NOVEL UMBELLIFERONE PHYTOSOMES: DEVELOPMENT AND OPTIMIZATION USING EXPERIMENTAL DESIGN APPROACH AND EVALUATION OF PHOTO-PROTECTIVE AND ANTIOXIDANT ACTIVITY

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INTRODUCTION

Phytochemical and phytopharmacological studies have been established for the compositions, therapeutics and overall health boosting capacities of various plants. But phytoconstituents have a disadvantage that they are limited in their effectiveness because they are poorly absorbed and their limited solubility both in water and in lipophilic phases, as well as hydrolytical instability at physiological pH values, makes them very poorly absorbed both orally and topically [1]. They cannot easily get absorbed and their passage through the lipoidal biological membrane is limited [2]. Many approaches have been developed to improve the bioavailability of these phytoconstituents, such as the inclusion of solubility and bioavailability enhancers, structural modification and entrapment with the lipophilic carriers [3].

Bioavailability can be improved by using novel drug delivery systems [4]. Among these, Phytosome process has been applied successfully to several popular herbal extracts. Phytosomes are phospholipid complexes of the herbal active drug constituent mainly formulated to improve the overall solubility and bioavailability in comparison to the plain herbal extract. They are ideally suited to optimise drug delivery and reduce toxicity [5]. It has been applied to flavonoids, polyphenols, terpenoids, tannins, catechins, etc. for example turmeric, ginseng, hawthorn, etc [6]. Phytosomes results from the reaction of a stoichiometric amount of the phospholipid (phosphatidylycholine) with the standardised extract in a non-polar solvent. Phytosome technology has been considered to be a major advancement in clinical research for active phytoconstituents with poor bioavailability [7].

The Phytosome technology, a novel drug delivery system, produces a little cell, better able to transit from a hydrophilic environment into the lipid-friendly environment of the enterocyte cell membrane and from there into the cell [8]. The hydrophilic moiety (choline group) binds with water soluble phytoconstituents and forms the body while the lipid soluble phosphatidyl moeity forms the tail and envelopes the choline bound material. As a result, a lipid compatible molecular complex is formed [9].

Coumarin is a plant flavonoid distributed widely in nature. Substituted coumarin derivatives were reported to have a variety of biological activities. Umbelliferone is yellowish-white in color and crystalline in nature. It absorbs ultraviolet light strongly at several wavelengths and acts as photoactive agent. Umbelliferone has antioxidant properties and can scavenge free radicals generated in the skin as a result of UV exposure when applied topically. Umbelliferone is found to occur in many plants from Apiaceae family such as carrot, contander and garden angelica, and also in other families, such as bigleaf hydrangea (Hydrangea macrophylla, Hydrangeaceae) or the mouse-eared hawkweed (Hieracium pilosella, Asteraceae). It can be isolated from the fruit of Aegle marmelos [10]. Umbelliferone has a polar hydroxyl group which has the affinity to bind with the phospholipid. Umbelliferone-phosphatidylcholine (UMB-HSPC) complex may be formed due to the interaction between the polar hydroxyl group of umbelliferone and polar choline group of phosphatidylcholine.

As umbelliferone has the desired properties, the present study aims to increase the solubility, permeability, antioxidant activity and the potent photoprotective effect by formulating it as a phospholipid complex.

MATERIALS AND METHODS

Materials

Umbelliferone was purchased from Sigma-Aldrich, Inc. (USA) having purity 99%. Phospholipon 90H was obtained as a gratis sample from Phospholipid Inc., USA.
Lipoid (Germany). All the other chemicals and reagents were of the analytical grade.

Methods

Preformulation studies

The presence of umbelliferone was studied by specific identification test by taking 0.5 g of moistened methanolic drug in a test tube. Filter paper treated with 1 N sodium hydroxide solution was used to cover the mouth of the tube. Then it was placed in boiling water for a few minutes, and the filter paper was removed and subjected to examination under UV light for fluorescence [11, 12]. Melting point was determined using Thiele tube method [13].

The analytical method development was done using UV spectroscopic analysis. Calibration of umbelliferone was performed in methanol, ultrapure water and phosphate buffer saline pH 7.4 (PBS). 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 Amon and calibration curves were obtained [10].

Characterization of the complex was done by obtaining FT-IR spectra using Shimadzu-IRAFFINITY-1 FT-IR spectrophotometer. The thermal behaviour of the drug was evaluated using differential scanning calorimeter and the enthalpy and melting point range was determined [14]. The various validation parameters like linearity, interday precision, intraday precision, robustness, ruggedness, limit of detection, limit of quantitation and % recovery/accuracy study were determined in accordance with the ICH guidelines [15].

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Preparation of umbelliferone-phospholipid complex

Accurately weighed amounts of umbelliferone and phospholipon 90H in stoichiometric molar ratios (1:1/1:2/1:3) were dissolved in 20 ml dichloromethane and refluxed using condenser at constant temperature (40/50/60 °C) for a defined period of time (1/2/3 h). The mixture was concentrated to 2-3 ml by evaporating the solvent. Complexes were retrieved by precipitation by adding an excess amount of n-hexane. The mixture was then allowed to stand and the residue was dried in a desiccator containing anhydrous calcium chloride [16].

Design of experiments-Box-Behnken design

The optimisation was carried out using 3 factors, 3 levels Box-Behnken design. Independent variables were the umbelliferone-phospholipid ratio (X1), process temperature (X2) and reaction time (X3) for two dependent variables i.e. complexation rate (Y1) and partition coefficient (Y2) as shown in table 1. A total of 15 batches were prepared and evaluated for complexation rate and partition coefficient. This data was statistically analysed and validated by Design Expert® (Version 8.0.0, Stat-Ease Inc., Minneapolis, MN) using ANOVA and polynomial equations to find an optimised set of process parameters. The optimised batch was selected using desirability functions. Various 3-D response surface graphs and contour plots were studied. A matrix design was constructed to generate a non-linear quadratic model equation as-

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

Where Y is the measured response for each factor level combination; b is an intercept; b1 to b13 are regression coefficients fig. d from the observed experimental values of Y; and X1, X2 and X3 are coded levels of independent variables [16].

| Types of variable | Variable | Optimisation levels used |
|------------------|----------|-------------------------|
|                  |          | Low (-1) | Medium (0) | High (1) |
| Independent      | X1(umbelliferone : phospholipon) | 1:1 | 1:2 | 1:3 |
|                  | X2(Temperature) | 40 °C | 50 °C | 60 °C |
|                  | X3(Time) | 1 h | 2 h | 3 h |
| Dependent        | Y1(complexation rate) | Maximize |
|                  | Y2(partition coefficient) | Minimize |

Table 1: Dependent and independent variables in Box-Behnken design used for optimisation

Characterization of the complex

Complexation rate of complex

The amount of complex equivalent to 10 mg of umbelliferone, was dispersed in 5 ml of chloroform. Both the complex and phospholipid were dissolved easily in chloroform but free umbelliferone remained practically insoluble in chloroform. The non-complexed umbelliferone was separated, dissolved in methanol and assayed using a UV-Spectrophotometer (Y-650 lasco 2000 series, Japan) at 324 nm. The free drug concentration was calculated from standard curves. The complexation rate of umbelliferone was determined by the formula:

\[ \text{Complexation rate} (\%) = \frac{(m_1/m_3) \times 100}{(1-(m_1/m_3)) \times 100} \]

Where \( m_1 \) is the total content of umbelliferone added, \( m_2 \) is the content of umbelliferone present as a complex and \( m_0 \) is free umbelliferone [17].

Determination of solubility by partition coefficient method

Complex equivalent to 10 mg of umbelliferone was taken in a beaker. To this 10 ml distilled water was added and stirred for 20 min on a magnetic stirrer. In a 60 ml separating funnel, 10 ml of n-octanol was added, and dispersion of complex in water was added to the funnel. The funnel was uniformly agitated for a period of 2 h and then kept aside for 30 min. When two distinct layers were formed, they were separated and the solution was diluted and assayed using UV spectrophotometer (Y-650 lasco 2000 series, Japan) by recording absorbance in triplicate. Similar procedure was used for the plain drug. Concentration in both the phases was calculated using calibration equation and partition coefficient was calculated by-

\[ \text{Partition coefficient} = \frac{C_o}{C_w} \]

Where \( C_o \) = concentration in oil phase, \( C_w \) = concentration in water phase [17]

Percentage practical yield

The percentage practical yield was determined by first calculating the theoretical yield of optimised batch and then weighing the batch to get the practical yield [16, 17]. The % practical yield was calculated using the formula-

\[ \% \text{Practical yield} = \frac{\text{Practical yield}}{\text{Theoretical yield}} \times 100 \]

Drug content

Complex equivalent to 10 mg of umbelliferone (101.15 mg) was dissolved in 10 ml methanol to form a solution of concentration approximately 1000 µg/ml. From this 100 µg/ml solution was prepared. Then again 0.2 ml was taken using a micropipette and volume was made to 10 ml with methanol to obtain 2 µg/ml and it was evaluated in UV spectrophotometer at 324 nm.

The blank was made by dissolving 10 mg of phospholipid in 10 ml of methanol to form a solution of 1000 µg/ml. From this 100 µg/ml solution was prepared and then subsequently 2 µg/ml solution by dilutions. This solution was used as blank. The drug content was calculated for the optimised batch [18].

In vitro antioxidant activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) antioxidant assay

Preparation of 0.2 mmol DPPH solution—Accurately weighed 7.9 mg of DPPH was dissolved in methanol and volume was made to 1000 ml to get a 0.2 mmol solution.

Procedure for assay—For the assay 1 ml of each concentration of drug and complex was mixed with 2 ml of 0.2 mmol DPPH solution and
was evaluated using UV spectrophotometer at 517 nm and the %
DPPH antioxidant activity was calculated and compared for the
complex and drug. The DPPH solution was used as the control and %
DPPH activity was calculated using following formula [19–22]-

\[
\text{% DPPH radical scavenging activity} = \left( \frac{A_t}{A_c} \right) \times 100
\]

A--Absorbance of control
A--Absorbance of test sample

Ferrozine antioxidant assay-
Preparation of 2 mmol ferrozine solution: Accurately weighed 24.7 mg of
ferrozine was dissolved in water and volume was made to 25 ml with
distilled water to obtain a solution of 2 Mm concentration. Preparation of
250 mmol ferric chloride solution: Accurately weighed 2483.8 mg of ferric
chloride was weighed and dissolved in water and volume was made up
to 50 ml with distilled water to get a solution of 250 mmol. Procedure
for assay—From each solution of drug and complex, 0.25 ml was
withdrawn. To it 1.75 ml of methanol, 0.25 ml of 2 mmol ferrozine
solution and 0.25 ml of 250 mmol ferric chloride solution were
added. The solutions were kept in the dark for 10 min and their
absorbance was noted at 562 nm against the blank solution. For
added. The solutions were kept in the dark for 10 min and their
absorbance was noted at 562 nm against the blank solution. For

High-performance thin layer chromatography (HPTLC)
A 10 µl volume of the standard solution of umbelliferone and
complex was applied using 100 µl Hamilton syringe in triplicate on an
HPTLC plate (20×10 cm, 0.2 mm thick pre-coated using silica gel
60 F254). The plates were developed in a solvent system of toluene–
methanol (9.5:0.5 v/v) at 25±2 °C temperature and 40% relative
humidity until a distance of 8 cm was achieved. After development,
the plates were dried and scanned at 351 nm for umbelliferone
using Camag TLC scanner 3. The peak areas were found and Rf
values were recorded and compared for the plain drug and complex
using WINCATS software [24, 25].

Particle size and zeta potential
For measurement of the particle size of phytosomal lipid complex, the
sample (5 mg) was dispersed in 10 ml deionized water and the mean
diameter, polydispersity index and zeta potential was
determined using a Delta Nano Zetasizer, Beckman coulter
(Government college of pharmacy, Aurangabad) [17].

Differential scanning calorimetry (DSC)
DSC curves of umbelliferone, phospholipid, and the complex were
recorded using a Differential Scanning Calorimeter, STAR SW 1.00
Mettler (R. C. Patel institute of pharmaceutical education and
research, Shirpur). The thermal behaviour was studied by heating
2.0±0.2 mg of each sample in a covered sample pan under nitrogen
gas flow. The analysis was carried out over the temperature range
0–300 °C at a rate of 10 °C min⁻¹ and the results were compared for
drug and complex [17, 26].

FT-IR spectroscopy
The interaction of umbelliferone and phospholipid due to the
formation of a complex between them was studied by FT-IR
spectroscopy. The FT-IR spectra of umbelliferone, phospholipid,
complex and the physical mixture were obtained using Shimadzu–
IRAFFINITY-1 FT-IR spectrophotometer (Gurunanak College of
pharmacy, Nagpur). The samples were triturated and mixed well
with potassium bromide in the ratio 1:100. Then the mixture was
introduced in the sample holder and scanned to obtain the graphs in
the range of 4500–500 cm⁻¹. Then the spectrum obtained for the
complex was compared to that of the plain drug, phospholipid and
their physical mixture [27, 29].

Scanning electron microscopy (SEM)
SEM imaging of the complex was performed by a scanning electron
microscope (JEOL model JSM 6390LV) using electron beam for
imaging from sophisticated analytical instrument facility, STIC,
Cochin [29].

X-ray diffraction study
The crystalline behaviour of umbelliferone, phosphatidylcholine, physical mixture and the complex was evaluated using X-ray powder
diffraction. Diffraction patterns were analysed with the help of
Bruker AXS D8 Advance (sophisticated analytical instrument facility,
STIC, Cochin). The X-ray generator was allowed to operate at 35 mA
tube current and 40 kV tube voltages. The scanning angle was
adjusted in the range from 3 to 60 ° in the step scan mode with a step
time of 0.28s [26].

Nuclear magnetic resonance
\[ H-\text{NMR}\]-The samples of umbelliferone and complex were dissolved in the solvent dimethyl sulphoxide and analysed with a Bruker
Avance II 400 NMR spectrometer (SAIF, Panjab University,
Chandigarh). The spectrum was obtained and compared for the drug
and complex.
\[ ^{13}\text{C}-\text{NMR}\]-The \[^{13}\text{C}\]-NMR spectrum was taken for confirmation of the
interaction between drug and phospholipid and the formation of the
complex. The sample of umbelliferone and complex was dissolved in the solvent dimethyl sulfoxide and then analysed with a Bruker
Avance II 400 NMR spectrometer (SAIF, Panjab University,
Chandigarh). The spectrum was obtained and compared for the drug
and complex [29, 30].

In vitro permeation
Dialysis membrane-60 (Himedia) of diameter 15.9 mm was
mounted on Franz diffusion cell. The receptor compartment was
filled with PBS. The diffusion cell was maintained at 37±0.5 °C with
constant stirring on a magnetic stirrer. Then 1 ml of umbelliferone-
phospholipid incorporated gel formulation and conventional
umbelliferone gel (0.1%) prepared similarly was spread evenly on
the skin in donor compartment. Then 1 ml sample of receptor
medium was withdrawn at definite time intervals and an equivalent
volume of fresh PBS were added in. All samples were filtered, diluted
and analyzed by UV spectrophotometer. The permeation of the
complex was compared with the plain drug [29].

Animal study
Animals-Adult Albino rats were procured from the central animal
house of UDPS, R. T. M. N. U. Nagpur and used for UV protectant
activity of the developed phytosome formulation as a gel. Animals
were kept in plastic cages with free access to food and water and
maintained at proper temperature and humidity. All the
experimental protocols were approved duly by the institutional
animal ethics committee (IAEC), Nagpur (India). All the experiments
were performed as per the guidelines of the committee for the
purpose of control and supervision of experiments on animals
(CPCSEA), India. Sanction letter number–IAEC/UDPS/2014/26.

Ex vivo skin permeation
Procedure-The abdominal region of rats was shaved using a soft hair
removing the cream. A full thickness abdominal skin was excised.
Any adhered tissue on the skin was removed and wiped with
isopropyl alcohol to remove adhered fat. It was then washed with
phosphate buffer saline (PBS) of pH 7.4.

On the Franz diffusion cell, the skin was mounted properly with
stratum corneum side facing the donor compartment and dermal
dside towards the receptor compartment. The receptor compartment
was filled with buffer. The cell was maintained at 37±0.5 °C and
constant stirring was implemented on a magnetic stirrer. Then 1 ml of
UMB-HSPC gel formulation and conventional UMB gel (0.1%)
which was similarly prepared was evenly spread on the skin in
donor compartment. After that, 1 ml sample from receptor medium
was withdrawn at a predetermined time interval which was
compensated by an equivalent volume of fresh buffer. All samples
were filtered, suitably diluted and analysed by UV spectrophotometer. The permeation of the complex through the skin
was compared with the plain drug [27, 28, 31, 32].
Evaluation of photoprotective potential of the phytosomal complex against UV exposure

Philips UV lamp (125 W) was used for the UV exposure. A demarcated area on the dorsal surface of (2 × 3 cm²) the rat was shaved using a soft hair-removing cream. The rats were observed for 48 h. This was done to identify and exclude those rats which show abnormal hair growth or an irritation reaction to depilatory preparation. UV exposure for 20 min was given to the rats in this study.

Six groups of the experimental rats were formed. A thin and uniform layer of gel formulation was applied to the demarcated shaved area of the rats. Animals of the control group and UVA irradiated group were applied placebo formulation. UMB1 and UMB2 groups were applied conventional umbelliferone gel (containing 0.1% drug). UMB–HSPC1 and UMB–HSPC2 groups were applied umbelliferone–HSPC loaded gel (containing complex equivalent to 0.1% drug). UV irradiated, UMB1 and UMB–HSPC1 groups were given exposure to UV radiation immediately after topical application for 7 d. UMB2 and UMB–HSPC2 groups were exposed to UV radiation after 4 h of topical application for 7 d. On the eighth day, diethyl ether induced anaesthesia was used to sacrifice all the rats. The UV-exposed portion of cutaneous tissue was removed quickly in ice-cold saline, washed with ice-cold saline and the homogenate was prepared in 0.1 M phosphate buffered saline (pH 7.4) [27, 33].

Estimation of antioxidant enzymes

The rat skin (2×3 cm²) was washed in ice–cold saline kept in PBS and cut into small pieces. It was homogenised immediately in a homogenizer under the cold condition and centrifuged (R 24 Remi research centrifuge) to obtain 10 % w/v supernatant skin tissue homogenate. The supernatant homogenate was used for the assay of antioxidant marker enzyme namely reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO) [29, 34-36].

Stability study

A short term chemical stability of the Umbelliferone-phospholipid complex was examined for three months at 30±2 °C at 65±5% RH. The complex samples were analysed at an interval of 30 d for 3 mo and the in vitro permeation was compared [39]. This data was statistically analysed and validated by using ANOVA.

RESULTS

Pre-formulation studies

Identification-When the filter paper was examined under UV radiation it showed blue fluorescence. The melting point range by Thiele tube method was found to be 232.1-234.3 °C. The λ<sub>max</sub> of umbelliferone in methanol was found to be 324 nm. The regression equation and correlation coefficient for calibration in methanol, water and 7.4pH PBS and calibration curves are shown in fig. 1. The FT-IR spectrum of umbelliferone showed characteristic peaks.

The DSC graph of umbelliferone showed an exothermic peak and phase transition from solid at 235.34 °C to liquid at 243.45 °C. The validation parameters were as per the ICH guidelines. The linearity range was 2-10µg/ml.

Preparation of umbelliferone-phospholipid complex i.e. phytosome (Box-Behnken design)-

The optimised batch was found by evaluating two responses-complexation rate (Y<sub>1</sub>) and partition coefficient (Y<sub>2</sub>). The results were as summarised in table 2 and the predicted and observed values in table 3.

The desirability for the optimised batch (F4) was maximum 0.992. Regression equation of the fitted quadratic model for both the responses was as follows-

\[ Y_1 = 99.95 + 0.017X_1 + 0.062X_2 - 0.063X_1X_2 - 7.35X_1X_3 + 1.325X_2X_3 + 0.12X_1X_2X_3 + 0.045X_1^2 - 0.079X_2^2 - 0.047X_3^2 \]

\[ Y_2 = 14.54 - 4.07X_1 - 2.34X_2 + 5.79X_3 - 2.285X_1X_3 + 6.5X_2X_3 + 1.52X_1^2 - 1.67X_2^2 + 2.81X_3^2 \]

The contour plots and 3D response curves were obtained as shown in fig. 2.

Complexation rate

On the basis of highest complexation rate i.e. 99.9572 %, batch F4 was found to be the optimised batch (table 2).

Determination of solubility by partition coefficient method

The comparative solubility was summarised for all the batches in table 3.
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Fig. 2: Contour plots and 3D surface curves for responses-complexation rate (a) and partition coefficient (b)

Table 2: Optimization of phytosomal formulation using Box-Behnken design

| Batch | X1 | X2 | X3 | Comlexation rate (%) | Concentration in water (Cw) (µg/ml) | Concentration in oil (Cv) (µg/ml) | Partition coefficient(K,Cw/Cv) |
|-------|----|----|----|----------------------|-------------------------------------|----------------------------------|---------------------------------|
| F1    | 1:2| 50 | 2  | 99.950±0.27          | 104.7±1.22                          | 668.4±1.43                      | 6.68                            |
| F2    | 1:3| 50 | 3  | 99.955±0.22          | 109.4±1.43                          | 954.0±1.50                      | 8.71                            |
| F3    | 1:2| 40 | 3  | 99.956±0.32          | 103.7±1.68                          | 1196.5±1.98                     | 11.53                           |
| F4    | 1:2| 60 | 3  | 99.957±0.21          | 140.7±1.55                          | 920.4±1.68                      | 6.52                            |
| F5    | 1:1| 50 | 1  | 99.943±0.24          | 137.7±1.82                          | 850.8±1.85                      | 6.77                            |
| F6    | 1:1| 40 | 2  | 99.940±0.21          | 100.8±1.44                          | 703.6±1.77                      | 6.98                            |
| F7    | 1:3| 60 | 2  | 99.948±0.22          | 82.0±1.29                           | 610.6±1.23                      | 7.44                            |
| F8    | 1:2| 60 | 1  | 99.940±0.19          | 78.1±1.55                           | 793.5±1.76                      | 10.14                           |
| F9    | 1:2| 50 | 2  | 99.940±0.24          | 53.1±2.04                           | 832.9±1.49                      | 15.65                           |
| F10   | 1:1| 50 | 3  | 99.856±0.18          | 34.7±1.98                           | 732.2±1.65                      | 21.09                           |
| F11   | 1:1| 60 | 2  | 99.893±0.22          | 33.4±1.74                           | 668.7±1.55                      | 19.97                           |
| F12   | 1:2| 40 | 1  | 99.930±0.36          | 46.7±1.43                           | 1071.4±1.83                     | 22.96                           |
| F13   | 1:2| 50 | 2  | 99.944±0.26          | 48.3±1.87                           | 1000.1±1.48                     | 20.69                           |
| F14   | 1:3| 40 | 2  | 99.948±0.28          | 39.1±1.32                           | 1136.3±1.06                     | 29.01                           |
| F15   | 1:3| 50 | 1  | 99.946±0.016         | 40.6±1.92                           | 904.8±1.59                      | 22.23                           |

Table 3: Observed and predicted values of optimized batch

| Response                  | Predicted values | Observed values (Optimized batch F4) |
|---------------------------|------------------|--------------------------------------|
| Comlexation rate          | 99.942±0.02      | 99.957±0.21                          |
| Partition coefficient     | 7.98             | 6.52                                 |

Table 4: In vitro antioxidant assay-% DPPH radical scavenging activity and % ferrozine antioxidant assay

| Concentration (µg/ml) | %DPPH scavenging activity for umbelliferone | %DPPH scavenging activity for complex | % Ferrozine antioxidant activity for umbelliferone | % Ferrozine antioxidant activity for complex |
|-----------------------|---------------------------------------------|---------------------------------------|---------------------------------------------------|---------------------------------------------|
| 10                    | 35.26±1.65                                  | 30.44±1.55                            | 95.70±1.03                                        | 99.48±0.23                                  |
| 20                    | 35.80±1.68                                  | 36.59±1.84                            | 96.65±1.26                                        | 98.9±0.3                                  |
| 30                    | 35.08±1.72                                  | 35.74±1.75                            | 97.20±1.12                                        | 98.2±0.2                                  |
| 40                    | 35.86±1.55                                  | 36.12±1.89                            | 94.12±1.54                                        | 97.0±0.1                                  |
| 50                    | 36.42±1.53                                  | 36.54±1.79                            | 93.22±1.48                                        | 97.8±0.1                                  |
| 60                    | 35.79±1.76                                  | 36.05±1.66                            | 90.20±1.62                                        | 98.0±0.1                                  |
| 70                    | 34.90±1.42                                  | 35.54±1.65                            | 81.16±1.29                                        | 97.4±0.1                                  |
| 80                    | 34.85±1.66                                  | 35.43±1.73                            | 73.13±1.49                                        | 96.9±0.1                                  |
| 90                    | 33.98±1.58                                  | 35.69±1.63                            | -                                                  | -                                         |

Data is presented as mean value±SD (n=3)

Characterization of complex

Percentage practical yield

The percentage practical yield of the optimized batch F4 was found to be 96.04%.

Drug content

The drug content of umbelliferone in the phytosomal complex for optimised batch F4 was found to be 97.40%.

In vitro antioxidant activity

The results for DPPH and Ferrozine antioxidant activity of newly prepared phytosomal delivery system are shown in table 4 and the comparative antioxidant activity for drug and complex is shown in the fig. 3.

HPTLC of the complex and drug

The peak for the plain drug was found to be sharp whereas the peak for the complex was found to be broader as shown in fig. 4. The Rf value of the complex was 0.32, and that of umbelliferone was 0.33.
Particle size and zeta potential

The average particle size was found to be 1139 nm and the range was between 200–2600 nm. The polydispersity index was found to be 0.717 indicating uniform particle size. The zeta potential of complex was -0.05 mV as shown in fig. 5.

Differential scanning calorimetry

Umbelliferone showed an exothermic peak ranging from 235.34–243.52 °C. Phosphatidylcholine showed a major peak at 84.83 °C and another peak at 67.16 °C. The physical mixture showed peaks at 283.73 °C indicating little interaction. The complex showed a peak at 75.17 °C. Moreover, the onset temperature was 63.55 °C only as shown in fig. 6.

FT-IR spectroscopy

The FT-IR spectrum of umbelliferone showed a sharp peak of OH group at 3182.55 cm⁻¹. It showed C=O stretch at 1726.29 cm⁻¹, aromatic C–H stretch from 3061.03–3084.18 cm⁻¹ and aromatic C=C stretch. The spectrum of phosphatidylcholine showed characteristic C–H stretching band of long fatty acid chain at 2918.3 and 2854.96 cm⁻¹, carbonyl stretching band at 1728.22 cm⁻¹ in the fatty acid ester, P=O stretching band at 1236.37 cm⁻¹, P–O–C stretching band at 1093.65 cm⁻¹, and N(CH₃)₃ stretching at 966.34 cm⁻¹. The spectrum of the complex showed significant changes, and the absorption peak of hydroxyl stretching of umbelliferone showed remarkable broadening from 3223.05–3331.07 cm⁻¹ as depicted in fig. 7.
Fig. 7: FT-IR spectra of umbelliferone (a), phosphatidylcholine (b), physical mixture (c) and complex (d)

**Scanning electron microscopy**

Fig. 8 depicts the micrograph showing the surface morphology of the complex and drug.

**X-ray diffraction study**

X-ray diffractogram of umbelliferone showed intense sharp diffraction peaks of crystallinity at a diffraction angle (2θ) of 15.834. The phospholipid showed a single intense peak at 2θ value of 21.242. The physical mixture showed a less intense and broader peak from 2θ values 13.901-32.635. X-ray diffraction pattern of the complex showed peaks from 2θ value 15.681 to 27.618 as shown in fig. 9.

**Nuclear magnetic resonance**

$^1$H–NMR- fig. 10 shows the H–NMR spectrum of umbelliferone with a value of $\delta$ 2.5328 indicating (d, 1H, H-3 equatorial) hydrogen at position 3. The value of $\delta$ 2.52 was for (dd, 1H, H-4 equatorial) hydrogen at position 4. The value of $\delta$ 10.45 was distinct for (s, 1H, 7-OH) hydroxyl group of umbelliferone. The H–NMR spectrum of complex showed $\delta$ 0.863.

$^{13}$C–NMR- fig. 10 shows the $^{13}$C-NMR spectrum of umbelliferone with the signal at $\delta$ 155.57 ppm for carbon with a hydroxyl group. It showed a signal at $\delta$ value 138.59 ppm for carbon with a carbonyl group. It also showed signals for aromatic rings from $\delta$ 107.32 to 155.57 ppm. The spectrum of the complex showed signals on the higher side indicating a chemical shift from $\delta$ 155.57 to 160.39 ppm.

Fig. 8: Scanning electron microscopy images of umbelliferone (a) and complex (b)

Fig. 9: X ray diffractograms of umbelliferone (a), phosphatidylcholine (b), physical mixture (c) and complex (d)
**Ex vivo and in vitro permeation**

The results for percent cumulative release of drug and phytosomal complex through membrane and skin are given in table 5 and comparison was depicted in fig. 11.

**Evaluation of photoprotective potential of the complex against UV exposure and estimation of antioxidant enzymes**

For each group, the mean±SEM values were calculated. The data was analysed using Graph pad software version 5 by one-way ANOVA and the groups were compared using Dunnet test. The amount of enzymes found were summarised in the table 6 and compared for all groups in the fig. 12.

**Stability study**

Table 7 shows the results obtained for the stability study.

**DISCUSSION**

Development of valuable drug delivery system from natural sources is very much necessary because of the beneficial role of herbal drugs in the management of various diseases [40]. Blue fluorescence is the characteristic property of coumarin and its derivatives. The coumarin ring allows the material to fluoresce under ultraviolet radiation making it a property for specific identification and as umbelliferone showed blue fluorescence it indicated its specific nature [10].

### Table 5: Ex vivo and in vitro permeation study

| Time (minutes) | Ex vivo %CR of umbelliferone | Ex vivo %CR of complex | In vitro %CR of umbelliferone | In vitro %CR of complex |
|----------------|----------------------------|------------------------|-------------------------------|-------------------------|
| 60             | 9.627±1.62                 | 5.860±1.87             | 21.762±1.23                   | 16.122±1.89             |
| 120            | 14.152±1.30                | 11.573±1.54            | 24.531±1.54                   | 21.924±1.65             |
| 180            | 20.906±2.11                | 17.607±1.43            | 28.278±1.65                   | 30.911±1.44             |
| 240            | 28.098±1.65                | 19.623±1.66            | 41.583±1.87                   | 36.371±1.76             |
| 300            | 33.620±1.69                | 24.7137±2.34           | 52.504±1.49                   | 43.503±1.54             |
| 360            | 39.496±2.23                | 30.149±1.98            | 56.935±2.06                   | 46.820±2.98             |
| 420            | 46.270±1.54                | 36.861±2.04            | 61.904±1.68                   | 49.092±1.02             |
| 480            | 61.017±1.84                | 42.7791±1.66           | 69.744±1.71                   | 56.918±1.51             |
| 540            | 72.0101±1.33               | 46.9396±1.84           | 77.9617±1.44                  | 63.770±1.69             |
| 600            | 100.071±1.76               | 54.2533±1.67           | 99.2683±2.11                  | 69.302±1.84             |
| 660            | –                          | 58.1376±1.49           | –                             | 76.963±2.14             |
| 720            | –                          | 67.724±1.98            | –                             | 89.266±1.23             |
| 1440           | –                          | 96.950±2.32            | –                             | 100.787±1.36            |

Data is presented as mean value±SD (n=3)

**Fig. 11: Ex vivo (a.) and in vitro (b.) permeation study of umbelliferone and complex**
The melting point by Thiele tube method complied with the standard value. The regression equation for the calibration curves in all the solvents followed Lambert-Beer's law and the correlation coefficient was found to be near 1 depicting linear relationship. The linearity parameter and the corresponding regression data indicated good linear relationship for quantification method. The validation parameters confirmed the adequate sample stability, accuracy and method reliability and the observed % RSD was<2% as per ICH guidelines [10, 15].

Phospholipids play a major role in drug delivery and have numerous advantages in addition to solubilizing property while considering them for a carrier system [40]. The umbelliferone-phospholipid complex was prepared using solvent evaporation method by applying the Box-Behnken design. The quadratic equation for each response was obtained and only statistically significant coefficients (p=0.0405 for Y1 and p=0.0483 for Y2) were included and a positive coefficient indicated that the response is favored, while a negative value indicated an inverse relationship between the factor and response. It was clear from the equations that umbelliferone: phospholipon ratio (X1), temperature (X2) and time (X3) had a positive relationship and phospholipon: umbelliferone ratio (X1) had an inverse relationship on partition coefficient (Y2) [16].

On the basis of highest complexation rate i.e. 99.9572%, batch F4 was found to be the optimized batch, and the complexation rate depicted a good affinity of phospholipid towards the drug. The solubility was found to increase for the complex as compared to the drug in both the phases. This may be attributed to the fact that the complex formed was amphiphilic in nature possessing hydrophilic choline group which bound with the hydroxyl group of the drug while the lipid soluble phosphatidyl moiety enveloped the choline...
UV exposure, the unit/mg of reduced glutathione (GS H) diffusion and matrix erosion [29]. The high drug content depicted maximum entrapment in the complex which indicated a stable complex of umbelliferone with phospholipid [18]. The % DPPH radical scavenging activity and the % Ferrozin antioxidant activity of the complex was better than that of the drug because the complex had a better ability for scavenging oxidative free radicals than the drug owing to its amphiphilic and stable nature [19-23]. HPTLC study depicted distinct Rs value for the complex and the drug, which indicated the successful formation of the complex [25]. The particle size range between 200–2600 nm indicated uniform particle size for the complex and the zeta potential of −0.05 mV indicated the formation of a stable complex [17].

The exothermic peak of umbelliferone indicated a phase transition. The thermogram of the physical mixture showed a little interaction. On the basis of the highest coefficient of correlation, the best fit was loaded gel was performed using Zero order, First order, Higuchi fitting of permeation profile of drug from the UMB gel and complex.

SEM showed that the crystalline drug was completely converted into the amorphous form on complexing owing to the change in visual sharpness to blunt structure. This can be attributed to the solubility data which showed higher solubility for the complex than drug [29].

The X-ray diffraction study indicated that the sharp peak of umbelliferone owed to its crystalline nature. The physical mixture showed less intense and broad peaks which indicated transformation into an amorphous form. Complete conversion into amorphous form was indicated by the large diffused peaks of complex [29].

H-NMR results indicated that the phenolic protons were found to shift (downfield) in the complex indicating the interaction of phospholipid with the phenolic–OH group which depicted the successful formation of the complex. Similarly 1H-NMR study indicated that the spectrum of the complex showed values of signals on the higher side depicting a chemical shift due to the interaction of hydroxyl group with the phosphatidylcholine hydroxy group causing a shift. The spectrum of complex showed very intense and fused signals indicating the formation of the complex [30, 42].

The in vitro and ex vivo skin permeation of complex from the gel was higher than the drug in ex vivo as well as in vitro permeation. Model fitting of permeation profile of drug from the UMB gel and complex loaded gel was performed using Zero order, First order, Higuchi model and Korsmeyer–Peppas model. On the basis of the highest coefficient of correlation, the best fit was shown by zero-order drug release for both the preparations. Using the Korsmeyer–Peppas model, the value of permeation coefficient (n) for the drug and complex was obtained to be 0.5<n<1 indicating a non-Fickian pattern of drug release i.e. a combination of drug diffusion and matrix erosion [29].

In the evaluation of photo protective potential of the complex against UV exposure, the unit/mg of reduced glutathione (GS H), superoxide dismutase (SOD) and catalase (CAT) was the highest in control group and the least in UV-irradiated group. The lipid peroxidation (LPO) was highest in UV-irradiated group and least in the group treated with complex gel. When the enzyme activity was compared for UMB gel and complex gel it was found that the enzymes were higher in the group treated with complex gel than those with UMB gel with immediate as well as with those with UV irradiation after 4 h. This may be attributed to the fact that the complex showed a better permeation than the drug. The comparison using Dunnet test was statistically significant. This indicated that the complex gel was better able to protect the skin and antioxidant enzymes from UV radiations than the plain UMB gel [34-38].

Stability study indicated that the optimised batch did not show any significant changes during the study period of three months which further indicated the formation of the stable complex on storage [39].

CONCLUSION
Umbelliferone was successfully complexed with phosphatidylcholine to form phytosome, a novel drug delivery system. The pre-formulation studies confirmed identification of umbelliferone and the validation parameters confirmed stability and method reliability. The complex was successfully formulated by solvent evaporation method using Box-Behnken experimental design and batch was optimised. The optimised batch was evaluated for % practical yield, complexation rate, drug content and the results were within the range. The complex showed better solubility than the drug in both phases. The complex was found to show higher in vitro antioxidant activity than the drug at the same concentrations. The HPTLC, DSC, FT-IR, SEM, XRD and NMR study confirmed the successful formation of the complex. The ex vivo and in vitro permeation studies showed better release for phytosomal complex than the drug. The animal study was carried out for the photoprotective effect of complex and the effect was evaluated by estimating the antioxidant enzymes. The phytosomal complex was better able to protect the skin and the antioxidant enzymes than the drug. The stability study revealed that there were no significant changes in the formulation over the period of three months. Hence it can be concluded that umbelliferone in novel drug delivery system i.e. phytosomal form, produces a better therapeutic effect than the drug alone.

CONFLICT OF INTERESTS
Declared none

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