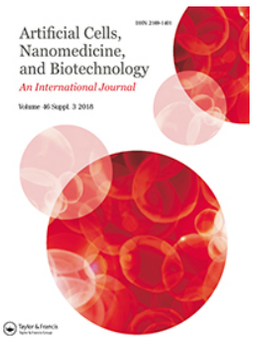

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Design, optimization, characterization and in-vivo evaluation of Quercetin enveloped Soluplus®/P407 micelles in diabetes treatment

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ABSTRACT

Quercetin (Qu), is a flavonoid known to have anti-diabetic effects owing to its antioxidant property, thus promoting regeneration of the pancreatic islets, ultimately increasing insulin secretion. But the therapeutic application of Qu is hampered by its low oral bioavailability and its unfavourable physicochemical characteristics. The present work aimed at formulation of Quercetin loaded Soluplus® micelles (SMs) so as to enhance its bioavailability and provide prolonged release for the management of diabetes. Box-Behnken response surface methodology was employed to optimize the formulation prepared using co-solvent evaporation method. Physicochemical characterization confirmed the nano-spherical nature of Quercetin loaded Soluplus® micelles (Qu-SMs) with average particle size ranging from 85-108nm, encapsulation efficiency of 63-77%. Solid state characterization confirmed the encapsulation of Qu in the micelles without any incompatibilities. Moving forward, the results of *in vitro* study revealed prolonged and slow release of Qu from the developed formulations. The *in vivo* pharmacokinetic study revealed improved bioavailability by enveloping the drug in SMs. Moreover, the study performed to evaluate the efficiency in diabetes treatment revealed an enhanced anti-diabetic effect. Thus, Qu-SMs can serve as potential carriers aimed at improving the anti-diabetic property of Qu.

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KEYWORDS
Quercetin; Diabetes; Soluplus®; Micelles; Box-Behnken Design

Introduction

The term diabetes mellitus can be delineated as a metabolic disorder indicated by hyperglycaemia in a chronic manner resulting either due to defective insulin action, insulin secretion or both of them. These defects, as suggested by literature are generally the accompanied effects of insulin resistance (IR) which serves as the major factor contributing to the pathogenesis of diabetes mellitus. IR is characterized by increased generation of reactive oxygen species(ROS), elevated levels of pro-inflammatory responses and glucolipotoxicity [1]. Subsequently, IR exerts oxidative stress on the β-islets cells of pancreas and the peripheral tissues, thus resulting in impaired insulin secretion or insulin action [2]. Moreover, elevated ROS and oxidative stress imparts damaging effects on various organs responsible for regulating the normoglycemic condition in the body. Hence, diabetes mellitus is accompanied with excessive oxidative stress on one hand and reduced levels of naturally occurring anti-oxidants on the other. Thus, the treatment approach which includes the administration of an anti-oxidant drug to overcome the oxidative stress upon various vital organs could be successful, as reported in previous studies where resveratrol, a phenolic antioxidant has exhibited significant therapeutic potential against diabetes mellitus [3, 4].

Quercetin (Qu), chemically known as flavan-3, 4-diol (3, 5, 7, 3′, 4′-pentahydroxyflavone) is flavonoid and has several pharmacological properties, such as anti-inflammatory, antiviral, antibacterial, and anticancer activities. Apart from this, Quercetin exhibits potential anti-oxidant activity by the means of several mechanisms, such as scavenging of reactive oxygen species, metal ions chelation etc.[11]. The anti-diabetic effect of Qu can be attributed to the reduction of oxidative damage to the pancreas followed by an increase in the β-islets cells, causing an increase in insulin secretion, thus reducing glycaemia. Another mechanism through which Qu exhibits its action is that Qu increases the levels of glucokinase in the liver thus leading to increased glucose storage [5, 6].

However, the clinical applications of Qu, which is a BCS class II drug, finds limitations due to its characteristics like high hydrophobicity, poor solubility, first pass metabolism, limited absorption through the gastrointestinal tract and low bioavailability[7]. Various approaches have previously been reported to solve this problem by formulating solid lipid nanoparticles[8], self-nanoemulsifying systems[9], polymeric nanoparticles[10], liposomes[11], nanocrystals[12], and cationic nanocarriers[7]. Recently, the use of nanomicelles as the carrier of hydrophobic drug has gained attention. Soluplus® is one such tri-block copolymer consisting of polyvinyl caprolactam (PCL)–polyvinyl acetate (PVAc)–polyethylene glycol (PEG) units with the ability to augment the solubility of hydrophobic drugs. Soluplus® has the CMC of 7.6 mg/L

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which is very low, hence considered suitable for *in vivo* applications. Moreover, Soluplus® has been found to have excellent solubilizing efficiency for the BCS class II drugs[13], by incorporating them in the hydrophobic core of the core-shell structure of micellar system [14]and inherent property to provide sustained release. Thus, in this study Soluplus® was used to formulate a micellar system which would enhance the bioavailability of Qu. Poloxamer 407 was used as the surfactant with an aim to increase the stability of the nanomicelles and enhanced solubilisation of the hydrophobic drug.

Hence, with an aim to increase the stability and bioavailability of Qu so that it can be administered via oral route, quercetin loaded Soluplus® micelles were prepared by co-solvent evaporation method, using P407 as surfactant. The formulation process was optimized using Box-Behnken Design (BBD). In addition to this, the physicochemical characterization, in vitro release, haemolytic activity, pharmacokinetics and efficacy of system in diabetes regulation was evaluated.

Methods

Materials

Soluplus® and Poloxamer 407 were supplied as gift samples by BASF India Ltd (Navi Mumbai, Maharashtra, India). Quercetin (Qu) was procured from Cayman Chemical Company. (New Delhi, India). All other reagents were procured from Sigma Aldrich and used as received.

Preparation of Qu loaded Soluplus® micelles (SMs)

Qu encapsulated Soluplus® micelles (Qu-SMs) were formulated by using co-solvent evaporation method using Poloxamer 407 as surfactant. Initially, a defined amount of Soluplus® was dissolved in semi-polar solvent acetone, followed by addition of accurately weighed amount of Qu. Simultaneously, aqueous solution of Poloxamer 407 was prepared using deionized water. Finally, the polymer/drug solution was injected to the aqueous solution stirred at 800 rpm, thus allowing the self-assembly of drug and polymer to form Qu loaded Soluplus® micelles. Excess of water and the co-solvent acetone were evaporated using rotary evaporator under reduced pressure. The micellar formulation was then lyophilised to obtain the powdered formulation. Different micellar formulations were prepared according to the design.

Experimental design

The response surface methodology (RSM) was employed to perform Quality by Design approach for constructing and investigating the polynomial models, using fewer experimental runs. Box-Behnken Design comprising of 3-factors and 3-levels was employed to examine the quadratic response surfaces by assessing the effect of pre-defined independent variables on different response variables wherein hydrodynamic particle size (PS) was coded as Y_1, Solubilisation Efficiency (EE) as Y_3 and Polydispersity Index (PDI) as Y_2 for every batch of Qu-SMs. Three independent variables namely polymer amount (V_1), volume of the organic solvent (V_2) and surfactant concentration (V_3) were chosen. Each of the variables was varied at three different levels, known as high, medium and low levels. All the finalized independent variables and the response variables are described in Table 1 with the corresponding coded and actual levels. Design-Expert® software (7.0.0, Stat-Ease Inc., Minneapolis, USA) was used to generate the experimental design matrix with a total of 17 runs, among which 12 were factorial points and 5 were centre points. 3D response surface plots were used to determine the interrelationship among two distinct independent variables and a particular response variable. All the experiments were implemented in a randomized way so as to augment the predictability of the used model while avoiding any possible cause of experimental bias[15].

Table 1. Coded levels of independent variables and the dependent variables with the defined constraints according to Box-Behnken Design.

	Coded level of variables		
Independent Variables	Low −1	Medium 0	High 1
V_1 Polymer: Drug Ratio (w/w)	5	15	25
V_2 Organic/Aqueous Phase Ratio (v/v)	0.2	0.35	0.5
V_3 Surfactant Concentration (%)	0.50	1.00	1.50
Dependent variables (response)	Constraints		
Y_1 Particle Size (nm)	Minimize		
Y_2 PDI	Minimize		
Y_3 Solubilisation Efficiency (%)	Maximize		

Characterizations of Qu–SMs

Analysis of the mean particle size

The mean particle size (PS) and the polydispersity index (PDI) of the formulated Qu–SMs were analysed using the DelsaNano C particle size analyzer (Beckman Coulter, UK) at the temperature condition of 25°C (See S1).

Zeta Potential

The zeta potential of the prepared Qu–SMs were determined using DelsaNano C (Beckman Coulter, counter, USA). The instrument measures the electrophoretic mobility of the micelles under the impression of an external electric field.

Solubilisation Efficiency (EE)

The amount of quercetin solubilised in the formulated Qu–SMs was determined using UV spectrophotometry technique (Shimadzu UV 1800, Japan) at λmax of 371.5nm. The Qu-SMs solution was suitably diluted using ethanol in order to extract all the Qu from the micelles and then the Qu content was calculated from the standard plot developed using UV spectrophotometer[16].

Solid state characterization

The FTIR spectra of Qu, Soluplus®, physical mixture, Qu–SMs and lyophilised micelles were analyzed to find if any significant change occurs in Qu due to interaction with the formulation ingredients, before and after the encapsulation of Qu.

The XRD patterns of Qu, Physical Mixture, Qu–SMs were analysed to characterize the crystallographic structure of the drug and the formulation as well as determine any change in the physical state of Qu during the formulation procedure (See S2).

Shape and surface morphology

The shape and size of the prepared Qu–SMs was evaluated by using scanning electron microscopy (SEM). The diameter of Qu-SMs were measured at 10 random locations of the obtained SEM micrograph with the help of Image J software.

Atomic force microscopy (AFM) (NT-MDT, Moscow, Russia) was used to determine the surface morphology of optimized Qu–SMs. Nova Px 3.1.0 software was used for data analysis (See S3).

In vitro *release study of Qu-SMs*

Slightly modified dialysis membrane diffusion technique was employed to study the release characteristics of the Qu-SMs. The receptor medium consisted of Ethanol–Water in the ratio (35:65)[8]. The extent of Qu released was estimated with using UV spectrophotometry at λmax of 371.5 nm (See S4).

The release data was further fitted to different release kinetic models with the aim to determine the kinetics and mechanism of Qu release. Regression analysis was implemented to find the best fitting model using correlation coefficient (R^2).

In vitro *haemolysis study in human blood*

A simple technique generally used for determining material induced haemolysis was used for evaluating the haemolysis index of the pure drug suspension and Qu-SMs. Blood samples were collected from consenting human volunteers and placed into heparinized tubes. The blood samples were then centrifuged at 4500 rpm for 10 min so as to separate the erythrocytes from the plasma and washed with physiological saline (0.9% w/v NaCl) gently 3 times.

In each case 100 µl of various drug concentrations of pure drug suspension or Qu-SMs was mixed with 900 µl of red blood cell suspension. These were gently mixed and incubated at room temperature for 1 h. After this, the mixture was mixed with 4 mL of physiological saline (0.9% w/v NaCl) and centrifuged at 10000 rpm for 15 min for erythrocytes to precipitate.

The optical density of the separated supernatant was quantified at 540 nm by a Shimatzu mini 1240 UV-VIS spectrophotometer, and the % haemolysis was calculated by the equation presented below.

$$\text{Haemolysis}(\%) = \frac{(\text{IDtestsample} - \text{ODnegctrl})}{(\text{ODposctrl} - \text{ODnegctrl})} * 100 \quad \text{(Eq.1)}$$

The positive control (pos ctrl) is the OD value obtained after the incubation of 0.5% Triton-X100/blood and the negative control (neg ctrl) is the OD value obtained after physiological saline/blood incubation.[17]

Pharmacokinetic studies

Experimental Animals

Pathogen free male Wistar rats weighing between 170-230 g were procured from Central Animal House (IMS, BHU, India). All the rats were accommodated in propylene cages with surrounding temperature of 26 ± 1° C, 45-55% relative humidity. The rats were fed with standard diet and water ad libitum while maintaining 12 h light/12 h dark cycle. All the protocols were performed after prior approval by Central Animal Ethical Committee of Banaras Hindu University (Dean/2018/CAEC/626).

Pharmacokinetic evaluation

The optimized Qu-SMs and Qu pure drug suspension were evaluated for pharmacokinetic parameters. Two groups, each consisting of 6 overnight fasted rats were formed. Qu pure drug suspension and Qu-SMs equivalent to dose of 10 mg/kg of Qu were orally intubated to the rats of group I and II respectively. At predefined time intervals (0.166, 0.333, 0.5, 0.75 1, 2, 4, 8, 12, 24hr) after dosing, blood samples (0.3 ml) were collected from retro-orbital puncture in the eye into a heparinized tubes. Each blood sample was centrifuged at 4500 rpm for 10 minutes at 4° C and the plasma was separated. Qu concentration in the separated plasma samples was analyzed by slightly modified validated RP-HPLC method (See S5)[18].

The pharmacokinetic parameters for both formulations were analyzed by performing non-compartmental analysis with help of Kinetica 5.0 software (PK-PD analysis, Thermofischer). The results were statistically analyzed by student unpaired t test using GraphPad Prism 5.0 (GraphPad Software, USA)[15].

Evaluation of optimized Qu-SMs in diabetes regulation

The effectiveness of optimized Qu-SMs was assessed in diabetic rats. The rat model for diabetes was established by i/p administration of 55mg/kg dose of streptozotocin (STZ) (toxic towards β pancreatic cells) in male Albino rats. After 3 days of STZ injection, glucose levels were determined using glucose meter (Accu-Chek®, Roche diabetes care, India) [19, 20] and the rats with high glucose levels were chosen for the study. The diabetic rats were divided into three groups (n = 6) (Diabetic control, Qu-SMs, Qu suspension group) and a group of 6 non-diabetic rats was used as normal control. The rats in Qu-SMs and Qu suspension group were orally administered with the formulations at a dose of 50 mg/kg[21]. Normal and diabetic control groups were administered saline.

The blood glucose levels in the rats in each group was determined at day 7 and day 15. Moreover, the percentage change in the body weight was also evaluated. At the end of 15 days of treatment, the animals were sacrificed and the levels of physiological antioxidants SOD and catalase were determined by excising the target organs (kidneys and pancreas) using standard procedures[22, 23].

Statistical Analysis

All the results are articulated in the form of mean ± standard deviation (SD). GraphPad Prism (GraphPad Prism Software, USA) was used to perform the statistical comparisons by the means of one-way/two way Analysis of Variance (ANOVA) and student (unpaired) t-test. The disparity was supposed to be statistically significant when $p < 0.05$. (95% confidence interval).

Results and Discussions

Optimization of Qu-SMs based on Box-Behnken Design

The BBD is considered to be efficient in determining the individualized as well as mutual effects of the factors on the desired responses. Moreover, BBD is also more effective and low-risk design as compared to other designs. Taking this into account, BBD was used to estimate the significance of independent variables V_1 (Polymer to Drug Ratio), V_2 (Organic phase volume/Aqueous Phase volume Ratio) and V_3 (Surfactant Concentration) on the selected response variables Y_1 (Particle Size), Y_2 (PDI), and Y_3 (Solubilisation Efficiency). Plackket-Burman design was employed to screen the factors actually influencing the selected response variables. According to the generated BBD design, 17 experimental batches were formulated and the responses were compiled in S6. The results were statistically evaluated by application of ANOVA with the aid of Design Expert® software. The model with highest F value was considered to be the best. 3D response surface frames were analysed to determine the mutual effects of two different factors on a single response (Figure 1).

Impact of independent variables on Y_1(hydrodynamic particle size) of Qu-SMs

The particle size of the prepared Qu-SMs batches according to BBD was found to be varying from a minimum of 83.5 ± 3.7 nm to a maximum of 108.1 ± 1.2 nm. Response surface analysis generated a statistical equation depicting the relationship between particle size and the independent variables which is as follows:

$$Y_1 = +95.46 + 8.79 * V_1 + 4.45 * V_2 + 1.21 * V_3 - 0.23 * V_1 * V_2 + 0.35 * V_1 * V_3 + 0.38 * V_2 * V_3 + 0.54 * V_1^2 + 0.020 * V_2^2 + 0.40 * V_3^2 \quad \text{(Eq.2)}$$

The obtained quadratic model had a *F-value* of 89.85 which suggests the suitability of the model with the results of the particle size.The R^2 value for the model was found to be 0.9914 which represents existence of good correlation between the predicted and the observed results for the particle size. Moreover, The "Pred R-Squared" of 0.8839 was found to be in rational consensus with the "Adj R-Squared" of 0.9804, thus suggesting the suitability of the selected model for the particle size prediction.

Influence of independent variables on Y2(PDI) of Qu-SMs

The PDI of the formulated batches of Qu-SMs was found to vary within a minimum of 0.25 ± 0.12 and a maximum of 0.32 ± 0.078. The quadratic model suggested the following expression depicting the correlation between the PDI and the defined independent variables:

$$Y_2 = +0.28 + 0.030 * V_1 + 7.250E{-}003 * V_2 + 7.000E{-}003 * V_3 - 2.500E{-}004 * V_1 * V_2 - 2.500E{-}004 * V_1 * V_3 + 0.010 * V_2 * V_3 + 6.125E{-}003 * V_1^2 + 4.125E{-}003 * V_2^2 + 3.125E{-}003 * V_3^2 \quad \text{(Eq.3)}$$

The *F-value* obtained was 16.90 indicative of the statistical significance of the suggested quadratic model. Also, The "Pred R-Squared" of 0.7411 was in rational concurrence with the "Adj R-Squared" of 0.8994 suggesting the fairness of the selected model to fit the obtained PDI results. The correlation coefficient was found to be 0.9560 indicating the goodness of fit of the model.

Impact of independent variables on Y3(Solubilisation Efficiency) of Qu-SMs

The solubilisation efficiency of the formulated micelles according to the BBD was found to be varying between a minimum of 63 ± 1.5% and a maximum of 77.5 ± 2.3%. The correlation between solubilisation efficiency and the defined independent variables was depicted by a polynomial equation generated based on the multiple regression analysis. The equation is as follows:

$$Y_3 = +70.00 + 4.79 * V_1 - 2.00 * V_2 - 2.56 * V_3 + 0.000 * V_1 * V_2 - 0.075 * V_1 * V_3 - 1.25 * V_2 * V_3 - 0.31 * V_1^2 + 0.56 * V_2^2 + 0.54 * V_3^2 \quad \text{(Eq.4)}$$

On the basis of *F-test* in the suggested model, the found *F-value* of 121.60 implied the suitability of the model for relating the solubilization efficiency of Qu-SMs with the selected independent value. The high correlation coefficent value (R^2) of 0.9936 represents good level of correlation and suitability. Moreover, the close values of "Pred R-Squared" of 0.8983 and the "Adj R-Squared" of 0.9855 confirms the acceptability of the presented model with favourable significance.

Optimization of Qu-SMs

After collection of responses for all batches, the technique of numerical optimization was employed for concurrent optimization of the process parameters which would generate

Table 2. Optimized values of the design parameters generated in accordance with BBD for Qu-SMs.

Independent variables		Predicted Values	
Results			
	Experimental values	Predicted Values	% bias
Polymer to Drug Ratio (V_1)		7.7 (w/w)	
Organic volume to Aqueous volume Ratio (V_2)		0.22 (v/v)	
Surfactant Concentration (V_3)		0.52	
Particle Size (nm)	87.5	84.87	−3.09
Solubilisation Efficiency (%)	68.2	70.70	3.53
Polydispersity Index(PDI)	0.271	0.260	−4.23
Overall desirability		**0.795**	
Drug loading		**8.33**	
Zeta potential (mV)		**−12.30**	

Qu-SMs with desired dependent responses. Required limits were applied for all the three process parameters in the Design Expert® software with the aim to achieve formula for Qu-SMs with minimum particle size, maximum solubilization efficiency and minimum PDI values. The optimized batch was designed in accordance with the predicted values of the independent variables, hence checking the predictability of Box-Behnken Design as depicted in Table 2. Qu-SMs optimized batch exhibited an overall desirability of 0.795. The authenticity of the BBD for statistical optimization was proved by the obtained low percentage bias values among the predicted and the experimental response datas[24, 25].

Particle Size and PDI

Particle size is considered to be an essential parameter that affects the drug release and in-vivo uptake of the formulation which ultimately influences the bioavailability of the formulations. In this study, the particle size of both blank SMs and Qu-SMs was found to be less than 100nm with a narrow distribution range. The average particle size of the blank SMs was established to be 63.3 nm, whereas the average particle size of the optimized Qu-SMs was found to be 87.5nm. Moreover, the particle size distribution was also slightly widened in the case of QU-SMs. The PDI in the case of blank SMs was found to be 0.108 which increased to 0.270 for Qu-SMs, but was still found to be in the acceptable range. A rise in the particle size and PDI of Qu-SMs as compared to that of SMs was observed due to drug Solubilisation in the core of micelles.

Soluplus® micelles exhibit the capability of enveloping the hydrophobic drug into the core of the micelles. This ability might be attributed to the interactions between the drug and the polymer. In this case, the phenolic –OH groups present in the Qu moelcule might interact with the terminal –OH and ether oxygen groups in Soluplus® and form hydrogen bonds.

Zeta Potential

The zeta potential value depicts the charge on the surface of the suspended micelles in the aqueous system. Higher is the zeta potential of the nanomicelles, higher would be the repulsion between the particles, and hence greater would be the stability of the system. The particles will not coagulate due to the high repulsive forces among themselves and thus will remain in the suspended form. The Zeta potential of the blank SMs and Qu-SMs was found to be −4.84 mV and −12.30 mV. The change in the zeta potential of Qu-SMs might be attributed to the addition of Qu into the micelles. Higher value of zeta potential indicated about the good stability characteristics of the Qu-SMs formulations.

Solubilisation Efficiency

The solubilisation efficiency of the optimized formulation was found to be 70.70 ± 2.3% at the drug loading of 8.33% while the solubilisation efficiency was found to be 62 ± 1.7% at the drug loading of 16.66%. Higher solubilisation efficiency at lower drug loading could be attributed to the solubilisation of drug in the hydrophobic core of the micelles. Higher drug loading leads to lower solubilisation efficiency. This might be due to the development of higher concentration gradient causing release of drug from the micelles through diffusion mechanism into the aqueous medium.

Solid State Characterization

The FTIR spectra of Qu, Soluplus®, physical mixture, Qu–SMs and lyophilised micelles are depicted in the Figure2 (A). The FTIR spectra of Qu exhibits the strong absorption peak at 1667 cm^{-1} belonged to –C = O stretch, indicating the carbonyl group, which is its characteristics peak. The absorption peaks ranging from 1200-1400 cm^{-1} were analogous to the phenolic –OH stretch. The absorption peak at 1012 and 1608 cm^{-1} was attributed to the characteristic aromatic bending and stretching. Soluplus® shows characteristics peak at 1750 and 1650 cm^{-1} corresponding to the carbonyl –C = O group, 2747, 2890 cm^{-1} for –C-H, and broad band in the range 3200-3400 cm^{-1} representing the terminal –OH groups. The spectrum of poloxamer 407 is characterized by strong absorption peaks at 2880 cm^{-1} signifying the aliphatic –C-H stretch, 1339 cm^{-1} (in-plane O-H bend), and 1098 cm^{-1} (C-O- stretch). All the distinctive IR peaks of Qu were spotted in the spectra of physical mixture for micelles, but the peaks were slightly shifted, hence suggesting that Qu is not involved in any physicochemical interactions with ingredients used in the formulation of Qu-SMs. The FTIR spectra of the lyophilized Qu-SMs exhibit all the characteristics peak of Qu with minor variations in the frequency, hence it can be concluded that the drug Qu is encapsulated into Soluplus® micelles without any chemical incompatibilities.

PXRD patterns of Qu, Soluplus®, Physical Mixture and lyophilized Qu-SMs are depicted in the Figure 2(B) Qu exhibits characteristic peaks at diffraction angle of 2θ, 10.7°, 12.33°, 15.8°, 24.4°, 26.3°, and 27.6°. The sharp and highly resolved peaks indicate the crystalline nature of Qu. The diffraction pattern of Soluplus® presented small diffused peaks. PXRD pattern of physical mixture for Qu-SMs exhibited the peculiar peaks of Qu at similar 2-theta values as that of pure

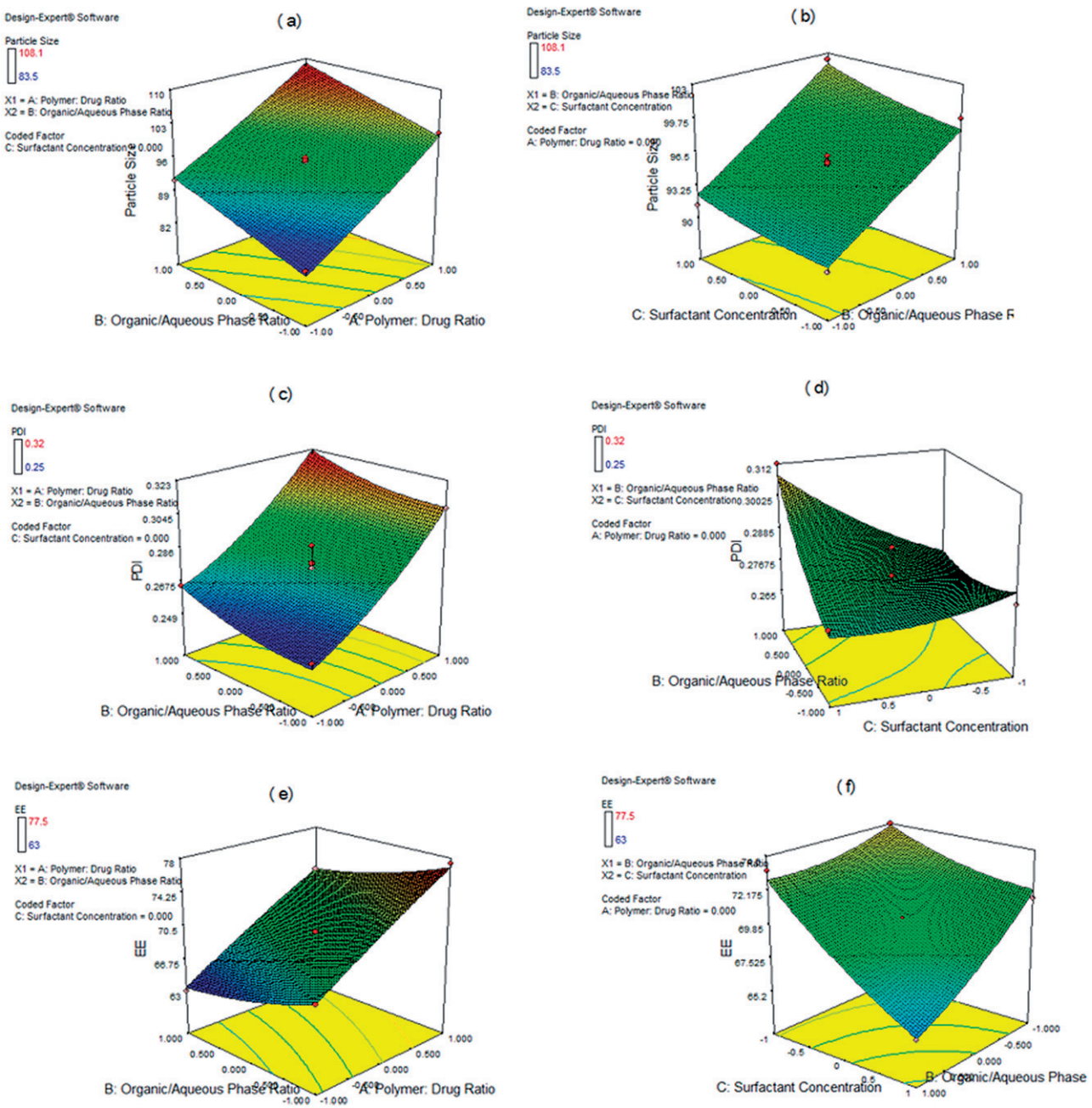

Figure 1. 3D contour plots interpreting the impact of Polymer to Drug Ratio (V_1) Organic/Aqueous Phase Ratio(V_2), and Surfactant Concentration (V_3) on dependent variables of Qu-SMs. (a & b) represent the influence on Particle Size, (c & d) represent the influence on Polydispersity Index (PDI) and (e & f) represent the influence on Solubilisation Efficiency (EE).

Qu, hence indicating no physical/chemical interactions between the drug Qu and excipients. Conversely, the PXRD pattern of the lyophilized Qu-SMs exhibited the peaks of Qu, but with reduced intensity, which suggests that the Qu has been converted to amorphous nature upon solubilisation inside the Qu-SMs.

Morphological Characterization

The morphological characterization using SEM micrographs established that Qu-SMs were spherical in shape, discrete and distributed uniformly throughout. The SEM micrograph is depicted in the Figure 3(c). The particle size of Qu-SMs was in agreement with the particle size distribution obtained through the particle size analyzer.

The surface morphology of Qu-SMs was further confirmed by analyzing the 2D and 3D AFM micrographs generated by the interaction of the sharp tip with the surface of Qu-SMs. The AFM micrographs are presented in the Figure 3(a) and (b) which reveal the results in agreement with the SEM micrograph. The Qu-SMs particles are spherical and separated from each other. The micelles have an average 3D roughness of 2.582 nm which was also measured by AFM using Nova Px 3.1.0 software.

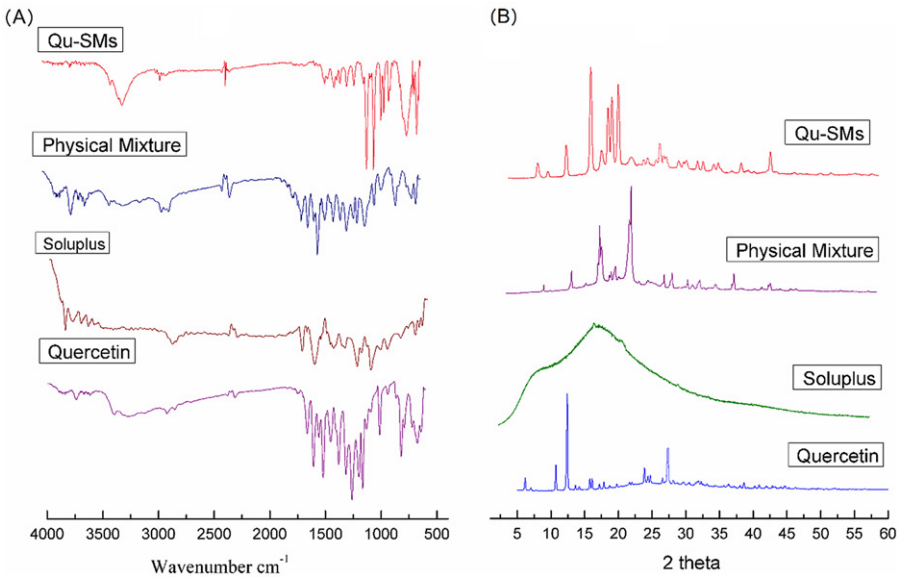

Figure 2. **(A)** Overlay of the FTIR spectra and **(B)** XRD spectra of Qu, Soluplus®, Physical Mixture and Qu-SMs

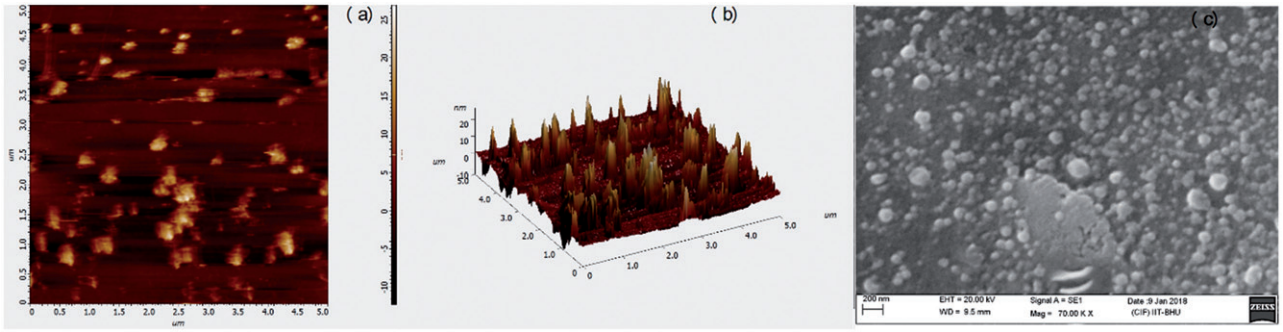

Figure 3. **(a)** 2D AFM (b) 3D AFM image of Qu-SMs (c) SEM image of Qu-SMs

In-vitro *release study of Qu-SMs*

The release behaviour of Qu from the Qu-SMs and Pure Qu drug suspension was studied using dialysis bag method at 37 °C in Ethanol-water as shown in the Figure 4(b) The Qu release from Soluplus® micelles exhibited biphasic behaviour showing burst release initially followed by extended release. Only 6.8% of Qu was released from the Qu-SMs during initial 8 hours while on the other hand, 49.84% of Qu was released from pure drug suspension in 8 hours. At the end of 24 h, only 28.75% of the total drug loaded in micelles was released, which leads to the conclusion that Qu-SMs exhibits sustained release property for the enveloped Qu. The mechanism of drug release was evaluated by fitting the obtained release data into various kinetic equations to get an idea about the mechanism of drug release. The r^2 correlation coefficient was found to be highest for Korsemeyer-Peppas model. This indicates that the drug release from Qu-SMs was through diffusion mechanism through the polymeric micelles.

In vitro *haemolysis study in human blood*

The haemolytic activity of free Qu and Qu-SMs (different drug and polymer concentrations) in human and rat blood are depicted in Figure 4(a). The haemolytic activity for Qu-SMs was found to be inversely proportional to the concentration of free drug. The haemolytic activity of free Qu was significantly higher than that of same concentration of Qu-SMs. Moreover, the haemolytic activity increased with the decrease in the polymer concentration, which may be attributed to the increased amount of free drug in the formulation.

In vivo *pharmacokinetic investigation*

The plasma drug concentrations obtained after the administration of Qu drug suspension and Qu-SMs were plotted against time, and the graph is presented in Figure 4(c). A significant difference was observed in the pharmacokinetic parameters of Qu drug suspension and Qu-SMs, as depicted in Table 3. The peak plasma concentration (C_{max}) of Qu from Qu-SMs was found to be 4.83 μg/ml, which was 10.7 ($p < 0.001$) times higher than that observed of Qu drug suspension value. The nano-sized micelles could have facilitated the drug absorption across GIT and this might be the reason for higher C_{max}.

The T_{max} of the Qu-SMs was found to be delayed 2 times in comparison to that of Qu drug suspension. This gives us confirmation about the sustained release of Qu from Qu-SMs

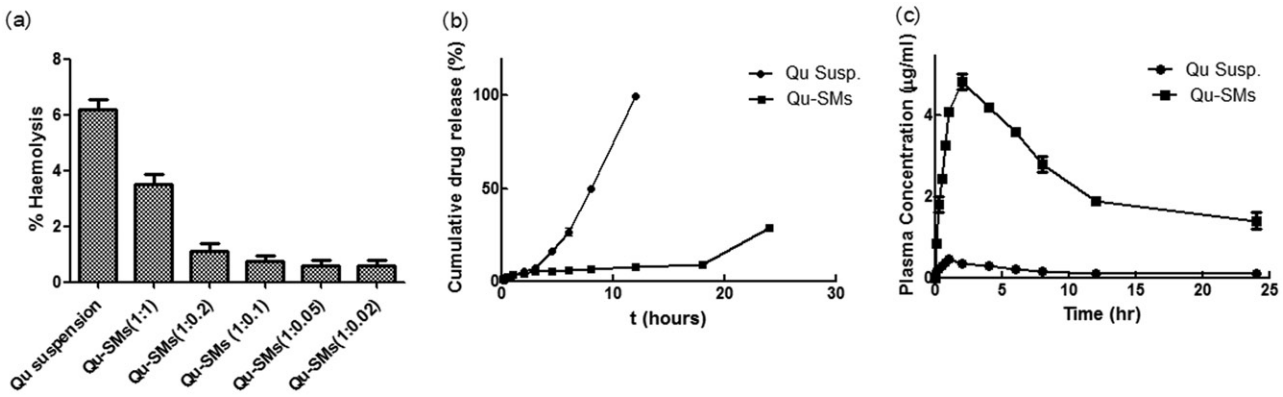

Figure 4. **(a)** Graph represents the haemolytic activity of the Qu suspension and QU-SMs with varying ratios of polymer to drug. The values are indicated in form of mean ± SD (n = 3) **(b)** *In vitro* release profiles of Qu-Susp and Qu-SMs. **(c)** Plasma drug concentration vs time profiles of Qu suspension and Qu-SMs. Each point on the graph (b) and (c) represents mean ± SD (n = 6).

Table 3. Pharmacokinetic parameters for Qu-SMs and Qu drug suspension.

Parameters	Qu-SMs	Qu pure drug suspension
C_{max} (µg/ml)	4.83	0.45
T_{max}(hour)	2.00	1.00
AUC_{last}	58.83	3.46
AUC_{extra}	55.01	0.86
AUC_{tot}	113.85	4.33
$t_{1/2}$(hour)	27.37	12.00
MRT(hour)	35.48	14.53

in agreement with the in vitro release study. The $AUC_{0\text{-}24h}$ and $AUC_{0\text{-}\infty}$ values were calculated for both Qu-SMs and Qu drug suspension, and a significant improvement ($p < 0.001$) in the values corresponding to Qu-SMs were observed. The relative bioavailability of Qu was evaluated by comparison of the AUC value of Qu-SMs to that of Qu drug suspension. The relative bioavailability was found to be 1676% which can be attributed to bypassing of the hepatic metabolism and enzyme degradation of the Qu-SMs owing to its direct uptake into lymphatic system due to the small micelle size, thus resulting in higher C_{max} and higher bioavailability.

Evaluation of optimized Qu-SMs in diabetes regulation

The rats in the normal control group which were non-diabetic acted as the negative control and the mean glucose levels of rats in this group was found to be 120 mg/dl. On the other hands, rats in the diabetic control group acted as the positive control and the mean glucose levels in these rats was found to be 380 mg/dl. The glucose levels in the group treated with Qu drug suspension and Qu-SMs was found to be significantly lower ($p < 0.05$) as compared to the diabetic control group. Moreover, comparison was made between the groups administered with Qu suspension and Qu-SMs on day 7 and day 14, and it was found that the rats in the latter group exhibited significantly lower blood glucose levels. Hence, indicating that Qu-SMs was therapeutically more effective ($p < 0.05$) than that of Qu pure drug suspension at day 7 and day 14 (Figure 5(a)).

Body weights of the rats in different groups were monitored and % change in body weight was statistically analysed. Significant difference in the pharmacological activity of Qu pure drug suspension and Qu-SMs was observed as depicted in Figure 5(b).

It has been reported that Qu has potential anti-oxidant and free radical scavenging property, which might be responsible for the anti-diabetic effects of the drug. Hence keeping this in mind, it was concluded that the level of naturally existing antioxidants catalase and SOD will be helpful in determining the extent of oxidative damage and, hence the efficacy of optimized Qu-SMs. Therefore, SOD and catalase levels in the pancreas and kidney were evaluated. It can be easily concluded from the graph in Figure 5(c & d) that the amount of SOD and catalase per mg of protein was significantly lowered in diabetic rats as compared to the rats of normal group, due the hyperglycemic oxidative stress. The groups treated with Qu drug suspension and Qu-SMs exhibited increased amount of SOD and catalase at the end of the treatment period. Moreover, these groups exhibited significantly ($p < 0.05$) greater amounts of SOD and catalase as compared to that of the diabetic control group. This increase can be attributed to the anti-oxidant effect of the drug Quercetin, which helps mitigate the action of reactive oxygen species.

Conclusion

In the present study Qu-SMs were successfully prepared by co-solvent evaporation method which was optimized using BBD. Optimized Qu-SMs exhibited PS in the desired nano size range (below 100nm) with high EE of 70.70%. The optimized Qu-SMs formulation exhibited uniformly distributed spherical particles coupled with intact FT-IR and XRD characteristics of Qu. *In-vitro* release profiles of optimized batch provided sustained release of Qu (28.75% in 24 hours) as desired to achieve its prolonged antioxidant activity. The haemolytic activity of the Qu-SMs formulations was found to be significantly ($p < 0.05$) lower than that of pure drug suspension and in the acceptable range. *In vivo* pharmacokinetic study revealed improved pharmacokinetic parameters, wherein the relative bioavailability of optimized Qu-SMs was found to be 1676%, when compared to pure drug suspension. The animal groups treated with Qu-SMs exhibited significantly lower glucose levels, higher SOD and higher catalase levels, when

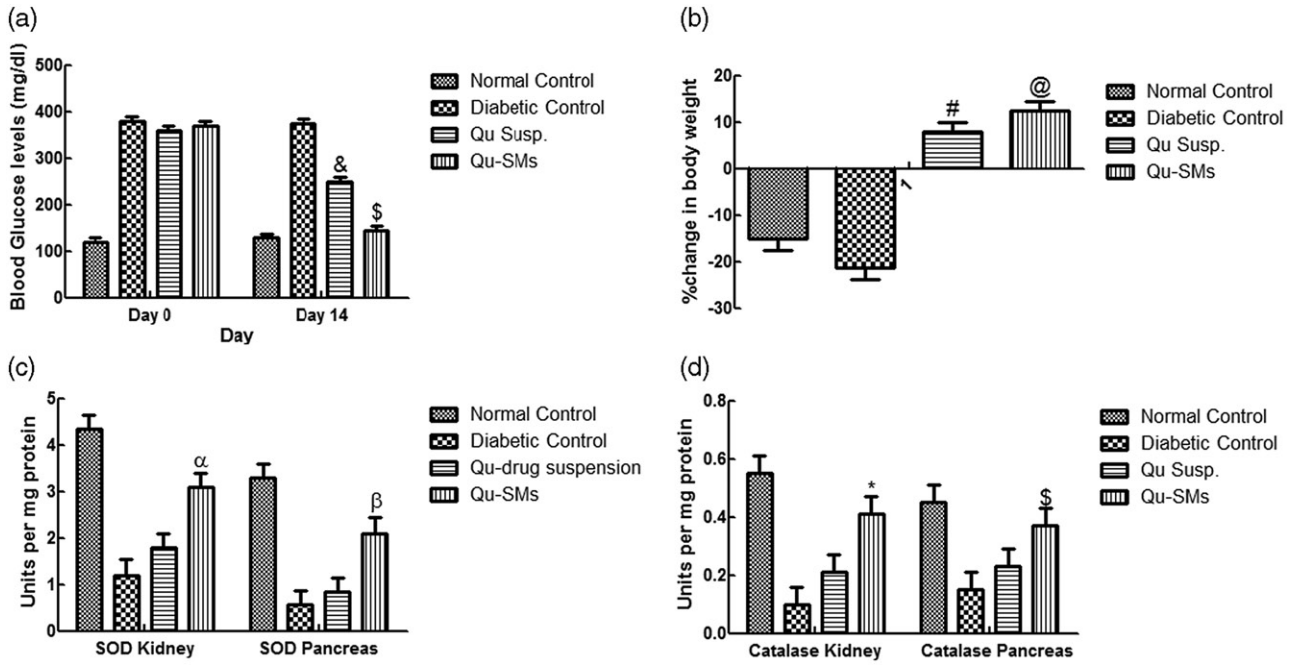

Figure 5. **(a)** Blood Glucose levels of different animal groups on day 7 and 14. & and $represents significant ($P < 0.05$) change in comparison to diabetic control (Two-way ANOVA followed by Bonferroni post tests), **(b)** % change in the body weight, # and @ represents significant ($P < 0.05$) difference in comparison to to diabetic control (One-way ANOVA followed by Tukey's multiple comparison test), **(c)** SOD levels of kidney and pancreas on day 14, α, β depict groups with significant ($P < 0.05$) difference in comparison to diabetic control (Two-way ANOVA followed by Bonferroni post tests), **(d)** Catalase levels in kidney and pancreas of the rat on day 14, *, $ represent significant change in comparison to diabetic control. Vertical bars represent mean ± SD (n = 6).

evaluated for diabetes treatment *in vivo*. These results lead us to conclude that formulated Qu-SMs exhibit the desired therapeutic potential in diabetes treatment. The obtained results also evidenced that Soluplus micelles provide us with an excellent platform that can be used for delivery of BCS class II drugs. The optimized system will further be evaluated in diabetic wound healing.

Conflict of interests

The authors confirm that this article has no conflict of interest.

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