Pharmacological and Technical Evaluation of Statistically Formulated and Optimized Dual Drug-Loaded Silica Nanoparticles for Improved Antifungal Efficacy and Wound Healing

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ABSTRACT: The current research aimed at designing mesoporous silica nanoparticles (MSNs) for a controlled coadministration of salicylic acid (SA) and ketoconazole (KCZ) to effectively treat highly resistant fungal infections. The sol–gel method was used to formulate MSNs, which were further optimized using central composite rotatable design (CCRD) by investigating mathematical impact of independent formulation variables such as pH, stirring time, and stirring speed on dependent variables entrapment efficiency (EE) and drug release. The selected optimized MSNs and pure drugs were subjected to comparative in vitro/in vivo antifungal studies, skin irritation, cytotoxicity, and histopathological evaluations. The obtained negatively charged (−23.1), free flowing spherical, highly porous structured MSNs having a size distribution of 300−500 nm were suggestive of high storage stability and improved cell proliferation due to enhanced oxygen supply to cells. The physico-chemical evaluation of SA/KCZ-loaded MSNs performed through powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), and thermal gravimetric analysis (TGA) indicates absolute lack of any interaction between formulation components and successful encapsulation of both drugs in MSNs. The EE, SA release, and KCZ release varied significantly from 34 to 89%, 36 to 85%, and 43 to 90%, respectively, indicating the quadratic impact of formulation variables on obtained MSNs. For MSNs, the skin tolerability and cell viability percentage rate were also having an extraordinary advantage over suspension of pure drugs. The optimized SA/KCZ-loaded MSNs demonstrated comparatively enhanced in vitro/in vivo antifungal activities and rapid wound healing efficacy in histopathological evaluation without any skin irritation impact, suggesting the MSNs potential for the simultaneous codeelivery of antifungal and keratolytic agents in sustained release fashion.

1. INTRODUCTION

Human health is greatly affected by fungal infections, while the discovery of ideal antifungal formulation is still a challenge for pharmaceutical scientists. The patients with acquired immunodeficiency syndrome (AIDS), transplant recipients, hematological malignancies, and other immunocompromised individuals are prone to fungal pathogens. Initially, fungi infect the skin surface and then invade the stratum corneum to avoid being shed from the skin surface by desquamation. However, major problems associated with systemic fungal infections include clinical resistance, microbiological resistance, emergence of new pathogens, and involvement of more immunocompromised patients. The discovery of the antifungal activity of imidazoles represented an important therapeutic development, and ketoconazole (KCZ) that belongs to imidazoles has proven to be an important antifungal agent. Only limited classes of antifungal drugs are available, and most of the fungi show less susceptibility to available drugs including the azoles. The emerging resistance to antifungal agents is greatly gaining attention toward public health issue, which might be due to decreased drug concentration at a cellular level, and such resistant topical fungal infections might affect the other vital body parts through contaminating blood. Therefore, fungal infections require immediate consistent and appropriate antifungal treatment having suitable patient compliance.

Despite timely diagnosis and appropriate antifungal therapy, clinical outcome might be disappointing, necessitating treatment with a combination of medicinal agents. Thus, development of a formulation having advantages of enhanced antifungal and keratolytic activity is ever needed. In this regard, salicylic acid (SA), oldest known keratolytics, and a well-
established treatment for many dermatologic conditions, including psoriasis, was selected to combine with KCZ. Salicylic acid breaks down skin clumps and relieves itching and flaking, and the carboxylic group of salicylic acid provides hydration to skin. It reduces intercellular cohesiveness of the horny cells by dissolving the intercellular cement material along with provisions of comfort and thinner plaques that allow enhanced penetration of other added topical drugs. The notified advantages of combination therapy are lessened possibility of resistance, decreased toxicity, and synergistic effect and minimum adverse effects of therapy. For treatment of skin infections, loading of drugs in silica nanoparticles is a novel approach for drug delivery. Mesoporous silica nanoparticles (MSNs) are highly promising drug carriers for controlled drug delivery due to their high specific surface areas, large pore volumes, high loading capacity, and favorable biocompatibility. Traditional drug delivery systems produce a number of local adverse effects such as burning sensation, skin irritation, greasiness, stinging, pruritic rash, erythema, and tenderness which make them less acceptable. The proposed silica nanoparticles are ranked higher because they can overcome all of the mentioned problems associated with conventional mode of treatment. Regarding application of MSNs, there is no issue of viscosity as observed with the conventional system, and such nanoparticles showed comparatively longer duration of action with better penetration of drugs through dermis without repetitive use.

Figure 1. FTIR patterns (A) and XRD spectra (B) of SA/KCZ-loaded optimized MSNs and their formulation components.
optimized observing their impact mathematically on entrapment efficiency (EE) and drugs released from SA/KCZ-loaded MSNs. SA/KCZ and MSN compatibility were evaluated with the help of Fourier transform infrared spectroscopy (FT-IR), thermal gravimetric analysis (TGA), and X-ray diffraction (XRD). The selected optimized SA/KCZ-loaded MSNs were also analyzed for particle size, morphological appearance, zeta size, zeta potential, in vitro/in vivo drug release, in vitro/in vivo antifungal studies, skin irritation, cytotoxicity, wound healing, and histopathology studies.

2. RESULTS AND DISCUSSION

2.1. Physicochemical Characterizations of MSNs. The FT-IR spectra of drugs (SA and KCZ) and carriers (MSNs) recorded in a stepwise procedure and comparative analysis of spectra have been presented in Figure 1A. FT-IR of SA showed characteristic peaks at 3233–3223 cm⁻¹ and 2972–2839 cm⁻¹ that were assigned to OH and C–H stretching, respectively. A peak near 1635 cm⁻¹ was due to stretching of the C=O bond of the acid group [–(C=O)–O–H]. A large peak near 1605 cm⁻¹ was due to a skeletal vibration of the benzene ring. Further, peaks appeared at 1570 cm⁻¹ were assigned to C=C (phenolic) multiple peaks.¹⁹,²⁰ The spectrum (KCZ) revealed the appearance of the peak at 3109 cm⁻¹ due to N–H stretching while peaks at 2978 and 2723 cm⁻¹ are due to aliphatic C–H stretching. Absorption peaks at 1786 and 1647 cm⁻¹ revealed C=O stretching frequencies. Two absorption peaks observed at 1255 and 1232 cm⁻¹ illustrated the presence of tertiary amine.²⁰ Comparative analysis of FT-IR spectra of pure drugs and silica nanoparticles loaded with drugs and combination of both drugs exhibited a stretching peak nearly at 1060 cm⁻¹. This peak is attributed to the Si–O–Si linkage. A shoulder peak appeared at 940 cm⁻¹ was attributed to free –OH groups present on the surface of silica. The two peaks at 1060 cm⁻¹ and 940 cm⁻¹ confirmed that the basic structure of nanoparticle formulation is composed of silica.²¹ Moreover, there was only a slight shift in the location of peaks of drugs in drug-loaded MSNs; this significantly indicates that there was no substantial chemical interaction of drug with excipients for all of formulations.

XRD is a very expedient tool to evaluate whether the degree of crystallinity of the drug is reserved or it is altered into the amorphous state after entrapment into formulation. XRD pattern obtained for salicylic acid presented in Figure 1B exhibited characteristic peaks at 11.07 and 17.31° 2θ,²²,²³ which were evident toward the crystalline structure of SA. The diffraction spectrum of pure ketoconazole showed 6 distinct peaks 15.97°, 17.58°, 19.88°, 23.82°, 25.7°, and 27.51° at 2θ angles which demonstrated the crystalline nature of the drug.²²,²³ As shown in Figure 1B, MSNs showed a broad peak at 21.74° at 2θ angle.²²,²⁵ Disappearing of pure drug peaks exhibited entrapment of drugs (SA & KCZ) into MSNs and specifies the amorphous state of the encapsulated drug.

DSC analysis is a tool to study thermal behavior of pure drugs, MSNs, and to determine the presence or absence of the crystalline form of drugs in the pores of nanosilica.²²,²⁵ As presented in Figure 2A, DSC of SA exhibited an intense peak at 195.18 °C which is attributed to its melting point, while DSC of MSNs loaded with SA exhibited an endothermic peak at 162.05 °C, which is related to depression in melting point of SA. This depression in the melting point indicated the presence of the crystalline state of the drug in SA-MSNs.²² The DSC curve for KCZ exhibited an endothermic peak with

\[ T_{\text{mel}} = 165.93 ^\circ \text{C} \]

which is attributed to the melting of KCZ, while DSC of MSNs loaded with KCZ exhibited no melting peak of KCZ. The absence of phase transitions owing to KCZ is evidence that KCZ is in the noncrystalline state.²⁵ Melting point peaks of SA and KCZ were found missing in the DSC curves of MSNs loaded with the combination of SA and KCZ (optimized formulation). The absence of phase transitions is evidence that drugs are present in noncrystalline states in the mesoporous silica matrix.²⁵ DSC outcomes are in agreement with the finding of XRD.

TGA was carried out to ascertain the encapsulated amount of SA and KCZ in the pores of MSNs.²⁶ As shown in Figure 2B, TGA of SA exhibited weight loss at two different points at 162.05 °C and at 198.07 °C linked to loss of humidity and the intense weight loss was due to melting of SA. A similar intense peak at 195.18 °C was observed in DSC of SA. The TGA curve of KCZ showed no mass loss at its melting temperature range. Literature studies showed that the drug is thermally stable up to 285 °C.²⁷ The weight loss due to drug uptake was 30.03, 41.11, and 55.41% for three different formulations SA-MSNs, KCZ-MSNs, and SA/KCZ-MSNs, respectively. The maximum weight loss found in case of combination of SA/KCZ-loaded MSNs revealed maximum encapsulation of both of the drugs in the MSNs matrix. The drug-entrapped values measured by HPLC and shown in Table 1 were in line with those obtained by thermal analysis. EE of optimized formulation of SA/KCZ-loaded MSNs was 85%. The result suggested that the drug loading capacity of MSNs is mainly dependent on porosity of MSNs. So, extensive physico-chemical evaluation performed through FTIR, XRD, DSC, and TGA have demonstrated the excellent compatibility of drugs with TEOS while the remaining physico-chemical parameters of finally formulated MSNs. Like percentage yield, entrapment efficiency, particles size, rheology, shape, morphology, surface charge, and in vitro drugs release have been discussed below.

2.2. Optimization of Drug Retention on MSNs Using CCRD. Formulations of SA and KCZ-loaded MSNs were formulated according to CCRD as shown in Table 1. The values of correlation coefficients \((R^2)\) of the applied quadratic model were found to 0.9826 for EE_s, 0.9901 for EE_KCZ, 0.9899 for SA, and 0.9832 for KCZ release. These values were found very close to the obtained outcomes of adjusted \(R^2\) which indicates the suitability of the applied model. The results
of ANOVA and regression analysis are presented in Table 2 where p-values suggested that the quadratic model was significant. From Table 1, it was observed that, there was an increase in EE of both drugs with an increase in pH, stirring time, and stirring speed (F3), but at higher levels of these dependent variables (F8, F9 and F19), a prominent decrease was seen in results of these responses. Moreover, it was observed at lower values of pH (F1), stirring time (F8), and stirring speed (F3), but at higher levels of these independent variables (F11, F12, and F17), a significant decrease in EE of both drugs was seen. The negative correlation between pH and EE of both drugs was quite reliable and reasonable. 

To validate the experimental model, the optimized batch of nanoparticles (Table 3) was prepared using suggested optimal levels of independent variables by software Design Expert version-12. The predicted values of all studied responses were well closely matched with the experimental values of responses, which suggested that the optimized nanoparticle formulation was quite reliable and reasonable. 

The optimized nanoparticle formulation was established for attaining higher EE_{PY} (87%), EE_{KCZ} (78%) and controlled drug release (40%) during the numerical optimization process. All of the suggested options regarding formulation’s conditions and the desirable results were prioritized at the basis of the desirability factor. So, software generated optimized nano-
particles having an average size of 500 nm and zeta potential of $-23$ mV with improved EE and significant control over drugs release were formulated (Table 3) and further evaluated for biomedical applications. Table 3 exhibited the most optimized formulation conditions for MSNs. For all of the dependent variables of MSNs, the desirability factor was observed close to one and the calculated PE was also observed to be less than 10% suggesting that the applied optimization process was quite successful.

The calculated PY ranges from 31 to 82% and affected by all of the factors including pH, stirring time, and stirring speed (Table 1). The values indicated that there is a fall in the PY value up to 31% at pH 4.5 (F1), whereas PY has increased up to 82% with pH value 5.8 (F3). So, a higher pH is associated with higher PY as reported in the literature. A shorter stirring time of 90 min showed reduced PY up to 42% (F13) but a higher stirring time of 120 min brought an increase in PY up to more than 70% (F3 and F12). This might be subjected to the fact that longer stirring time leads to more PY of MSNs as there would be more time available for solvent diffusion hence leading to increased yield and inverse will be true for lower stirring time and percentage yield. Similarly, increasing the stirring speed has enhanced the PY (F3 and F11) because of achievement of lower size of nanoparticles at higher speed.

But, at very high speed, an irregularity was observed because of turbulent impact which tends to lower the yield as observed in F9.

EE for both drugs was found to be within the range of 36 to 85%. Polynomial equations 1 and 2 for EE were given as

\[ EE (SA) = 65 + 14.77A + 4.58B + 4.64C + 2.50AB + 2AC + 0.75BC - 3.2A^2 - 0.62B^2 - 3.2C^2 \]

(1)

\[ EE (KCZ) = 60 + 14.3A + 4.67B + 4.21C + 1.6AB + 1.5AC + 1.38BC - 1.3A^2 - 0.72B^2 - 1.9C^2 \]

(2)

The equations signified that a normal level of pH (A), stirring time (B), and stirring speed (C) and their interacting terms had a direct correlation and synergistic impact with the results of EE of both drugs. A negative coefficient with $A^2$, $B^2$, and $C^2$ indicated that very higher values of pH, stirring time, and stirring speed antagonized the results of EE as observed in formulations F8, F9, and F14 (Table 1). Similar studies regarding the effect of higher stirring speed and stirring time on reduced EE for both medicaments have also been reported in the literature. It can be noted in formulations (F2 to F12) that an increased pH value has raised values of EE more than 50% which could be associated to higher stirring time and speed. These results have been supported by previous studies. The highest EE (89% for SA and 85% for KCZ) was observed at stirring time 120 min as compared to other lower stirring speed-generated nanoparticles. The reason may be linked to the availability of sufficient time for proper tightening of silica network surrounding the particles which would definitely be not possible at lower stirring time. At lower stirring speed of 1000 rpm, EE for both drugs was found as low as 40% for SA and 42% for KCZ (F16) because at...
reduced stirring, coalescence become more and more evident. At higher stirring speed of 1940 rpm, EE was observed to be 60% for SA and 64% for KCZ (F9). It happened because particles have displaced toward the walls of flask due to higher rotation and contact time between polymer and drug reduces. It was observed in F3 that at an optimum stirring speed of 1700, EE for SA and KCZ was found to be 89 and 85%, respectively. The reason might be that there was no sticking of particles with walls, and contact time between the polymer and drug was quite higher.30 Further, the three-dimensional response surface plots for significant interactive impact of independent variables on EE of both drugs are shown in Figure 3. Studies showed that EE is greatly affected by size of particles which in turn was strongly impacted by higher stirring time and higher stirring speed and thus leading to greater EE of both drugs.

2.3. Drugs Release. Drug release studies were carried out for all SA/KCZ loaded MSNs (Table 1). Its value ranged from 39 to 88% for SA and from 43 to 90% for KCZ. Quadratic equations 3 and 4 generated by software for drug release are given below.

\[
DR_{SA} = 49 - 13.3A - 5.2B - 4.81C - 2.0AB - 1.2AC + 0.51BC + 6.5A^2 + 4.5B^2 + 5.5C^2
\]  

(3)

\[
DR_{KCZ} = 51 - 12.8A - 5.4B - 5.71C - 3.1AB - 1.5AC + 0.75BC + 6.3A^2 + 5.5B^2 + 6.8C^2
\]  

(4)

It was evident in above equations that pH (A), stirring time (B), and stirring speed (C) were presented with negative sign, which showed an indirect relationship between these variables and drug release. Moreover, it was seen that increasing these values of experimental parameters had directly influenced the release of both drugs from MSNs. In the case of F13 and F16 formulations, higher release for both of the drugs (more than 80%) was observed. A high release profile of drugs can be attributed to lower pH and reduced particle size.31 It can be observed that higher stirring time led to higher release rate of both drugs more than 50% (F12, F16, and F19). Similarly, a higher stirring speed (F18 and F20) has increased the release of both drugs up to more than 70%. The three-dimensional response surface plots for significant interactive impact of independent variables on release of both drugs are shown in Figure 4. Studies showed that drug release is affected by size of particles; higher stirring time and higher stirring speed were thought to be associated with particle size reduction, thus leading to greater release of both drugs.30 Moreover, in some formulations, it has also been observed that an increase or decrease in release of both drugs was not totally dependent on individual factor alone; all the three factors like pH, stirring time, and stirring speed concomitantly affected the results of release of both drugs.

The experimental data showed that MSNs exhibited a slow initial release of the drugs from respective suspension. The release of drugs from MSNs was gradually increased with the passage of time demonstrating the sustained pattern for release.

Figure 4. 3D plots showing the combination impact of pH, stirring time, and stirring speed on SA release (A–C) and KCZ release (D–F).
of SA/KCZ from MSNs as given in Figure 5 which depicts the release of both drugs from selected optimized formulation of nanoparticles remained continue for 14 days and about 50% of both drugs were released in almost 8 days. As compared to pure drug suspensions, the results of in vitro release studies of MSNs showed a better control over the release of drugs for an extended period of time suggesting applicability and acceptability of SA/KCZ-loaded MSNs for topical delivery of the drugs. Data obtained from experiments was analyzed to study the release mechanism by applying various release kinetic models (zero order, first order, and Higuchi model and Korsmeyer-Peppas model). The values of correlation coefficients ($R^2$), rate constants ($K$), and release exponent ($n$) for various kinetic models were comparatively analyzed. The release kinetic suited best in zero order because the value of the correlation coefficient ($R^2$) for the zero order kinetic model was close to unity which indicated that release of drug was independent of remaining drug concentration. The values of $n$ calculated by the Korsmeyer-Peppas model depicted that the release mechanism follows the diffusion along with erosion mechanism.

2.4. Rheological Properties. Rheological studies concern with evaluation of flow properties of MSNs. Appropriate flow behavior of MSNs is mandatory if MSNs are to be converted in tablet, transdermal patch, capsule, or any other dosage form for their appropriate administration to patients. Table 4 presents the outcomes of rheological analysis of all formulations. The selected parameters such as stirring time, stirring speed, and pH have significant effect on MSN formulations. Optimum speed, optimum time, and mild acidic conditions have positive influence on the flow behavior of MSNs. The value of the Car’s index for all MSN formulations ranged from 9 to 18 indicating a better flow character of MSNs. The outcomes from the angle of repose had also verified the excellent flow character of MSNs because for most of the formulations and the value of angle of repose remained less than 20°. Similar findings from the results of Hausner’s ratio had confirmed the good rheological behavior of MSNs. It remained less than 1.5 for all of the formulations suggesting the good flow behavior of MSNs.

2.5. Determination of Particle Size, Size Distribution, and Zeta Potential. The particle size is a good indicator toward determination of physical stability of formulations. Particles with reduced size have more surface area and have more tendencies to cross the skin barrier layers. The particle size, size distribution, and zeta potential of optimized MSNs formulation were shown in Figure 6A,B, respectively. The size distribution of MSNs ranged from 200 to 900 nm while the major fraction (54%) of the MSNs has an average size of 450 nm (Table 4). The particle size was greatly influenced by pH of the medium and stirring speed. The optimized formulation was prepared at pH-5.8 and higher stirring speed. The polydispersity index (PDI) value was observed to be 0.564, which identified that the drug delivery system has a rationally wide-ranging size distribution.

Measurement of zeta potential is a significant characterization method for nanoparticles to estimate their surface

![Figure 5. Comparative in vitro release study of SA/KCZ from MSNs.](image)

### Table 4. Rheological Properties, Size Range, Zeta Potential, and Percentage Yield of all MSN Formulations

| Formulations | Hausner’s ratio | Carr’s index | Angle of repose | Size (nm) | Zeta potential (mv) | Percentage Yield (%) |
|--------------|----------------|--------------|-----------------|----------|---------------------|----------------------|
| F1           | 1.15 ± 2.72    | 13 ± 2.75    | 20 ± 1.94       | 895 ± 6.66 | 22 ± 3.43           | 31 ± 3.72            |
| F2           | 1.09 ± 2.86    | 9 ± 1.95     | 17 ± 2.36       | 580 ± 5.56 | 23 ± 5.17           | 59 ± 3.47            |
| F3           | 1.20 ± 2.34    | 17 ± 2.34    | 16 ± 2.91       | 450 ± 3.98 | 25 ± 2.97           | 82 ± 2.19            |
| F4           | 1.18 ± 3.17    | 14 ± 2.78    | 20 ± 1.81       | 540 ± 5.12 | 22 ± 5.21           | 56 ± 3.37            |
| F5           | 1.09 ± 2.56    | 9 ± 2.54     | 17 ± 1.89       | 580 ± 3.65 | 23 ± 3.65           | 58 ± 3.67            |
| F6           | 1.16 ± 2.67    | 14 ± 2.66    | 19 ± 2.91       | 760 ± 5.84 | 24 ± 4.45           | 53 ± 3.16            |
| F7           | 1.09 ± 2.85    | 9 ± 1.92     | 17 ± 3.08       | 575 ± 5.45 | 25 ± 5.22           | 61 ± 3.22            |
| F8           | 1.10 ± 1.92    | 10 ± 2.32    | 16 ± 2.52       | 480 ± 3.94 | 24 ± 5.13           | 78 ± 3.28            |
| F9           | 1.08 ± 2.52    | 8 ± 2.87     | 18 ± 1.96       | 590 ± 4.69 | 23 ± 3.77           | 63 ± 2.19            |
| F10          | 1.17 ± 2.69    | 15 ± 1.96    | 21 ± 2.41       | 810 ± 3.43 | 21 ± 3.50           | 49 ± 4.24            |
| F11          | 1.19 ± 3.04    | 16 ± 2.43    | 16 ± 1.95       | 390 ± 3.83 | 24 ± 2.96           | 75 ± 2.54            |
| F12          | 1.21 ± 2.88    | 18 ± 2.67    | 15 ± 3.17       | 345 ± 4.41 | 24 ± 3.652          | 71 ± 2.53            |
| F13          | 1.19 ± 2.24    | 17 ± 2.31    | 25 ± 1.78       | 740 ± 4.28 | 26 ± 4.79           | 42 ± 3.83            |
| F14          | 1.09 ± 1.97    | 9 ± 2.54     | 17 ± 2.67       | 577 ± 5.62 | 23 ± 4.08           | 63 ± 1.67            |
| F15          | 1.09 ± 2.65    | 9 ± 1.85     | 17 ± 2.65       | 580 ± 5.19 | 23 ± 5.31           | 60 ± 3.81            |
| F16          | 1.21 ± 2.39    | 18 ± 2.13    | 24 ± 1.94       | 695 ± 3.64 | 22 ± 2.9            | 45 ± 2.54            |
| F17          | 1.09 ± 1.59    | 9 ± 2.19     | 17 ± 2.34       | 577 ± 4.67 | 23 ± 4.18           | 66 ± 3.53            |
| F18          | 1.25 ± 1.22    | 20 ± 1.82    | 23 ± 1.97       | 735 ± 4.77 | 24 ± 5.73           | 39 ± 2.87            |
| F19          | 1.18 ± 2.23    | 16 ± 2.19    | 18 ± 2.67       | 250 ± 4.54 | 25 ± 2.99           | 71 ± 2.58            |
| F20          | 1.23 ± 2.57    | 19 ± 2.29    | 22 ± 3.17       | 690 ± 5.88 | 21 ± 4.51           | 45 ± 3.31            |
charge which can further be used to understand the physical stability of nanoparticles. Nanoparticles with high zeta potential (negative or positive) are considered as electrically stabilized systems while particles with low zeta potentials have a tendency to aggregate or coagulate leading toward poor physical stability. The magnitude of zeta potential may be positive or negative. A formulation with its all components exhibiting either positive or negative potential is considered as more stable and exhibits less agglomeration. In contrast, a formulation having some particles with negative potential and some particles having positive potential is regarded as less stable. The reason attributed to less stability of later formulation is associated to attraction of opposite charges toward each other. Furthermore, in-between same charges repulsion forces exist consequently; formulation remains stable for long duration of time. As shown in Figure 6B, zeta potential analysis of SA/KCZ-loaded MSNs exhibited only a single peak at $-23.1 \text{ mV}$, which covers 100% area. A negative potential (Table 4) was observed due to the presence of negatively charged silanol groups ($-\text{OH}$). The nanoparticles were found to be stable and hence there was no sign of aggregation in the graph. The zeta potential curve was observed to be in the defined limit and verified the stability of formulation. The steady and stable silica nanoparticles have tendency of facile dispersion and enhanced solubility in the media.

2.6. Scanning Electron Microscope. The study of the surface structure, either it is porous, rough or smooth and distribution of drugs in nanoparticles remained critical factors to make ultimately drugs available at the target wound site. In this regard, SEM analysis was carried out for surface morphology determination of drug loading in MSNs. As shown in Figure 6CD, MSNs were nearly composed of monodispersed spherical nanoparticles of about 500 nm average size. The size distribution of particles ranges from 300 to 900 nm. The study also revealed a porous structure of nanoparticles which is quite favorable for cell proliferation, cell attachment, and migration of cell which favors supply of oxygen particularly for the topical healing process. The SEM analysis of drugs-loaded MSNs exposed a very fine distribution of drugs throughout the MSNs. The results of SEM were in agreement with the outcomes of zeta particle size distribution analysis.

2.7. Evaluation of MSNs for Biomedical Applications.

2.7.1. In Vitro Anti-Fungal Performance. In vitro antifungal activities of pure SA/KCZ and SA/KCZ-loaded optimized MSNs were investigated at 3rd day, 5th day 7th day, and 14th day. Differences were observed in between the zone of inhibitions of CA by pure drugs and by optimized MSNs as presented in Figure 7A. On 3rd day, zone of inhibition was found 16.20 mm by pure drugs suspensions and 16.90 mm by optimized MSNs. Observations at 5th day depicted that the zone of inhibitor was 16.30 mm by drug suspensions and 17.30 mm by MSNs. Similarly, an increased value zone of inhibition was recorded that at 7th day that was 16.60 mm by pure drugs and 17.60 mm by MSNs. At 14th day, the maximum zone of inhibitor was observed about 16.70 and 17.90 mm by pure...
Comparative in vitro antifungal studies (A) and cytotoxicity studies of pure drugs and SA/KCZ-loaded optimized MSNs (B).

Table 5. Comparative in Vitro Antifungal Analysis of MSNs

| treatment/formulation               | zone of Inhibition (mm) | 3rd day | 5th day | 7th day | 14th day |
|-------------------------------------|-------------------------|---------|---------|---------|----------|
| SA/KCZ loaded MSNs                  |                         | 16.90   | 17.30   | 17.60   | 17.90    |
| pure SA/KCZ suspension.             |                         | 16.20   | 16.30   | 16.60   | 16.70    |

2.7.2. Cytotoxicity Studies. Cytotoxicity is one of the most important indicators for biological evaluation of formulation and formulation components in vitro studies. Cytotoxicity studies were assessed using the MTT assay. The principle of the assay is based on the ability of healthy cells to reduce MTT to the purple-colored formazan, while unhealthy/dead cells cannot. Cytotoxic effects of suspensions of pure SA, pure KCZ, and optimized MSNs formulation were studied on cancerous cells MCF7, whereby MCF7 cells were treated with the various formulations for 6 and 24 h. Cells that were treated with pure drug suspensions did not have a significant effect on cell viability. As expected, cells treated with optimized MSNs had a significant reduction in cell viability. After 6 h, about 86% cells were living for SA and KCZ. About 93% MCF-7 cells showed survival for SA/KCZ-loaded optimized MSNs as shown in Figure 7B. After 24 h, cytotoxicity was again evaluated and there was a little bit difference in the survival rate of cells as compared to the survival rate of cells at 6 h. The cell viability percentage for salicylic acid was 83 and 83.5% for ketoconazole. The cell viability percentage was 89% for TEOS and 92%, for SA/KCZ-loaded optimized MSNs. These cytotoxicity studies revealed that drug-loaded MSNs were more acceptable formulation with negligible harm to cells as they exhibited the lowest cytotoxicity as compared to pure drug suspensions and MSNs, respectively. The results of vitro antifungal studies revealed that activity of SA/KCZ loaded in MSNs is better formulation as compared to the pure SA/KCZ suspension (Table 5). The greater zone of inhibition measured for MSNs makes them superior as compared to pure suspensions with greater acceptability.

Table 6. Mean Erythema Scores Found for Various Formulations and in vivo Antifungal Studies in Three Groups of Rabbits

| sr. no. | formulation and treated groups | mean erythema scores | in vivo antifungal efficacy |
|---------|--------------------------------|----------------------|-----------------------------|
|         |                                | 1st day  | 7th day  | 14th day | rabbits having positive test/total no of rabbits | infected sites/log CFU |
| 1       | group I (control group)        | 0        | 0        | 0        | 6/6 | 4.24 ± 0.52 |
| 2       | group II (KCZ/SA suspension)   | 1        | 3        | 4        | 4/6 | 3.15 ± 0.37 |
| 3       | group III (drugs loaded MSNs)  | 0        | 1        | 0        | 0/6 | 0          |

2.7.3. Skin Irritation Studies. Ideally, the drug delivery system of SA/KCZ must be able to reduce these drawbacks. It was assumed that SA/KCZ-loaded MSNs would decrease its uninterrupted interaction with the skin stratum corneum. The outcomes of these trials showed optimized MSNs caused significantly less skin itching as compared to the SA/KCZ suspension as described in Table 6. The skin irritation continued to rise even after 14 days in the group applied with pure KCZ/SA suspension; however, no sign of skin irritation was detected in case of optimized MSNs (Figure 8a). Consequently, optimized formulation confirmed an extraordinary advantage as compared with pure drugs, thus improving the tolerability of the skin, representing their prospective in refining patient compliance and combined drug delivery of SA/KCZ.

2.7.4. In Vivo Antifungal Studies. The in vivo efficacy of optimized formulation of SA/KCZ-loaded MSNs was examined in the rabbit’s model (1.5–2 kg). Fungal infection in rabbits was induced by using isolated colonies of CA. Table 6 represents the effectiveness of SA/KCZ-loaded MSNs in rabbits against infection as compared to SA/KCZ suspension. The isolated colonies of viable organisms were detached from the lacerations of treated animals. It was verified that SA/KCZ-loaded MSNs represents superior efficacy in the eradication of infection, as zero animal from six presented a positive culture test, whereas in the case of SA/KCZ suspension, four animals out of six (Log CFU 3.15 ± 0.37) and in control group, 6/6 animals showed positive culture test with an average value of log CFU 4.24 ± 0.52 (Figure 8b). Rapid recovery from the fungal infection was observed in case of SA/KCZ-loaded MSNs, and no rabbit shows the sign of positive culture test (Figure 8b,C). Such notable effectiveness of the SA/KCZ-loaded MSNs was probably due to improved biodhesiveness nature, high occlusive property, improved oxygen supply to cells due to porosity of silica nanoparticles, persistent keratolytic action along with antifungal property, lack of bacterial growth under the atmosphere of silica particles, and continuous controlled availability of drugs at the site of action.

2.7.5. Wound Healing Studies. The significant morphological changes occurring during the wound healing process were observed at 1st, 3rd, 7th, and 14th day. Each wound was deep down into the subcutaneous layer of tissue; therefore, evident bleeding was detected throughout excision of the skin wound. Consequently, the skin area adjacent to the wound was inflamed and distinguishably red. After 24 h, inflammation in the skin area nearby the wound was detected. In MSNs-treated group, the edges of wounded skin were tending toward the middle and the wound seem contracted and more healed as compared to the skin area nearby the wound was detected. In MSNs-treated group, the edges of wounded skin were tending toward the middle and the wound seem contracted and more healed as compared to the skin area nearby the wound was detected.
compared to the control group. On the 3rd day of injury, the superficial area of the wounded skin in both SA/KCZ suspension and optimized MSNs treated groups was observed dry, inflammation around the wounded skin lessened, and it progressively instigated to prepare a pale yellow layer of crust; wounded area had reduced in both the groups but wound in control group was still circular and larger in extent; however, in treated group, wound had softer texture and irregularly oval or circular with a smaller diameter as compared to diameter of control group wound. On 7th day, inflammation in both

Figure 8. Skin texture images of rabbits obtained during skin irritation studies and in vivo antifungal studies in control group I (A), group II treated with suspension of pure drugs (B), and group III treated with SA/KCZ-loaded optimized MSNs (C).

Figure 9. Wound healing effect in (A) control group I, (B) group II treated with suspension of pure drugs, and (C) group III treated with SA/KCZ-loaded optimized MSNs at day-1, day-3, day-7, and day-14.
treated groups disappeared and the wounded edges had diminished. The crust pale color of wound was still observed in the control group, and wounded area was more persistent and deep in the control group as compared to that of treated groups which had harder texture. At 14th day, the wounded skin surface of the control group turned into hard crusts of pale black appearance; a portion of that might be shed and found skin tissue was uncovered beneath. In the treated group, the wounded area had virtually absolutely healed with a little crust in the SA/KCZ suspension-treated group and no crust in the MSN-treated group.

2.7.6. Histopathology Studies on Rabbits. Histopathology slides presented in Figure 10A revealed evidence of hemorrhage, accumulation of extracellular matrix, RBCs and blood clots. At day-3, thin sheet of the ECM without macrophages infiltration in the control group (D), ECM thin sheet with decreased macrophages infiltration in pure drug suspension-treated group (E), ECM thick sheet with heavy macrophage infiltration indicating the healing process started in the MSNs-treated group (F), at day-7, less macrophage infiltration with decreased mitosis in the control group (G), obvious macrophage infiltration, but without formation of follicular bases in the suspension treated group (H), heavy macrophages infiltration with cell rearrangement, fast mitosis and trans-differentiation in MSNs treated group (I) and at day-14, ECM sheet with macrophages but no appearance of follicular cell bases in the control group (J), wavy fibrous strands with evident macrophages infiltration, cells trans-differentiation and follicular bases formation in pure drugs suspension treated group (K), highest proliferation, differentiation, and revamping of skin tissue in the MSN-treated group (L).

Figure 10. Histopathological changes indicating at day-1, evidence of hemorrhage, accumulation of extracellular matrix, RBCs and blood clots (A–C), at day-3, thin sheet of the ECM without macrophages infiltration in the control group (D), ECM thin sheet with decreased macrophages infiltration in pure drug suspension-treated group (E), ECM thick sheet with heavy macrophage infiltration indicating the healing process started in the MSNs-treated group (F), at day-7, less macrophage infiltration with decreased mitosis in the control group (G), obvious macrophage infiltration, but without formation of follicular bases in the suspension treated group (H), heavy macrophages infiltration with cell rearrangement, fast mitosis and trans-differentiation in MSNs treated group (I) and at day-14, ECM sheet with macrophages but no appearance of follicular cell bases in the control group (J), wavy fibrous strands with evident macrophages infiltration, cells trans-differentiation and follicular bases formation in pure drugs suspension treated group (K), highest proliferation, differentiation, and revamping of skin tissue in the MSN-treated group (L).
filaments with obvious and heavy macrophage infiltration. Rearrangement of cells form squamous cell layer, rapid cell division, and trans-differentiation was also quite visible in the MSN-treated group.

At Day-14, as clear from Figure 10J, still sheet-like ECM and some of wavy fibrous strands were found. However, no appearance of follicular cell bases appeared in the control group. In the SA-KCZ suspension-treated group, most of the sheet-like extracellular structure had been replaced by wavy fibrous strand-like structures with evident infiltration by macrophages. Follicular base formation and trans-differentiation of cells have been started (Figure 10K). Figure 10L illustrated that proliferation and revamping of the skin tissue has been undoubtedly appeared. Comparatively, more differentiated cells as compared to the control group and SA-KCZ suspension treated were found. Over all, the tissues in the MSN-treated group (Figure 10L) were healing rapidly, and skin was growing at a faster pace as compared to the control group and drug suspension-treated group. Among all three groups, the growth of the skin tissue in the control group was found to be highly slow.

3. CONCLUSIONS

The unmet need of new therapeutic alternatives can successfully be fulfilled by designing SA/KCZ-loaded MSNs by the simple convenient in-situ sol gel approach for prolonged co-delivery of antifungals to deal an emerging resistance against antifungal agents. The software generated polynomial equations and applied statistical model clearly predicted the optimized formulation parameters for having better values of EESA (89%), EEKCZ (85%), and well controlled release SA (39%) and KCZ (43%). Physicochemical characterization exhibited an excellent compatibility of TEOS with both drugs with a transformation of crystalline forms of drugs to the amorphous nature in MSNs and uniform distribution of drugs in MSNs was also evident in SEM studies. As compared to pure drugs which exhibited cell viability of 83% after 24 h, SA/KCZ-loaded MSNs proved as less cytotoxic because they show cell viability of 92% revealing that drug-loaded MSNs were more acceptable formulation with negligible harm to cells as compared to pure drugs. It was further verified that SA/KCZ-loaded MSNs represent superior efficacy in eradication of fungal infection as zero animal from six presented a positive culture test, whereas in case of SA/KCZ suspension, four animals out of six and in control group, 6/6 animals showed positive culture test. A rapid recovery from the fungal infection along with improved wound healing effectiveness of the SA/KCZ-loaded MSNs was probably due to improved bioadhesive and occlusive properties of MSNs and a consistent controlled supply of medicaments at target wound. Similarly, greater zone of inhibition measured at 14th day for MSNs (17.90 mm) as compared to pure drug suspensions (16.70 mm) makes them superior with greater acceptability which was further confirmed from skin irritation studies where at day 14 and MSNs showed zero erythema score as compared to drug suspension which showed an erythema score of four. So, the mild skin itching, dry skin, rashes, and such drawbacks linked with SA/KCZ treatments would be overcome by MSNs, which will consequently improve tolerability and compliance by patients. In histopathology, comparatively more follicular bases formation, Proliferation, trans-differentiation of cells, and revamping of skin tissues as compared to the control group and the SA/KCZ suspension treated group were found in the MSN-treated group suggesting the most rapid wound healing in this group. Consequently, the combination of therapy-loaded MSNs seems to be a potential strategy to improve patient compliance by enhancing synergistic antifungal efficacy at reduced dose, reducing the toxicity of therapeutic agents and accelerating the healing of fungal infections. In future, the potential of drug-loaded MSNs may further be tested against fungus species other than CA.

4. MATERIALS AND METHODS

4.1. Materials. KCZ was obtained as a kind gift sample from Nabi Qasim Pharmaceuticals (Pvt.) Ltd. Lahore, Pakistan. Tetraethoxysilane (TEOS), SA, ammonium hydroxide (NH₄OH), ethanol (95%), calcium chloride (CaCl₂), and hydrochloric acid (HCl) were purchased from Sigma Aldrich. All the other chemicals, solvents and reagents used in the study were of analytical grade.

4.2. Preparation and in Vitro Characterizations of Drug-Loaded MSNs. 4.2.1. Central Composite Rotatable Design. The formulation of nanoparticles is greatly affected by different variables such as concentration of polymer, pH, surfactant, stirring time and stirring speed. To optimize the formulation variables, the old conventional optimization techniques allow the variation of single variable while keeping all other variables at a constant level. Moreover, these conventional procedures may not elaborate the concurrent interactive influence of different variables and may also cause a huge consumption of time and excipients. So, in such situation, the statistical optimization procedure like CCRD seems to be an encouraging technique to examine mathematically the simultaneous impact of different independent formulation variables on features (Responses) of nanoparticles without any wastage of time and materials and it can best be employed with the help of software like Design Expert. A three-factor, five-level CCRD was developed to statistically investigate the individual and combined effects of selected formulation variables like pH (A), stirring time (B), and stirring speed (C) on dependent variables of nanoparticles such as EEₐ (Y₁), EEKCZ (Y₂), SA releases (Y₃), and KCZ release (Y₄) were selected. Table S1 presents the actual and coded values of independent factors. In current study, the software design expert version-12 was used which has suggested total 20 experimental MSN formulations.

4.2.2. Preparation of MSNs. The sol-gel method was used for the preparation of drug-loaded MSNs. Distilled water and freshly prepared 0.1 M HCl were added in ethyl silicate, while adjusting the speed of a magnetic stirrer at 200 rpm for 5 min. Solutions of SA (3%) and KCZ (10%) were prepared in ethanol separately and then were added in above solution with continuous mixing. The reaction mixtures were subjected to cooling at 4 °C while maintaining pH=5.83 using 0.08 M NH₄OH and gelation time was observed. Afterward, the resulting sol was added dropwise into 100 mL of vegetable oil under constant stirring at the speed of 1700 rpm till nanoparticles precipitated at the bottom of the beaker. The nanoparticles were filtered, rinsed with distilled water, and allowed to dry at room temperature. Twenty different formulations of drugs-loaded nanoparticles were prepared with the change of variables as suggested by CCRD (Table S1).

4.2.3. Selection of Optimized Formulation. After analyzing the PY, EE, and drugs release from twenty formulations suggested by CCRD, the numerical optimization technique
was implemented to achieve desirable outcomes of the studied responses by creating optimum conditions of formulation variables. In this technique, detailed feasibility investigation was also performed; the optimized formulation conditions were chosen to achieve maximum PY, higher EE, but slow release of both drugs. These responses of MSNs were also examined on the desirability scale (0–1) using design expert, and formulation with higher desirability factor was considered as optimized. The suggested optimized MSNs were then prepared, evaluated, and subjected to further in vivo/in vitro antifungal studies, skin irritation, cytotoxicity, wound healing, and histopathology studies. For optimized nanoparticles, a comparative analysis of calculated and predicted outcomes of all studied responses was made to calculate the prediction error (PE) using following eq 5.

\[
PE(\%) = \frac{\text{experimental value} - \text{predicted value}}{\text{predicted value}} \times 100
\]

(5)

4.2.4. Physicochemical Characterizations of MSNs. FTIR studies were carried out to investigate the loading of drugs with respect to structural interactions between SA, KCZ, and MSNs. FTIR spectra were recorded using the shimadzu instrument, IR prestige 21. The region for scanning was in the range of 400–4000 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\) for 20 scans.\(^{37}\)

XRD analysis for SA, KCZ, and SA/KCZ-loaded MSNs was carried out to determine the changes in the crystalline nature of drugs. The samples were exposed to monochromatic X-rays generated by Cu Ka using the D8 advance X-ray diffractometer (Bruker AXS, Madison, WI, USA) at a current of 40 mA by using rays with a voltage of 40 kV. The samples were scanned at a rate of 20/min in the diffraction angle (2θ) range from 0 to 45°. The samples were subjected to irradiation with monochromatized X-rays.\(^{37}\)

Thermograms of pure drugs, SA, and KCZ as well as SA/KCZ-loaded MSNs were recorded on a TGA/DSC-1 Star system (Mettler Toledo, Switzerland). The samples were sealed into hermetic pans of aluminum and exposed to the instrument to study thermal behavior of the samples combined for DCS and TGA. The scanning rate was 20 °C/min covering the temperature range between 40 and 300 °C. The analysis was carried out under an inert atmosphere by flowing nitrogen at a rate of 50 mL/min.\(^{38,39}\)

The shape and surface morphology of optimized formulation of drug-loaded MSNs were studied by the scanning electron microscope (MIRA 3 TESCAN). The samples in the form of solutions were placed on a double adhesive tape, and adhesive tape was then struck to an aluminum stub. After the evaporation of the solvent, the prepared samples were coated with gold to make them electrically conductive under an inert atmosphere. The photomicrographs of SA/KCZ-loaded MSNs were obtained at various magnifications at 20 kV.\(^{40}\) The shape, particle size, surface charge, yield of nanoparticles, drug excipients compatibility, crystallographic changes in drugs, thermal stability, release of drugs from MSNs, and entrapment efficiency of nanoparticles were extensively studied during physicochemical analysis of MSNs.

The equations to calculate the percentage yield and entrapment efficiency are discussed in Supporting Information (S1, S2). Methods and equations to calculate kinetics of drug release are discussed in Supporting Information (S3). The measurement of particles size and surface charge of SA/KCZ-loaded MSNs are discussed in Supporting Information (S4), whereas in vitro release study is discussed in Supporting Information (S5).

4.2.5. Rheological Properties. Rheological studies deal with the flow properties of formulations and can be studied with the help of different formula discussed below. After finding bulk volume (\(V_b\)) and tapped volume (\(V_t\)), the calculation of Carr’s index (I) was conducted using eq 6\(^{39}\)

\[
I = \frac{V_b - V_t}{V_t} \times 100
\]

(6)

The Carr’s index value ranges from 12 to 19% represent good flow character and higher than 21% suggest poor flow properties.\(^{39}\) Hauser’s ratio is a ratio between tapped density (\(\rho_t\)) and bulk density (\(\rho_b\)) of a material and was calculated as per eq 7

\[
\text{Hauser ratio} = \frac{\rho_t}{\rho_b}
\]

(7)

The ratio higher than 1.25 depicts poor flow characteristic, and a value lower than 1.25 presents good flow property.\(^{39}\) For the angle of repose, a specified amount of MSNs was passed through the funnel on a plain sheet of paper. The falling MSNs made a heap on the sheet of paper. The height (h) and radius (r) of the heap were measured, and these values were used to determine angle of repose with the help of following formula

\[
\tan \theta = \frac{h}{r}
\]

(8)

Free flow behavior of MSNs is confirmed from a value of angle of repose less than 30°.

4.3. Evaluation of MSNs for Biomedical Applications. 4.3.1. In vitro/In Vivo Antifungal Performance. For antifungal performance, Candida albicans (CA) was taken as standard strain for current study. Diffusion test was applied on Mueller Hinton agar with slight modification, and culture of CA strains was prepared according to standards.\(^{41}\) CA cells were prepared by shifting single colony of CA from agar plates to sabouraud dextrose broth by inoculation, which was placed overnight in aerobic conditions at 28 °C. In different Petri plates of Mueller Hinton agar, then, inoculation of CA strains was done with sterile cotton swab presoaked in suspension of adjusted strength. The streaking of the swab was done over a whole surface of agar, and process of streaking was repeated 2 times by rotating the agar plate at approximately 60°. After this process, 3–4 mm well cavity was punched and 50 μL SA/KCZ suspension and 50 μL SA/KCZ-loaded MSNs were introduced into cavities of separate plates. The agar plates were placed at room temperature for 2–3 min and then incubated at 28 °C under air for 24 h. Measurement of the zone of inhibition from a sharp decline in growth density was carried out with calipers.

Accurately weighed rabbits were categorized into three different groups with six rabbits in each group. Fungal infection using CA was induced on rabbits by minor alteration of a previously described method.\(^{31}\) Briefly describing, the hair from the back of the rabbits was shaved (using dermatologic hair removing cream) from an area of 4 × 4 cm². On the next day, the skin area was slightly scratched with the help of sandpaper and 600 mg of earlier prepared inoculum of candida was applied on hair free skin area by using a glass rod. Formulations like pure SA/KCZ suspensions and SA/KCZ-loaded optimized MSNs were applied for 14 days, initiating
from the postinfection day to rabbits of two treated groups. The animals that were present in the control group were also infected but ensured not to receive any drug treatment. The rabbits were uninterruptedly detected visually for any alterations in skin texture of the infected skin area later to the commencement of therapy. The antifungal activity of all groups to treat mycosis was compared with the control group, texture of skin was examined, and treatment time was noted. The antifungal activity of the groups was compared with the control group. After 14 days of respective therapy, the skin area was removed from the treated site and homogenized in 4 mL of saline using the tissue homogenizer. A portion of the homogenate was spotted on the solidified sabouraud dextrose medium. All petri plates were incubated in an incubator at 25 °C for 6 days. The numbers of colony forming units (CFUs) were counted, and for each infected site, the logarithm of number of CFUs was designed. Rabbit was measured as fungus positive after more than one colony of fungus was observed.

4.3.2. Skin Irritation Testing. Some drawbacks linked with the SA and KCZ treatments are mild skin itching, dry skin, and rashes, which limits their applications and tolerability by patients. The skin irritation probability of SA/KCZ suspension in comparison with optimized nanoparticle formulation was evaluated by performing Draize patch test on male and female rabbits (1.5–2.5 kg). Animals were divided into three groups, each group having 3 rabbits.

Group I: control group (no treatment was given).
Group II: group treated with the suspension of SA/KCZ.
Group III: group treated with SA/KCZ-loaded MSNs.

The hair from the back of the rabbits was shaved 24 h prior to the formulation application. Optimized MSNs were applied by uniform spreading on a hair-free skin area of 3 cm². Then, skin was examined for any type of erythema on 1st day, 7th day, and 14th day after the application of drugs containing suspension and drug-loaded MSNs. The average erythema scores were then documented as:

- No sign of erythema = 0, minor erythema = 1, modest erythema = 2, modest to severe erythema = 3, and severe erythema = 4.

4.3.3. Cytotoxicity Studies. The safety of nanoparticles and its formulation components was evaluated by cytotoxicity studies which were performed on MCF-7 cell lines. In order to study the cytotoxicity effect, MCF-7 cells (breast cancer cell line) were cultured in 96-well plates in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). DMEM was exchanged after every 48 h. One day before the cell viability studies, the cells were fed with DMEM without FBS. To study the potential cytotoxic effects, 0.5% dispersions of various formulation such as pure drug suspensions and optimized formulation of SA/KCZ loaded MSNs were prepared in DMEM. MCF-7 cells were incubated with these dispersions for 6 h and 24 h. Pure DMEM served as a positive control, Triton X-100 was used as a negative control. After completion of incubation, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Samples were removed, and the cells were washed three times with isoticophosphate buffered saline (PBS). Afterward, working solution of MTT (500 μL) in FBS-free DMEM (0.5 mg/mL) was added to each well, and the cells were incubated for another hour. Subsequently, the supernatants were removed. The amount of MTT dye reduced to formazan crystals was dissolved in 500 μL dimethyl sulfoxide (DMSO). This solution was then transferred to 1.5 mL tubes and centrifuged at 13,400 rpm for 2 min. The absorbance of the resulting solution was recorded immediately at λ = 570 nm after dilution of the samples with an equal volume of dimethyl sulfoxide (DMSO). Cell viability rates were calculated by relating all values to 100% cell viability of cells treated with DMEM.

Cell viability rates were calculated according to the following equation:

\[
\text{cell viability (\%) = } \frac{A_i}{A_d} \times 100
\]

Here, \(A_i\) is the absorbance measured after treatment with tested sample dispersions, and \(A_d\) is the absorbance measured after treatment with DMEM.

4.3.4. Wound Healing Studies on Rabbit Skin. This experiment was approved by the Ethical Committee of College of Pharmacy, University of Sargodha, Sargodha, Pakistan. A group of 8-weeks old male and female rabbits (1.5–2 kg) were individually housed in controlled conditions into hygienic cages (12 h dark—light cycle at 65–70% relative humidity 20–25 °C temperature) for two weeks to allow time to become acclimatize. The backs of the rabbits were clipped free of hair, 24 h earlier to the process. On the next day, the hair free area was cleaned germ-free with 5% iodophor and 76% ethanol, and a full-thickness excision on hair free skin was surgically made. For this operation, rabbits were first anesthetized with an IV pentobarbital sodium injection, to mark the circular area of 2 cm. The rabbits were divided into three groups that is control group, SA/KCZ suspension treated group, and SA/KCZ-loaded MSN-treated group. In the control group, the wounded area was treated with Vaseline topically and dressed with the thin surgical gauze fortified by a paragon bandage. The wounded areas of all the three groups were sensibly observed, and images were taken after different time intervals that is, 1st, 3rd, 7th, and 14th day.

4.3.5. Comparative Histopathology. Wounded area tissue samples of about 1.5 cm from all the three groups were harvested on 1st, 3rd, 7th, and 14th day respectively for histological evaluation after the wound injury. Excised tissues were retained in 3.7% formaldehyde preservative solution at pH 7.4 for about 24 h. All tissues were processed by using conventional histochemical techniques, dehydrated, and cleaned, and about 3 μm thick section were fixed on the adhesive glass slides by applying standard measures and stained. The wounded tissue segments were evaluated, and images were taken under a light microscope (Olympus BX3-CBH).

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c06242.

Percentage yield; entrapment Efficiency; kinetics of drug release; particle size and zeta potential determination; in vitro release study; and formulation variables and dependent factors of SA/KCZ-loaded MSNs (PDF)

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Notes

The authors declare no competing financial interest.

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