Understanding the Role of Response Surface Methodology in Development of Quercetin Loaded Phytocomplex for Wound Healing

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Abstract

Aim: It has been proved that optimized quercetin-loaded phytocomplex (QLP) system using response surface methodology occupied the desired characteristic for wound healing. Materials and Methods: The phytocomplex was prepared using complexation method. Quercetin and phosphatidylcholine were mixed by adding 20 mL of dichloromethane. The whole solution was refluxed for 2 h by maintaining the temperature below 60°C. The resulting solution was evaporated to 2-3 mL, and 10 mL of n-hexane was mixed with continuous stirring to form complex. Results and Discussion: According to entrapment efficiency (EE), drug release (DR), and percentage yield (PY) of phytocomplex concentration of quercetin (100 mg) and phospholipids (100 mg) were in optimum quantity. Physical characteristic were evaluated through in vitro characterization of phytocomplex including the EE, DR, and PY the resulting values of 82.10%, 61.96%, and 76.36% were found to be standard characterized values respectively. Conclusion: Optimized QLP will provide us an innovative platform as a novel addition to achieve high therapeutic efficacy on wound space.

Key words: Box-Behnken design, optimization, phytocomplex, quercetin

INTRODUCTION

Wound may be defined as a disturbance in the cellular and anatomic continuity of tissue. Wound can be classified accordingly various insult to the skin and its tissue including physical, chemical, thermal, microbial, or immunological insult. The process of wound healing covers the various cellular and biochemical mechanism leading to re-establishment of structural and functional integrity in injured tissues.[1] Wound-healing agents derived from plants need to be identified and formulated for the effective management of wounds. Various herbal products have been used in management and treatment of wounds over the years. Bioflavonoid and its glycosides playing an important role in wound healing without any side effect, i.e., Rutin, quercetin, etc. Quercetin phytoconstituents is bioflavonoid polyphenolic compound having potential of anti-inflammatory and antioxidant activity.[2] Quercetin has been extensively used as a therapeutic agent, and it has been incorporated in some novel additions for the treatment of different disease, i.e., skin disease, cardiovascular disease, and carcinoma.

In spite of better characteristic quercetin containing certain limitation of low lipophilicity of drugs, which leads to low bioavailability and therefore high concentration are required in designing of novel formulation.[3] Effective wound healing can be achieved by designing various factor dependent optimized formulations. These formulations possess the potential of therapeutic agent, textual properties and integrity of the formulation, drug pharmacokinetic profile, and drug retention efficacy at the site of application.[4] For better and effective delivery of formulations, it needs to be optimized using experimental design technique. Recently, lipoidal formulations are being extensively explored for the topical drug delivery due to its distinctive phospholipid complex. Phytocomplex containing phospholipids composition in its complex structure which resemble similar to the lipid cell membrane of the human body. In addition, lipophilic and

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hydrophilic drug can be loaded in lipoidal formulation which shows biocompatibility with the site of action. In the present research, we focused the enhancement of poorly aqueous solubility and bioavailability of drug so that high therapeutic efficacy can be achieved.[5] The present investigation aimed to develop, optimize, and characterize the phytocomplex formulation for the treatment of wound healing.

**EXPERIMENTAL**

**Materials**

Quercetin, phosphatidylcholine, and n-hexane were purchased from Hi-Media chemicals (Mumbai, India). HPLC grade solvents were purchased from Merck (Mumbai, India). All other materials and solvents used were of analytical grade.

**Preparation of QLP**

The phytocomplex was ready with quercetin and phosphatidylcholine at a molar ratio of 1:1 on the basis of our earlier reported method. The active constituents and phosphatidylcholine were mixed in flask by adding 20 ml of dichloromethane. The solution was refluxed for 2 h at temperature below 60°C. The resulting solution was evaporated to 2-3 ml, and 10 ml of n-hexane was mixed to it with continuous stirring. The quercetin-loaded phospholipids complex was precipitated and it was collected by filtering it.[6]

**Optimization of formulation parameters for phytocomplex**

As a preliminary study, the formulation technique was optimized by studying various formulating parameters such as phytoconstituents concentration ($X_1$), phospholipids ($X_2$), and rotation time ($X_3$) in Table 1. The optimum concentration of drug and lipid was found to be 75 and 150 mg and rotation time was 40 min. The complex obtained after rotary evaporation was kept overnight under vacuum to dry and remove. Further, it was observed that phytocomplex prepared. The above two observations were further corroborated by the values for entrapment efficiency (EE) of the formulation. Optimized formulation parameters were then used to formulate further batches. Suitable batches were then prepared to study intricately the interactions of quercetin with the lipids and its effects on the EE, drug release (DR), and percentage yield (PY) of the final formulation using experimental designing techniques.

**Experimental design**

A Box-Behnken design was applied using three independent variables to optimize the conditions and to analyze the sensitivity of responses to the changes made in the settings of experimental design. Therefore, a Box-Behnken design comprising of three factors was selected. A mathematical and statistical technique which enumerates the functional relationship between the variables which are measurable and many illustrative factors, to acquire an optimal response by using a sequence of tests is known as response surface methodology (RSM). A three-level factorial-RSM was used to examine the effect of different dependent variables on the characterization of the formulation, such as %PY, %DR, and %EE of the prepared phytocomplex and independent variables including quercetin concentration ($X_1$) and phospholipids concentration ($X_2$). Statistical analysis showed that $P < 0.05$ which was considered the significant best-fitted model. The predicted $R^2$ value and analysis of variance (ANOVA) were pursued to confirm the best-fit of the model. The correlation between independent variables and dependent variables were studied by three-dimensional (3D) response surface curves.[7]

To assess the reliability of the model, a comparison between the experimental and predicted values of the responses is also presented in terms of %bias.

The value of bias was determined by the following equation:

$$\% \text{Bias} = \frac{\text{Predicted value} - \text{Experimental value}}{\text{Predicted value}} \times 100$$

(1)

**Infrared (IR) spectroscopic analysis of phytocomplex**

Fourier transformed IR (FTIR) spectra were acquired using FTIR spectrometer (8400 Shimadzu FTIR) through KBr pelletizing techniques. Briefly, 500 mg of potassium bromide and 5 mg of phytocomplex sample, quercetin and phospholipids were taken into a mortar, mixed and grinded to a fine powder. The homogeneous disc was arranged by compacting the powder in palletizer and inserted into IR sample holder carefully.[8]

**Differential scanning calorimetry (DSC) analysis**

DSC employed to identify the interactions between quercetin and ingredients. The possibility of any type of interface between the phospholipids and QLP, during preparation, was

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**Table 1: Coded value and actual values for $X_1$, $X_2$, and $X_3$ for QLP**

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Low (−1)</th>
<th>Medium (0)</th>
<th>High (+1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoconstituents concentration ($X_1$)</td>
<td>50 mg (1%)</td>
<td>75 mg (1.5%)</td>
<td>100 mg (2%)</td>
</tr>
<tr>
<td>Phospholipids concentration ($X_2$)</td>
<td>100 mg</td>
<td>150 mg</td>
<td>200 mg</td>
</tr>
<tr>
<td>Rotation time (min) ($X_3$)</td>
<td>20</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

QLP: Quercetin-loaded phytocomplex
assessed by DSC. The 5 mg phytocomplex and quercetin were retained in pan separately. The pan was heated up to 305°C, and heating rate was 5°C/min. The nitrogen gas passed at a flow rate of 20 ml/min. The thermogram curve was obtained for phytocomplex formulation.[9]

X-ray diffraction (XRD) analysis

XRD was used to identify the crystalline or amorphous nature of QLP. X-ray powder diffraction studies of pure quercetin, physical mixture phospholipids, and lyophilized quercetin-loaded-phytocomplex were carried out using powder X-ray diffractometry. Samples were placed in sample stage and scanned from 2θ to 60θ with an operating voltage of 40 kV and current 30 mA.[10]

Transmissions electron microscopy (TEM) study

The negative stain electron microscope was used for morphological study of the vesicles of the optimized batch. The size and surface morphology phytocomplex were evaluated by TEM. Phytocomplex aqueous dispersion was employed on copper grids. Before placing in grids, it was treated with 1% aqueous phosphor tungstic acid and dehydrated at room temperature. After drying the specimen, it was assess under microscopy at 10-100 fold enlargement. The magnification for the TEM images was ×150,000.[11]

Determination of PY of phytocomplex

Free and quercetin encumbered phytocomplex were alienated by ultracentrifuge which was performed for 4 h the supernatant was collected and then analyzed the amount of free drug by HPLC. The total amount of quercetin in phytocomplex was determined by dissolving the QLP in phosphate-buffered saline (pH 7.4) and calculated of PY.[12]

Determination of quercetin content in the phytocomplex

The quercetin present in the phytocomplex was evaluated by HPLC method. The 5 mg of the complex was dissolved in 2 ml of methanol, and the volume was makeup up to 10 ml with methanol. The volume of sample injected was 20 µl to column. The acetonitrile and methanol (50:50 v/v) solvent was used as mobile phase for separation of the component. The flow rate of mobile in column was maintained at 1.0 ml/min at 30°C. The retention time was measured by PDA detector at wavelength of 256 nm.[13]

In vitro DR from phytocomplex

The release of quercetin from optimized phytocomplex was determined by diffusion cell apparatus (EMFD-08 Orchid Scientific and Innovative India Pvt. Ltd. Nasik, Maharashtra, India) using dialysis membrane (molecular weight cut-off 10,000 Da). Dialysis membrane was treated with double distilled water for 24 h earlier utilizing in diffusion cell apparatus. The formulation was placed in the donor compartment and temperature was maintained at 35 ± 0.5°C. The pH 7.4 phosphate buffer was filled in the receptor compartment. The 2 ml samples were withdrawn from cell at an interval of 0, 1, 2, 3, 4, 5, 6, 12, 16, and 24 h. The concentration of quercetin present in the sample was evaluated by HPLC at a wavelength of 256 nm.[14]

In vivo wound healing study

Wound creation Healthy, adult male Wistar rats (170-200 g) were utilized for the in vivo wound studies. The wounds were neither dressed nor covered. After anesthesia recovery, the animals were housed individually and closely monitored. The animals were grouped into three groups. The animals of Group I were not treated with any formulation and it are known as control group. The animals of Group II were treated with marketed formulation (Mega Heal hydrogel). The animals of Group III were treated with QLP formulation. All animal experiments were approved by Institutional Animal Ethical Committee, (Ref No.1413/PO/a/11/PCSEA) of Sapience Bioanalytical Research Lab, Bhopal (Madhya Pradesh). Percentage wound contraction was measured using the formula.

\[
\text{Percentage of wound contraction} = \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \times 100
\]

Collection of tissue and histopathological analysis

On days 4, 8, 12, and 16, three animals from each group were sacrificed with anesthesia overdose to collect the granulation/healing tissue. It was preserved in 10% formalin for histopathological evaluation. Hematoxylin and eosin (H and E) staining were used to determine the gross morphological changes at the wound site. The granulation/ healing tissues fixed in 10% formalin were embedded in paraffin. Rotary microtome (Leica Biosystems, India) was used to obtain 5 m thick tissue sections and stained with H and E, as per the standard method and visualized under a light microscope (Olympus CX31, Japan) at ×40 magnification.

Statistical analysis

The numerical data were expressed as mean ± standard deviation (SD). Two-way ANOVA followed by Tukey’s post hoc test was performed to obtain the statistical significance. GraphPad Prism version 9.01 (San Diego, CA, USA) was used to generate the statistical significance and the values having \( P \leq 0.05 \) was considered statistically significant.
Storage stability studies

The storage stability studies were carried out with the optimized QLP. A 10 mL of QLP with 2 mg/ml drug concentration was taken into glass vials and stored at 4°C and 25°C for 3 months. The stability test was analyzed on the basis of PY and %EE determination in the dispersion with a sampling frequency of 1 month.[15]

RESULTS AND DISCUSSION

Preparation and optimization of QLP

Each phytocomplex were formulated according to the experimental design comprising 17 trials of phytocomplex (QP1-QP17) and displayed in Table 2. Preparation of QLP nine batches of phytocomplex was prepared by complexation method. This method helps in determining the concentration of quercetin in each formulation and also phospholipids concentration in the laboratory production of phytocomplex.

Analysis of optimization data for the QLP

The observed responses of all formulations were applied to various models using Design-Expert software trial version 9.0.1. It was observed that the quadratic models were the best fit for the reactions studied, that is PY, %EE, and %DR. The following quadratic equations were formed:

\[ %PY = +77.80 + 3.06X_1 + 1.57X_2 + 2.00X_3 - 3.76X_1X_2 + 1.11X_1X_3 - 3.60X_2X_3 - 11.23X_1 - 1.35X_2 - 12.71X_3 \]

\[ %DR = +89.20 - 0.62X_1 + 0.31X_2 - 1.93X_3 + 4.02X_1X_2 + 3.23X_1X_3 + 3.63X_2X_3 - 9.55X_1 - 13.54X_2 - 3.33X_3 \]

\[ %EE = +75.11 + 4.97X_1 - 1.42X_2 + 1.82X_3 + 3.71X_1X_2 + 0.45X_1X_3 - 0.17X_2X_3 - 7.99X_1 - 3.88X_2 - 8.92X_3 \]

where \( X_1, X_2, \) and \( X_3 \) represent the coded values of the lipid concentration and drug concentration of the phytocomplex and rotation time of rotary evaporator, respectively. The affirmative value of a factor of equations points out the enhancement of that response and vice versa. All values of the correlation coefficient \( (R^2) \), SD, percentage coefficient of variation and results of ANOVA are exhibited in Table 3. A value of \( R^2 \) and results of ANOVA for the dependent variables confirmed that the model was significant for the response variables observed.[16]

The experimental results of QLP exhibited a wide variation in yield of phytocomplex from 45.64% to 82.10%, and in the %DR in 24 h the phytocomplex ranging from 55.96% to 88.31%. Further the %EE of the formulations was 52.14% to 76.36%. The outcomes of the quadratic response surface, the phytocomplex were formulated utilizing the optimal formulation variable taking, i.e. Quercetin and lipid concentrations were to 75 and 150 mg and rotation time 40 min, respectively. Table 4 exhibits that the value of predicted formulation was nearer to the value of observed

### Table 2: Independent variables along with their coded level, actual level, and respective response values of different batches of QLP

<table>
<thead>
<tr>
<th>Standard</th>
<th>Run</th>
<th>( X_1 )</th>
<th>( X_2 )</th>
<th>( X_3 )</th>
<th>( X'_1 )</th>
<th>( X'_2 )</th>
<th>( X'_3 )</th>
<th>PY%</th>
<th>DR%</th>
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<td>1</td>
<td>100</td>
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<td>73.38</td>
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<td>1</td>
<td>0</td>
<td>100</td>
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<td>66.69</td>
<td>71.85</td>
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<tr>
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<td>4</td>
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<td>-1</td>
<td>0</td>
<td>100</td>
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<td>0</td>
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<td>0</td>
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<td>82.10</td>
<td>61.96</td>
<td>76.36</td>
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<td>60</td>
<td>55.21</td>
<td>62.51</td>
<td>58.69</td>
</tr>
</tbody>
</table>

EE: Entrapment efficiency, DR: Drug release, PY: Percentage yield, QLP: Quercetin‑loaded phytocomplex
formulation. Thus, it confirming that the optimized formulations were suitable and also produce the desired outcomes.[17] Further, the relationship between the dependent and independent variables was determined by making the response surface plot. The Design-Expert software (trial version 9.0.1) was used to determine the 3D response surface graphs for the most statistically significant variables on the estimated parameters are displayed in Figure 1a-c.

IR spectroscopic analysis

The outcomes of FTIR exhibited that the no significant peak changes on pure quercetin and phospholipids but in the phytocomplex formulation quercetin peak position were slightly changed which indicated phytocomplex was prepared. The peak observed at 3411 and 1663/cm represent the presence OH of the phenolic groups and C=O group in heterocyclic ring, respectively. The phospholipids indicated characteristic bands at 3619/cm and 2994/cm. The QLP of FTIR spectra exhibited to shift in phenolic –OH to 3480/cm [Figure 2a-c].

DSC

QLP can be examined by DSC, shows the DSC thermograms of quercetin phospholipids complex and physical mixture of quercetin and phospholipids. The DSC thermogram of quercetin displayed a single peak with an onset of 119.24°C and maximum occurrence at 145.33°C. The QLP thermogram exhibited shifting of the melting peak of quercetin at 114.5°C and 178.62°C. From the findings of thermogram it has been concluded that faintly alterations between physical mixture and individual component peaks and displayed in Figure 3a-d. These outcomes expressed that drug were in amorphization and/or inclusion complex formation.[18]

X-ray powder diffraction

XRD was most reliable method used to assess the phytocomplex formation. The sharp diffraction peak was observed for quercetin at 12.5, 28.0, and 29.1, phospholipids displayed characteristic peak at 20 value of 11.3, 19.5, and 23.5. The crystalline peak of mixture for quercetin was clearly evident Figure 4a-d. Whereas the phytocomplex formulation showed deformed peak for quercetin, indicating the relative reduction in the diffraction intensities in the phytocomplex.[19] The appearance of sharp peak is strongly characteristic of the pure quercetin used in the experiments. As for crystallinity, each samples are amorphous, an indication they are probably easy to be complexation.

TEM

TEM was employed to evaluate the morphology and size of QLP. The outcomes of the TEM photomicrograph are displayed in Figure 5. The finding suggests that the phytocomplex appear pear-like and small. The nonspherical
shape with a particle size of about 1-2 μm for optimized QLP was observed in the formulation, which is almost the formulation consists of a mixed population of unilamellar and small multilamellar vesicles.\[20\]

%PY in the complex

The values for %PY of the QLP are displayed in Table 2. From outcomes, it has been observed that the linear effect of
phosphatidylcholine and quercetin concentration was significant (0.0065). The effect of independent variables on QLP is that at higher quercetin concentration, %PY was increased, due to which more quercetin was complex. Besides, increased ratio of quercetin and phosphatidylcholine increased the %PY. The values for %PY of each formulation were observed, and seen to be in the range of 45.64-82.10%, respectively. The quercetin plays a significant role in the determining the %PY, because the quercetin is complexed in the lipid phase therefore the %PY was found to be prominently higher.[21]

%EE

The values for %EE of the QLP are displayed in Table 2. From outcomes it has been observed that the linear effect of phosphatidylcholine and quercetin concentration was significant (0.0065). The effect of independent variables on QLP is that at higher quercetin concentration, EE was increased, due to which more quercetin was complex. Besides, increased ratio of quercetin and phosphatidylcholine increased the EE. The values for %EE of each formulation were obtained, and found to be in the range of 52.14-76.36%, respectively.

In vitro release studies

The findings of in vitro release curve of QLP are exhibited in Figure 6. The cumulative %DR of the optimized QLP dispersion was 61.96%, in 24 h. The outcomes exhibits that the formulation represents burst release of drug from phytocomplex during first 2 h. However, after 2 h release of drug from phytocomplex was retard and it represent the sustained release pattern. The findings of in vitro release suggested the burst release of drug was due to availability of the free quercetin in the external phase and on the surface of the phytocomplex. The sustained release of the drug was due to quercetin and could be the reason for the sustained release of the drug from the internal lipid phase after the initial burst release. The kinetic model was used to determine the mechanism of quercetin release from phytocomplex formulation. Consequently, linear regression was also analyzed by least square method to estimate the release rate.

In vivo animal study (excisional wound model)

In vivo performances of the prepared formulations QLP were evaluated on excision wound model on Wistar rat. Wound contraction, epithelization time, hydroxyproline estimation, and histopathological study were selected as parameters to evaluate the prepared delivery systems.[22]

Wound contraction

Wound contraction was analyzed in each group as a percentage of the reduction in wounded area at days 4, 8, 12 and 16 [Table 5]. In this study, the QLP treated animals completely healed the wound of animals on 12 days. The marketed formulation-treated animals showed complete wound contraction on 14 days. QLP showed complete contraction on the day of 12th similar to control group with smooth skin and negligible scar formation.[23] Among the treated groups, animals treated with formulation QLP showed more significant ($P < 0.001$) wound contraction than animals having treatment with other formulation. The mean percentage of wound contraction of the QLP treated group was significantly (96.10% at day 12, $P < 0.001$) higher compared with those of the control (73.83% at day 12, $P < 0.01$) and marketed formulation-treated (92.97% at day 12, $P < 0.01$) groups after wound creation.

Histopathological observations

Histological studies play significant role in investigating the mechanism of the wound healing. As result revealed that Figure 7 exhibited that healing of wound was due to beginning of remodeling of skin, treated with QLP after 12 days. Figure 7 indicates the normal histological topographies of skin, mice treated with marketed formulation after 16 days. Moreover, the collagen was noticeably seen in control groups. Histopathological studies conceived that accumulation of keratinocytes in the basal lamina of
epidermis. The whole in vivo outcomes suggested that the animals treated with QLP exhibited higher wound healing activity compared to control and marketed formulation treated group of animals. The prepared phytocomplex enhanced the wound healing activity in animals due to its occlusive property.

Present results showed an accelerated re-epithelialization applying QLP with good cosmetic effect. Phytocomplex also showed a high percentage of cumulative DR in a sustained manner. This sustained manner of DR through hydrogel supported to long-lasting antibacterial, debriding action and maintaining a natural moist environment to achieve the effective therapeutic effect at the wound site.

**Storage stability studies**

Storage stability of prepared QLP the stability studies were carried out according to ICH guidelines for residual drug content of QLP1, QLP3, QLP6, and QLP9 phytocomplex system exhibiting more than 70% entrapment. Results of stability studies showed that all phytocomplex lost around 1-2% of drug content in the 1st month under room temperature and around 5% in 3 months. This loss was also marginal in case of accelerated conditions where system lost more than 2% drug in 1 month and around 3-4% in 3 months. Thus, prepared systems were found to be stable under both normal and accelerated conditions.

**CONCLUSION**

The effects of the concentration of phosphatidylcholine and quercetin on preparing QLP were studied and optimized using the miscellaneous design-RSM by fitting a second order model to the response data. Second-order polynomial models were obtained for predicting PY, DR and encapsulation efficiency. While increasing the quercetin concentration decreased the PY and encapsulation efficiency. The effect of three variables, including drug concentration ($X_1$), phospholipids concentration ($X_2$) rotation time ($X_3$), were selected to see the responses on selected three dependent variables. The best result involved the maximum EE (76.39%) and PYs (82.10%) were found at concentration drug 75 mg and lipid 150 mg.

The DR study of the prepared phytocomplex were performed using phosphate buffer 7.4 pH media, and it showed prolonged release in 24 h, up to 61.96% release. The DR behavior from the phytocomplex exhibited a biphasic pattern showing burst release at the initial stage and sustained release subsequently. These results indicated that the optimized phytocomplex could potentially be exploited as a carrier with an initial dose and prolonged release for accelerated wound healing. The quercetin-loaded-phytocomplex showed an acceptable stability. Thus, the present work giving the concluding mark that optimized QLP will provide us innovative platform as novel addition to achieve high therapeutic efficacy on wound space.
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