diffraction (XRD) Study: Fig. 4 showed the powder X-ray diffraction (PXRD) patterns of

(A) SPE, (B) PC, (C) PM, and (D) KP. The powder of X-ray diffraction patterns of SPE displayed partial sharp crystalline peaks, which is characteristic of a molecule with some crystallinity.

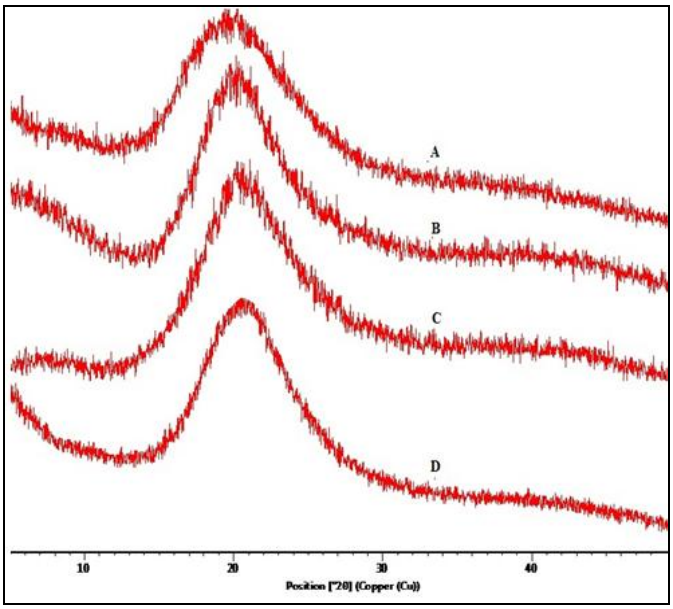

FIG. 4: XRD SPECTRA OF (A) SPE, (B) PC, (C) PM AND (D) KP

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 KP revealed the disappearance of most of the crystalline peaks associated with the SPE and when compared with the physical mixture (PM). These results were in concord with reported studies, where the disappearance of the peaks was associated with the formation of drug phospholipid complex [38]. The disappearance of SPE crystalline peak confirms the formation of SPE-phospholipid complex.

Scanning Electron Microscopy (SEM): SEM photographs of SPE and KP are shown in **Fig. 5**. The crystalline state of SPE was visualized in the SEM photograph as numerous crystals in **Fig. 5A**. In **Fig. 5B** the drug was completely converted into phyto-phospholipid (KP) complex where SPE was physically enwrapped by phospholipids imparting amorphous nature to the complex due to which crystals disappeared.

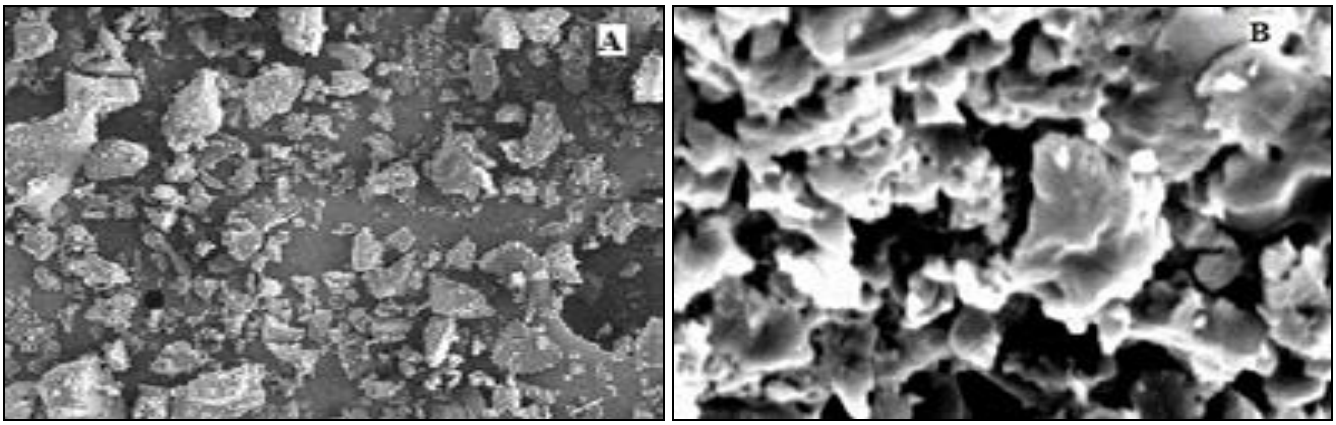

FIG. 5: SEM OF (A) SPE AND (B) KP

FT-IR Study: Fig. 6 shows Fourier transform infrared spectroscopy (FTIR) of the SPE, PC, the physical mixture of SPE with PC (PM), and the prepared KP. These spectrums have studied the interaction between SPE and PC. The FTIR spectrum of SPE showed broad peak at 3537 cm^{-1} representing alcoholic (−OH) group, 3051 cm^{-1} (Ar CH stretching), 1712 cm^{-1} (C=O stretching), 1037 cm^{-1} (C-O-C stretching). FTIR spectrum of PC **Fig. 6B** revealed the characteristic absorption at 3483 cm^{-1} (NH stretching), 2920 cm^{-1} (CH stretching), 1739 cm^{-1} (C=O stretching), 1234 cm^{-1} (P=O stretching), 1087 cm^{-1} (P–O–C stretching) and 972 cm^{-1} (C-C-N stretching). The FTIR spectrum of the prepared KP **Fig. 6D** is quite different from that of SPE and PC. Some absorption peaks were shielded by phospholipids. The absorption peak of hydroxyl stretching of KP showed remarkable broadening from 3278 -3464 cm^{-1}.[39]

Differential Scanning Calorimetry (DSC): DSC is a fast, reliable method to investigate the interaction between multiple components and drug excipient compatibility. These interactions are observed as the elimination of endothermic peak, the appearance of a new peak, the change in peak shape, onset temperature, melting point, relative peak area, or enthalpy [40-41]. **Fig. 7** showed DSC

thermograms of SPE, PC, the physical mixture of SPE with PC (PM), and the prepared KP. The Thermogram of SPE revealed a broad endothermic peak at 197.3 °C. PC shows an endothermic peak at 167.4 °C. PM showed a major peak at 143.5 °C. The thermogram of the complex showed a potentially broad endothermic peak at 210.5 °C. This peak differs from extract and phospholipid. The original peaks of SPE and PC disappeared from the thermogram of complex, thus indicating the successful formation of the complex.

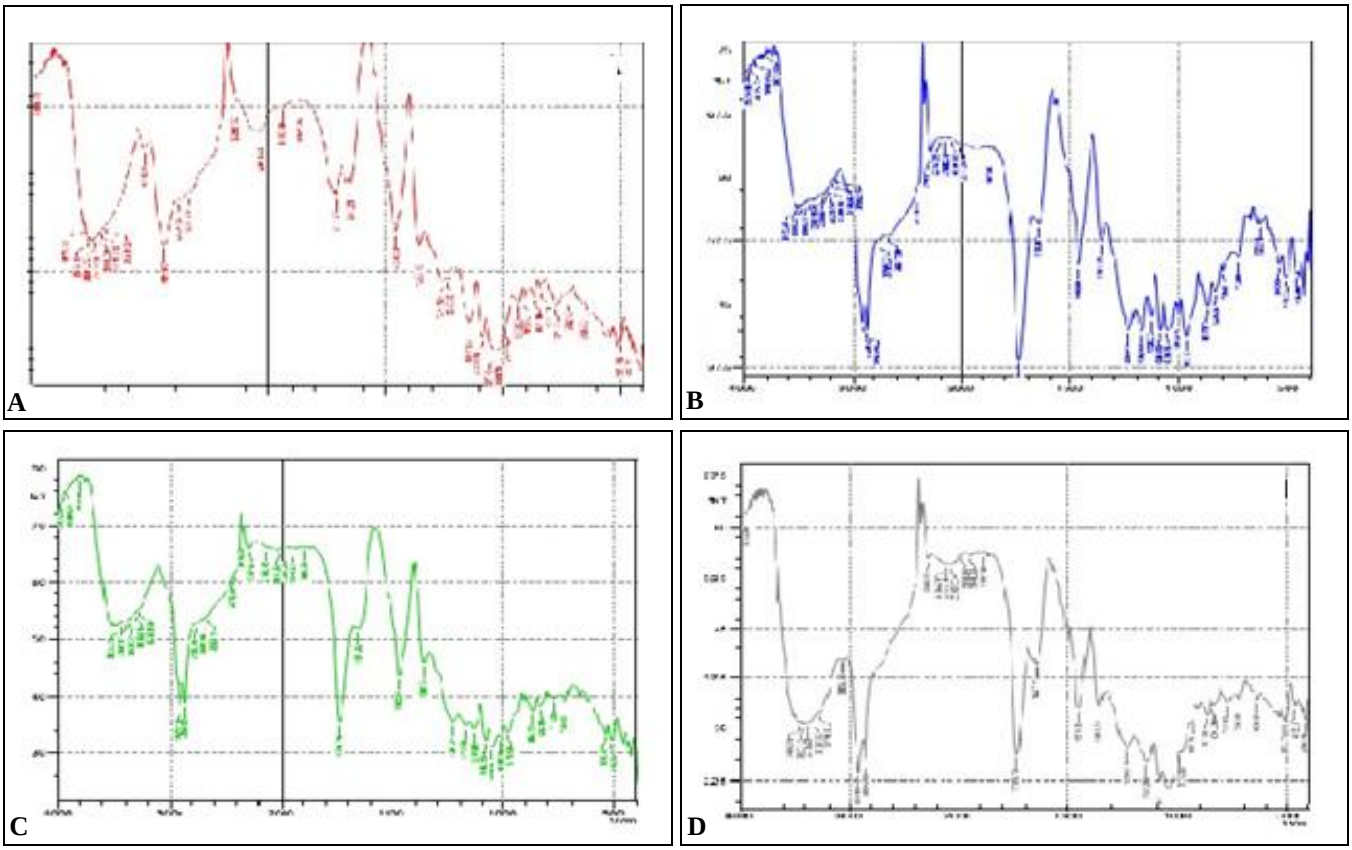

FIG. 6: FTIR SPECTRA OF (A) SPE, (B) PC, (C) PHYSICAL MIXTURE AND (D) KP

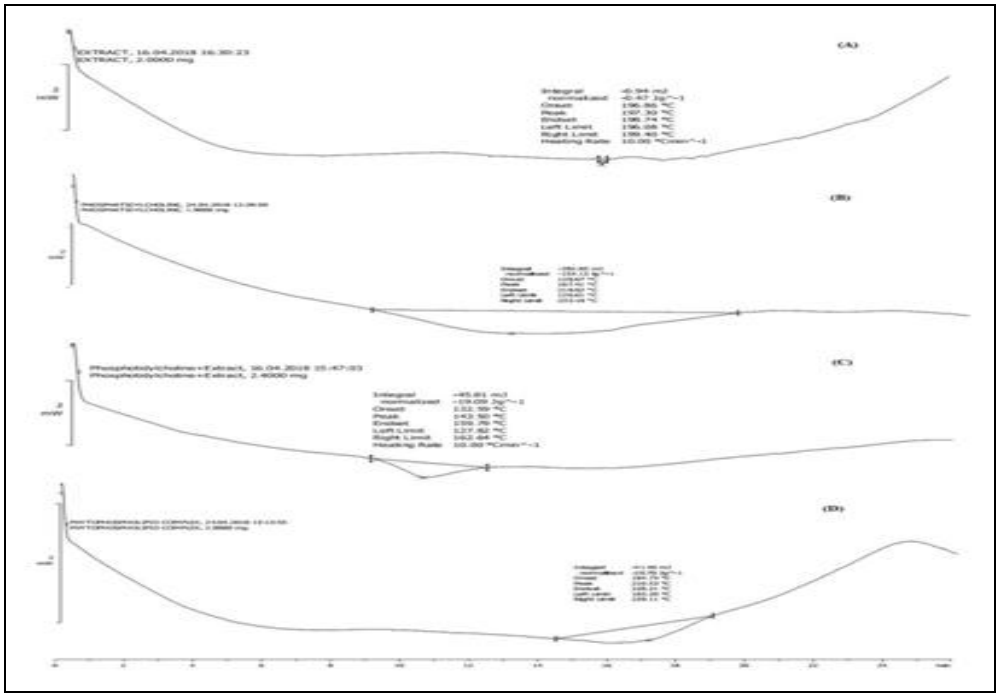

FIG. 7: DIFFERENTIAL SCANNING CALORIMETRY OF (A) SPE, (B) PC, (C) PM AND (D) KP

Dissolution Study (*in-vitro* Drug Release): The results of *in-vitro* drug release studies are shown in **Fig. 8**. The 12-h dissolution in the phosphate buffer (pH 6.8) revealed that the pure SPE showed the slowest rate of dissolution, *i.e.*, at the end of the dissolution period, only about 43.35% w/w of SPE was dissolved. The dissolution rate of the physical mixture was found not to be significantly different (45.43% w/w dissolved in 12 h) compared to the pure SPE. The prepared KP revealed a significantly faster release of SPE at the end of the dissolution period. At the end of 12 h, over 88.34% w/w SPE was observed to be released from KP. The dissolution rate is largely influenced by the crystal morphology of the solids, [42,] and the improved dissolution rate of SPE from KP may be explained by the improved solubility and the amorphous form in the prepared complex. The relatively higher amorphous state of the phytosome and the increased aqueous solubility may have a positive impact on the cumulative release of the drug [43].

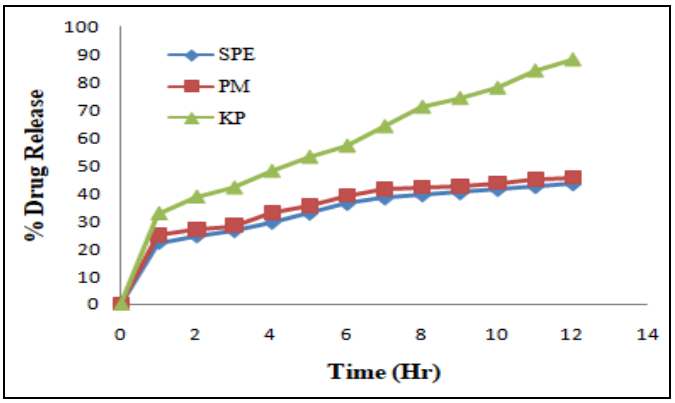

FIG. 8: % DRUG RELEASE PROFILE OF SPE, PM AND KP

Stability Studies of KP: Stability studies of optimised KP under two storage conditions were carried out, and the changes in entrapment efficiency were evaluated as parameters. The results showed that there was a slight change in entrapment efficiency during three months of storage at 4 °C and 25 °C. The percentage of % EE of the optimised phytosome was found to be 95.67 ± 0.68% and after 3 month storage at 4 °C and 25 °C, it was found to be 91.05 ± 0.72% and 89.27 ± 0.11% respectively. **Fig. 9** shows the changes in entrapment efficiency against storage time. The percentage of EE also decreased significantly during storage at 4 °C. Thus, the optimized KP was found to be stable at two different storage temperature conditions for 3 months.

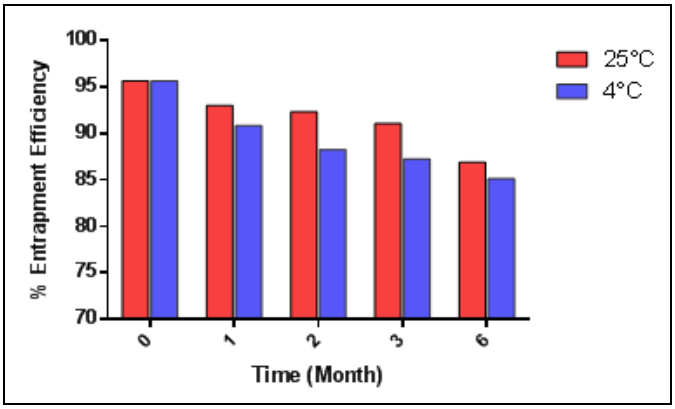

FIG. 9: EFFECT OF STORAGE CONDITION ON % ENTRAPMENT EFFICIENCY OF KP

Assessment of Antioxidant Activity: DPPH has been widely used to evaluate the free radical scavenging effect of various antioxidant substances. In the DPPH assay, the antioxidants are able to reduce the stable radical DPPH to the yellow colored diphenylpicryl hydrazine. DPPH is used as a reagent to evaluate free radical scavenging activity of antioxidants [44]. This method was possible to determine the antiradical power of an antioxidant by measuring the absorbance of DPPH. The % scavenging effect of SPE on the DPPH radical increase as the concentration of KP, SPE, and vitamin C is increased in the order of vitamin C >SPE > KP, which were 93.56%, 91.34%, and 74.43%, at the concentration of 1000 µg/mL, respectively **Table 5**.

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity [45]. The reducing power assay is used to test the reducing capability of standardised extract SPE and phytosome KP to convert the potassium ferricyanide (Fe^{3+}) complex to form potassium ferrocyanide (Fe^{2+}). The potassium ferrocyanide will then react with ferric chloride to form a ferrous complex, which can absorb maximally at 700 nm. **Table 5** showed increment in reducing power with an increase in the concentration of SPE, KP, and vitamin C. Higher absorbance of the reaction mixture indicates higher reductive potential [46].

Hydrogen peroxide is highly important because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because of it may give rise to hydroxyl radical in the cells [47]. Thus, removing of H_2O_2 is very important for the protection of food systems.

The ability of standardized extract SPE and KP to scavenge H_2O_2 was determined. **Fig. 10** shows the H_2O_2 scavenging activity of SPE, KP, and vit C. KP had strong H_2O_2 scavenging activity when compared to control ($p<0.01$). Moreover, this activity was close to standard antioxidant vit. C. These results showed that KP had effective H_2O_2 scavenging activity. H_2O_2 scavenging activity of those samples followed the order: vitamin C >KP > SPE.

TABLE 5: DPPH RADICAL SCAVENGING ACTIVITY AND REDUCING POWER ASSAY

S. no.	Concentration (µg/ml)	DPPH (% Inhibition) [a]			Reducing Power (Absorbance) [a]		
		SPE	KP	Vit C	SPE	KP	Vit C
1	100	12.21±0.23	22.28±1.23	23.16±0.13	0.421±0.52	0.785±0.12	0.985±1.81
2	200	32.23±1.31	41.34±0.43	54.68±0.14	0.812±1.02	0.932±0.44	1.150±0.54
3	400	52.57±0.67	61.32±1.21	68.04±1.17	1.034±1.03	1.283±0.32	1.920±0.89
4	600	60.32±1.02	72.18±1.21	74.48±0.15	1.481±0.34	1.481±0.8	2.193±1.54
5	800	70.08±0.22	82.62±1.32	85.34±0.46	1.950±1.25	2.342±1.0	3.143±0.32
6	1000	74.43±0.34	91.34±1.42	93.56±1.26	2.411±1.82	3.414±0.15	3.992±1.02

[a]All values are mean ± SD (n=3)

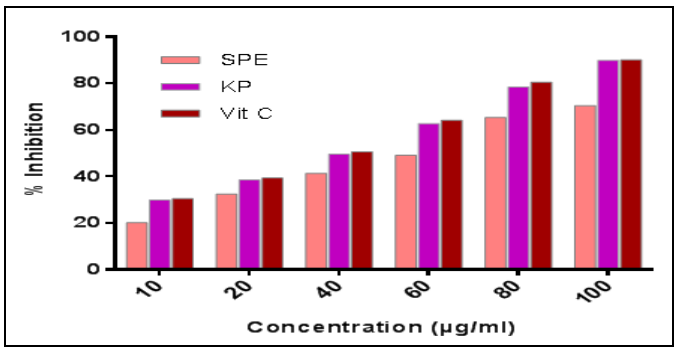

FIG. 10 % H_2O_2 INHIBITION OF SPE, PM AND KP

CONCLUSION: Standardized *Picrorrhiza kurroa* extract (SPE) was successfully complex with phosphatidylcholine (PC) to form phytosome KP. An attempt was made to enhance the solubility of SPE by its complexation with phospholipids. The complex was formulated using solvent evaporation technique using central composite design-response surface methodology and formulation was optimized. The prepared phytosome (KP) was characterized by FTIR, DSC, PXRD, and SEM.

These studies indicated the successful formation of the vesicular drug-phospholipids complex. The apparent solubility and *in-vitro* dissolution studies indicated a significant improvement in the solubility and the drug release of SPE from KP, respectively. The stability studies revealed that there were no significant changes in the formulation over a period of three months. The *in-vitro* evaluation revealed a significantly higher antioxidant activity of the prepared KP compared to the pure SPE.

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