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Enhancing pharmaceutical potential and oral bioavailability of *Allium cepa* nanosuspension in male albino rats using response surface methodology

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ABSTRACT

Objective: To enhance the pharmaceutical potential and oral bioavailability of quercetin contents of *Allium cepa* peel extract by novel nanosuspension technology.

Methods: Nanoprecipitation approach was successfully used for the formulation of nanosuspension. To obtain pharmaceutical-grade nanosuspension with minimum particle size and polydispersity index, sodium lauryl sulphate was selected as a stabilizer. Important formulation parameters were statistically optimized by the response surface methodology approach. The optimized nanosuspension was subjected to stability and *in vitro* dissolution testing and characterized by scanning electron microscopy, atomic force microscopy, Fourier transform infrared spectroscopy, and zeta sizer. To evaluate the preeminence of nanosuspension over coarse suspension, comparative bioavailability studies were carried out in male albino rats. The pharmaceutical potential of developed nanosuspension was evaluated by antioxidant, antimicrobial, and toxicity studies.

Results: The optimized nanosuspension showed an average particle size of 275.5 nm with a polydispersity index and zeta potential value of 0.415 and −48.8 mV, respectively. Atomic force microscopy revealed that the average particle size of nanosuspension was below 100 nm. The formulated nanosuspension showed better stability under refrigerated conditions. Nanosuspension showed an improved dissolution rate and a 2.14-fold greater plasma concentration of quercetin than coarse suspension. Moreover, the formulated nanosuspension exhibited enhanced antioxidant and antimicrobial potential and was non-toxic.

Conclusions: Optimization of nanosuspension effectively improves the pharmaceutical potential and oral bioavailability of *Allium cepa* extract.

KEYWORDS: *Allium cepa*; Nanoprecipitation; Pharmaceutical potential; Particle size; Polydispersity index; Stability; Toxicity; Quercetin

1. Introduction

Formulation of drugs having poor water solubility always remained a challenging task for pharmacists[1]. Almost 40% of the drugs that are produced in drug discovery programs have poor water solubility[2]. Drugs having poor solubility and low permeability in the gastrointestinal tract result in poor oral bioavailability[3]. Along with synthetic drugs, there are many natural substances, including plant constituents (flavonoids, essential oils, and flavors), which

Significance

Quercetin is a principal bioactive constituent present in *Allium cepa* peels and is of prime importance in the pharmaceutical industry. However, poor aqueous solubility and low oral bioavailability limit its efficacy. The present study showed that the pharmaceutical potential and oral bioavailability of quercetin contents of *Allium cepa* peels extract were enhanced by novel nanosuspension technology.

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are pharmaceutically important but suffer from poor aqueous solubility[4]. Therefore, new technological formulation techniques are required to improve the water solubility of pharmaceutics[5]. Nanosuspension technology has proved to be an efficient approach in this regard which helps to enhance the solubility and bioavailability of drugs due to their simplicity and greater benefits compared to other techniques[3]. Nanotechnology-based herbal drugs possess efficient biopharmaceutical properties and desirable target characteristics[6] because of their smaller size and greater surface area available for dissolution[7].

Response surface methodology (RSM), as a collection of mathematical and statistical methods, provides a suitable experimental design with a minimum number of experimental runs[8]. This experimental design can decrease the experimentation time and cost and increase reliability with better process output[9]. In the present study, central composite design (CCD) of RSM was used for the optimization study.

Allium cepa L. (*A. cepa*), commonly known as onion is a bulbous herb belonging to the family Alliaceae. It is one of the most frequently consumed vegetables and has numerous health benefits owing to its diverse phytoconstituents[10]. In onion, a large number of polyphenols and flavonoids such as quercetin, kaempferol, and their glycosides are present that have a defensive effect against coronary heart diseases and cancer[11]. The antioxidant and free radical scavenging potential of onion increases from inner to outer layer and maximum quercetin contents are reported in the outermost layer (peels) of onion[12]. Due to its rich quercetin contents, *A. cepa* peel extract has been used to treat numerous diseases[13,14] and is regarded as an important ingredient in pharmaceutics[15].

In the present study, nanoprecipitation approach was used to formulate *A. cepa* nanosuspension and the CCD of RSM was used for optimization. The optimized nanosuspension was characterized by microscopic techniques and evaluated for *in vivo* oral bioavailability in rats. The pharmaceutical potential of formulated nanosuspension was evaluated by determining *in vitro* antioxidant, antibacterial, and antifungal activities. In addition, a mutagenicity assay was performed to determine any toxicity related to nanosuspension.

Present research is significant because, in this study, an attempt is made to enhance the oral bioavailability of quercetin contents of *A. cepa* peel extract to improve its pharmaceutical potential. Such detailed optimization using RSM approach to formulate pharmaceutical-grade nanosuspension of *A. cepa* peel extract was scarce in earlier studies. However, *A. cepa* was used to prepare metal nanoparticles in many previous studies[16–19]. Metal nanoparticles are different from herbal nanosuspension/nanoparticles. In metal nanoparticles, the particle size of metal is reduced and converted to nano-range using plant extract as a stabilizing and reducing agent. The drug (plant extract/synthetic drug) is converted into nanoparticles using different techniques and excipients in nanosuspensions.

2. Materials and methods

2.1. Preparation of plant extract

A. cepa peels were purchased from the local vegetable market and identified by a plant taxonomist of the Botany Department and a voucher specimen (228-3-2016) was placed at the herbarium of the Botany Department, University of Agriculture, Faisalabad. Peels were washed with double distilled water, dried under the shade, and ground to a fine powder. Excessive oil or fat contents were removed by using *n*-hexane. For the extraction of crude quercetin, an oil-free sample was extracted with ethanol (95% HPLC grade) for about 6-8 h using a Soxhlet extractor (Behr Labor-Technik, Germany). The resultant extract was concentrated by using a rotary evaporator (Buchi, CH-9230 Flawil 1, Switzerland) and the residue obtained was used for the formulation of nanosuspension.

2.2. Preparation and statistical optimization of nanosuspension

Nanoprecipitation method was employed for the preparation of nanosuspensions by following the method of Zafar *et al.*[6] with some modifications. For this purpose, the quercetin-rich *A. cepa* peel extract was fully dissolved in an organic phase (ethanol) and filtered. The resulting solution was gradually inserted (1 mL/min) into an aqueous phase containing stabilizer sodium lauryl sulfate (SLS) with constant mechanical stirring (Lab Mechanical Stirrer JJ-1, China) at 6 000 rpm for 6 h at room temperature. To obtain nanosuspension with the smallest particle size and polydispersity index (PDI), imperative formulation parameters such as amount of plant extract (A), concentration of stabilizer (B), and antisolvent to solvent (AS/S) ratio (C) were statistically optimized by employing the most emergent CCD of RSM using design expert software (version 7.1, Stat-Ease, Inc. USA). A total of twenty experiments (including 6 replicates) were carried out to optimize the formulation parameters according to the experimental design given in Supplementary Table 1. Results were evaluated by applying the analysis of variance (ANOVA) and by three-dimensional (3D) response surface plots.

2.3. Preparation of coarse plant suspension and standard quercetin solution

For comparative study, coarse suspension of *A. cepa* was prepared by simply dissolving highly concentrated *A. cepa* peel extract (0.13 g) in 100 mL distilled water[20] to keep the concentration of plant (drug) same as used in optimized nanosuspension (*i.e.* 0.13%). Similarly, standard quercetin solution (0.13%), was prepared by dissolving 0.13 g of pure quercetin in 100 mL of distilled water.

2.4. Characterization of nanosuspension

Mean particle size (z-average; nm) and PDI values of the prepared

nanosuspensions were determined by dynamic light scattering (DLS) technique, whereas zeta potential (ZP) was determined by measuring electrophoretic mobility with a field strength of 20 V/cm employing Malvern zeta sizer Nano ZS (Malvern Instruments, UK). For further characterization, optimized nanosuspension (nanosuspension with minimum particle size and PDI value, prepared by using 0.13% plant extract, 0.48% stabilizer, and AS/S ratio of 11.69) was frozen and then lyophilized for 72 h at −40 ℃. Atomic force microscopy (AFM) (Shimadzu WET-SPM 9600, Tyoto Japan) analysis was carried out for three-dimensional (3D) characterization of nanosuspension. Scanning electron microscopy (SEM) (JEOL, JSM-6400, Japan) was used to determine the particles' surface morphology. Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer Spectrum Version 10.4.3) analysis was carried out to determine the drug excipient interactions.

2.5. Stability studies

The stability of optimized nanosuspension (nanosuspension with minimum particle size and PDI value, prepared by using 0.13% plant extract, 0.48% stabilizer, and AS/S ratio of 11.69) was measured at two different temperatures, at 4 ℃ (in a refrigerator) and 25-30 ℃ (at room temperature) for three months[21]. Parameters like physical appearance, particle size, PDI, and zeta potential were determined after storage for three months. Particle size, PDI, and zeta potential values of freshly formulated optimized nanosuspension were used as a reference[22]. Results were also compared with coarse suspension of *A. cepa* peel extract.

2.6. Determination of antimicrobial activity

The antimicrobial potential of standard quercetin, optimized nanosuspension, and coarse plant suspension was determined by using two bacterial strains (*Escherichia coli* and *Bacillus subtilis*) and one fungal strain (*Aspergillus niger*) by standard disc diffusion method[23]. Fluconazole was used as a positive control for antifungal activity. Methanol and rifampicin were employed as negative and positive control, respectively for antibacterial activity.

2.7. Determination of antioxidant activity

Antioxidant activity of standard quercetin, coarse plant suspension,

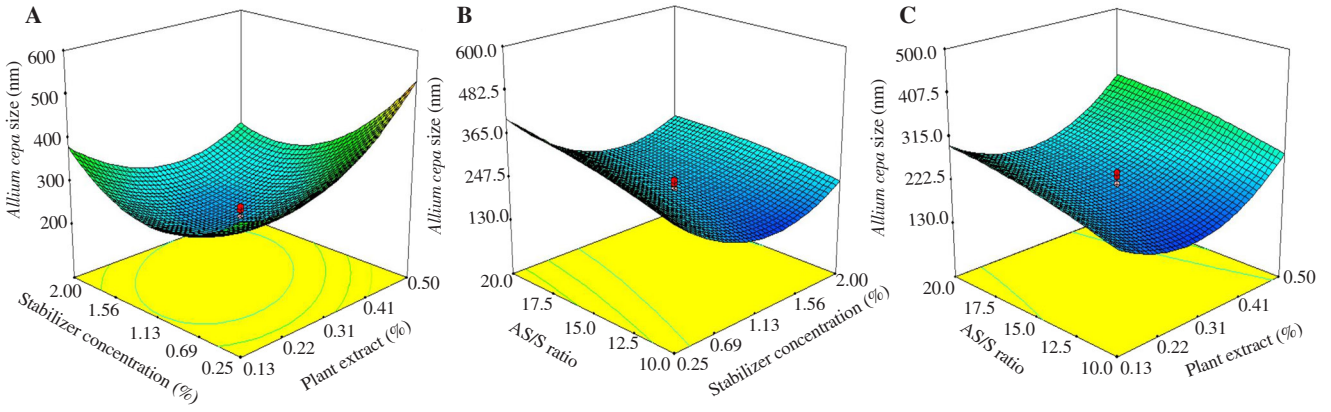

Figure 1. Three-dimensional response surface plots illustrating the combined effect of (A) plant and stabilizer, (B) stabilizer and AS/S ratio, and (C) plant and AS/S ratio on particle size reduction of *Allium cepa* nanosuspensions. AS/S: antisolvent to solvent.

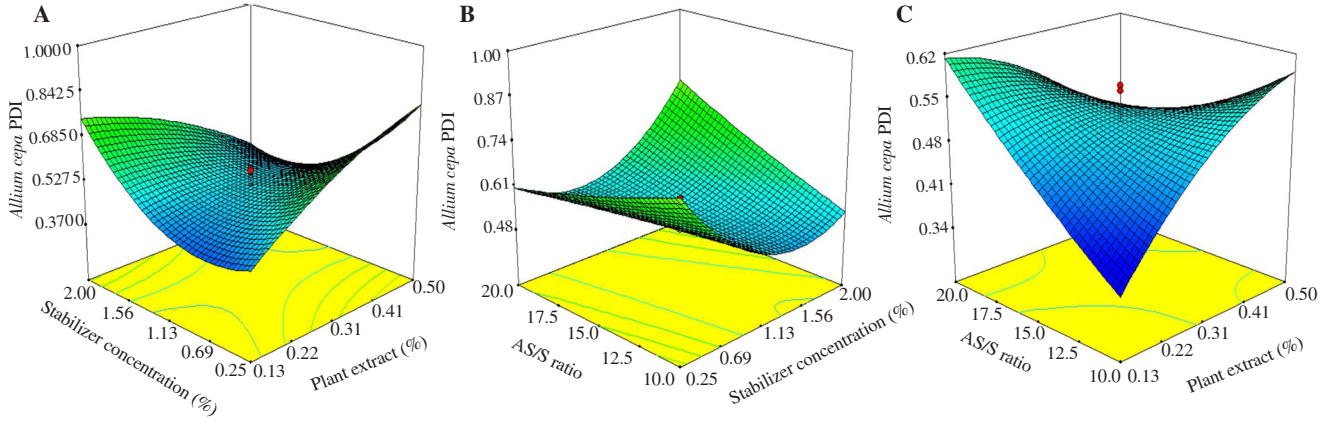

Figure 2. Three-dimensional response surface plots illustrating the combined effect of (A) plant and stabilizer, (B) stabilizer and AS/S ratio, and (C) plant and AS/S ratio on PDI reduction of *Allium cepa* nanosuspensions. PDI: polydispersity index.

and optimized nanosuspension was evaluated by DPPH assay following the method of Zafar *et al*[24]. Ascorbic acid was used as a standard compound to compare results. The formula employed for the calculation of percentage inhibition of DPPH radical was given as follows:

Percentage inhibition of DPPH $=(1-A_1/A_0) \times 100$

Where A_1 = Absorbance of samples, A_0 = Absorbance of control.

2.8. Toxicity testing

To determine the *in vitro* toxicity of formulated nanosuspension, mutagenicity assay (Ames test) was used. The test was based on the "Ames bacterial reverse mutation assay" and carried out in liquid culture. Two mutant strains, *Salmonella typhimurium* (*S. typhimurium*) TA100 and *S. typhimurium* TA98 were used to determine the toxicity related to *A. cepa* coarse plant extract, nanosuspension, and standard quercetin. For mutagenicity assay and for interpreting the results, method of Zafar *et al.*[25] was followed.

2.9. Dissolution testing

Dissolution testing was performed by employing USP dissolution apparatus type Ⅱ (pharma test de, ISO 9001, Germany) by following the method of Gera *et al*[26]. For dissolution studies, lyophilized optimized nanosuspension (nanosuspension with minimum particle size and PDI value, prepared by using 0.13% plant extract, 0.48% stabilizer, and AS/S ratio of 11.69) and coarse plant extract (500 mg each) were separately filled in capsules and placed in dissolution medium (0.1 M phosphate buffer at pH 7.4). Temperature of the dissolution media and paddle speed were set to (37±0.5) ℃ and 50 rpm, respectively. Aliquots (5 mL) were collected from dissolution media after specific time (0, 15, 30, 45, 60, 75, 90, 105, and 120 min). The sink conditions were maintained by regularly adding the pre-warmed dissolution medium to the dissolution vessel. Pure quercetin was used as a standard compound and samples were analyzed at 373 nm wavelength (λ_{max} of quercetin) spectrophotometrically. The experiment was carried out in triplicate, and the results were expressed as percentage of drug dissolved for nanosuspension and coarse plant extract.

2.10. In vivo bioavailability study

Male Wistar albino rats weighing 250-300 g were used for bioavailability studies and kept for a one-week acclimatization period before the experiment and were treated as per principles of 3R's. Rats were divided into two groups with five rats in each group and fasted overnight before administering the dose but provided with free water access. For comparative bioavailability studies, rats of group 1 were administered orally with coarse suspension of *A. cepa* extract (50 mg/kg body weight), whereas rats of group 2 were orally administered with *A. cepa* nanosuspension at a similar dose. After specific time intervals of 0.5, 1, 2, 4, 6, 12, and 24 h, blood samples (0.5 mL) were collected into sodium heparinized tubes by cardiac puncture. Tubes were immediately centrifuged (model 800, China) at 170 ×*g* for 20 min and separated plasma was preserved at −20 ℃ for further analysis.

For extraction of quercetin from plasma, separated plasma (200 μL) was added to eppendorf tube containing methanol (400 μL) and HCl (25%, 200 μL). The mixture was vortexed for 90 s and incubated at 50 ℃ water bath for 15 min. The resulting mixture was centrifuged (Bolton scientific, UK) at 1 064 ×*g* for 15 min and the separated clear supernatant was injected into an HPLC column (Supelco analytic HS, C-18) for detection of quercetin contents in plasma samples. The flow rate of mobile phase [acetonitrile 80%, methanol 20%, and trifluoroacetic acid 3% (for acidification)] was set to 1 mL/min. The effluents were detected at 370 nm wavelength using a UV-visible detector (SPD-10A, Shimadzu). For analyzing the chromatograms, acquisition software (Class LC-10, Shimadzu) was used. Important pharmacokinetic parameters such as C_{max}, T_{max}, and area under the curve ($AUC_{0\text{-}24h}$) were determined to compare the oral bioavailability of coarse suspension and nanosuspension.

2.11. Ethical statement

Animal studies were performed according to the rules and regulations of international ethical committee under the guidance of veterinary doctors of Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad. The protocol was approved by the graduate study research board on 9 March 2016 *via* letter-number GDS/15501-4.

2.12. Statistical analysis

To optimize the best conditions for the preparation of pharmaceutical-grade nanosuspension with the least particle size and PDI, CCD of RSM was successfully used. The impact of independent variables on response parameters was checked using ANOVA. Results of antioxidant and antimicrobial assay and dissolution study were expressed as mean ± SD (n=3). Outcomes of pharmacokinetic parameters were expressed as mean ± SD (n=5).

3. Results

3.1. Optimization of nanosuspension

In the present study, nanoprecipitation approach was used to formulate *A. cepa* nanosuspension using a SLS stabilizer. To

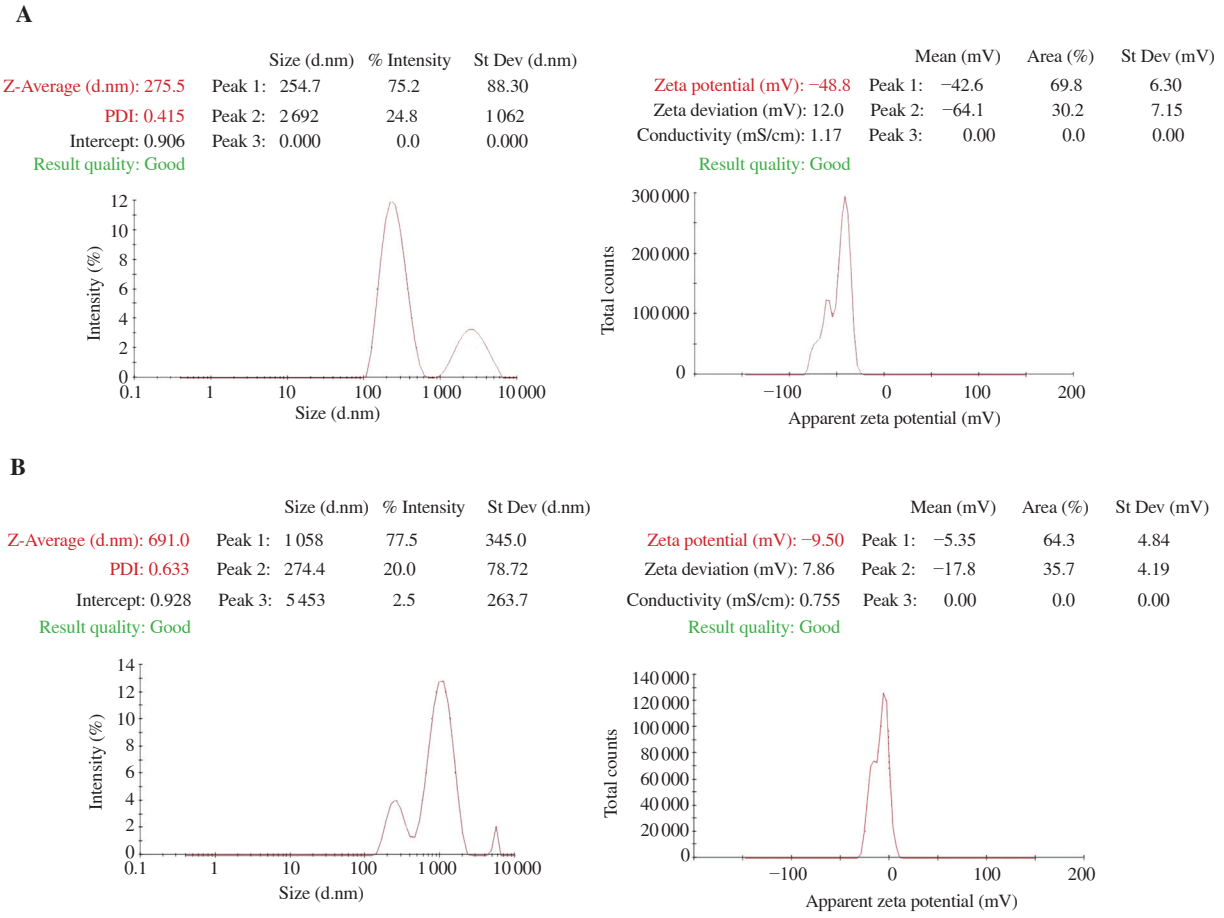

Figure 3. Particle size, polydispersity index (PDI), and zeta potential results of (A) optimized *Allium cepa* nanosuspension and (B) *Allium cepa* coarse suspension. d.nm: diameter in nanometer.

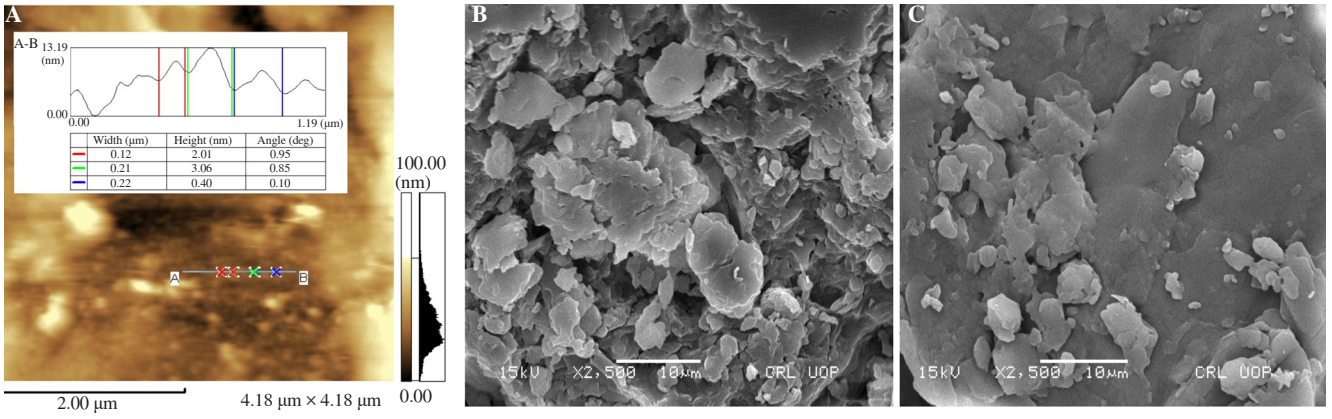

Figure 4. Atomic force microscopic profile (A) and scanning electron microscopic image (B) of *Allium cepa* nanosuspension, (C) *Allium cepa* coarse suspension.

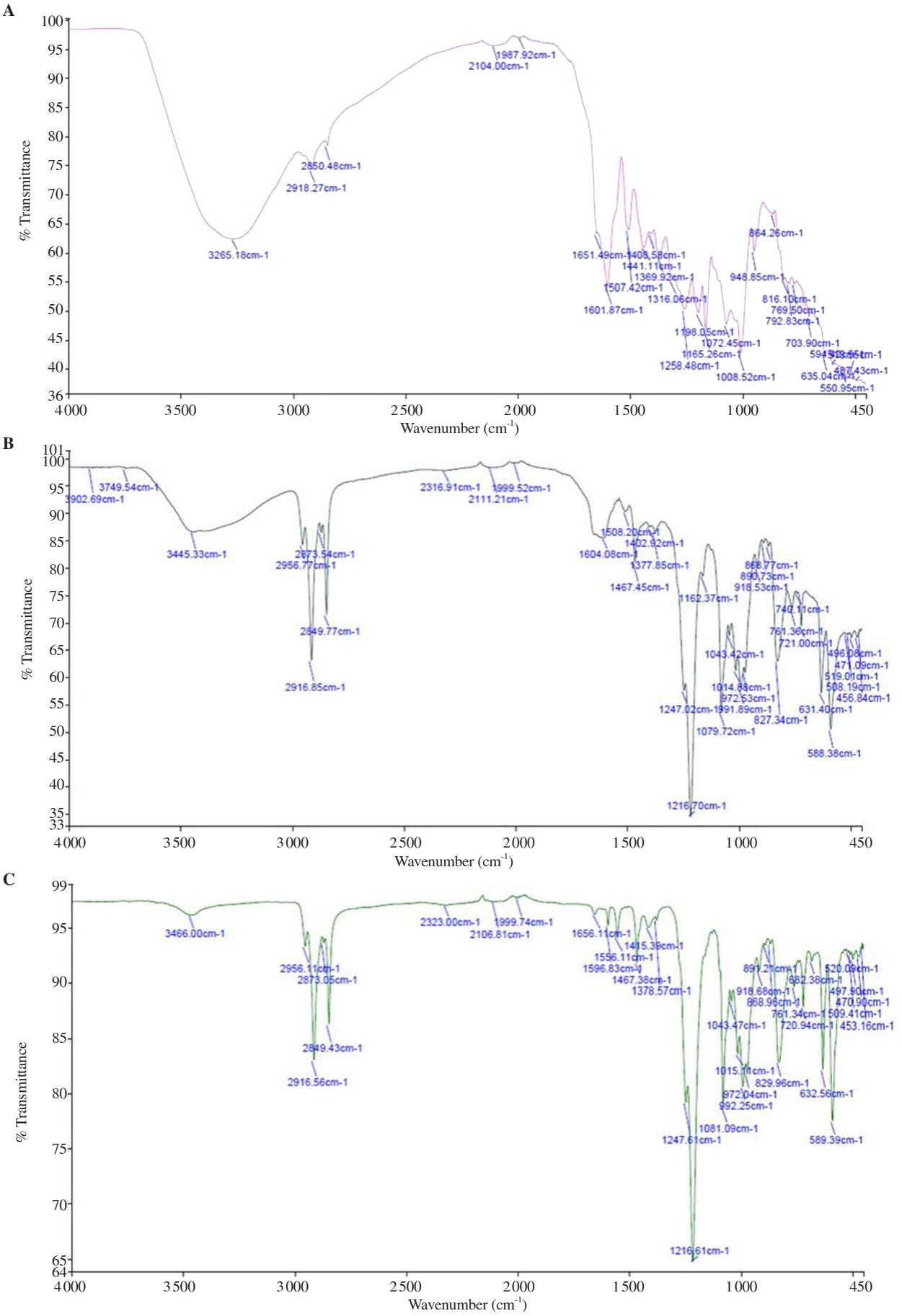

Figure 5. Fourier transform infrared spectroscopic spectra of *Allium cepa* (A) coarse plant extract, (B) nanosuspension and (C) stabilizer.

optimize important formulation parameters, CCD of RSM was successfully used, which proposed quadratic model as most suitable to elucidate the relationship between independent variables [amount of plant extract (A), concentration of stabilizer (B), and antisolvent to solvent (AS/S) ratio (C)] and response variables (particle size and PDI). Model selection was made based on the smallest probability values (*P* values) with larger *F* values. Regression equations for the response variables, particle size (R1), and PDI (R2) were established (Equations 1 and 2) to explore the positive or negative effect of independent variables on dependent variables. The coefficients of the equation with a positive sign indicated the synergistic effect of independent variables on R1 and R2, whereas the coefficients with a negative sign showed an inverse relation.

A. cepa (Size-nm) (R1)= +218.14+27.32A−48.62B+35.40C−59.80AB −1.82AC−17.82BC+80.09A^2+98.67B^2−11.07C^2 (Equation 1)

A. cepa (PDI) (R2)= +0.52+0.010A−0.015B+0.021C−0.16AB− 0.11AC+0.10BC−0.04A^2+0.14B^2+0.011C^2 (Equation 2)

ANOVA study was performed to determine the linear, interactive and quadratic effect of independent variables on responses (R1 and R2). *P* values ($P<0.05$) were used as a tool to determine the significance of model and model terms. Very small *P* values for R1 and R2 ($P<0.000$) illustrated that quadratic model was highly significant for both responses (Supplementary Tables 2 and 3). The model terms with the *P* value less than 0.05 were regarded as significant, whereas the model terms having *P* values greater than 0.05 were categorized as non-significant. Results of the present study demonstrated that all liner and quadratic coefficients (A, B, C, A^2, B^2, C^2), as well as the cross-product coefficients AB and BC, had constructive effect on particle size reduction of *A. cepa* nanosuspension (Supplementary Table 2). ANOVA study for second response (R2) revealed the significant impact of AB, AC, BC, A^2, and B^2 on PDI reduction ($P<0.05$) (Supplementary Table 3). The remaining terms showed no/negative effect on particle size and PDI reduction.

The three dimensional (3D) response surface plots for particle size and PDI reduction of *A. cepa* nanosuspension illustrated that all the formulation parameters had a significant effect in reducing the particle size and PDI; however, the impact of A and B was more pronounced in reducing the particle size (Figures 1 and 2).

Based on experimental results, the software provided desirability and overlay plot which proposed the optimum experimental conditions (0.13% plant extract, 0.48% stabilizer, and AS/S ratio of 11.69) for the development of stable nanosuspension (also considered as optimized nanosuspension) with a mean particle size of 275.5 nm, PDI value of 0.415 and zeta potential value of −48.8 mV (Figure 3A). In addition, the optimized nanosuspension showed improved particle size, PDI, and zeta potential value compared with the coarse suspension (Figure 3B).

3.2. Characterization of nanosuspension

AFM analysis of *A. cepa* nanosuspension showed that particles were below 100 nm in height, although some larger aggregates were also present (Figure 4A). The selected area (A-B) of the figure illustrated the presence of particles with an average height of less than 13 nm. SEM image of *A. cepa* nanosuspension revealed the presence of a little bit spherical and flower-shaped particles (Figure 4B) and showed better surface structure than *A. cepa* coarse suspension (Figure 4C). The FTIR spectrum of coarse plant suspension (Figure 5A) was different from the spectra of nanosuspension (Figure 5B) and stabilizer (Figure 5C). The spectrum of nanosuspension (a mixture of plant extract and stabilizer) showed almost similar peaks to the spectrum of stabilizer. This similarity in peak position and peak intensity (peak to peak correlation) indicated that during preparation of nanosuspension stabilizer interacted physically as well as chemically with plant extract.

3.3. Stability studies

Optimized nanosuspension of *A. cepa* illustrated an increase in the particle size (from 275.5 nm to 381.4 nm), PDI values (from 0.415 to 0.541), and zeta potential (from −48.8 mV to −50.7 mV) after storage at room temperature for three months (Figure 6A) as compared to freshly prepared optimized nanosuspension (Figure 3A). Regarding the physical stability, stored nanosuspension was found clear at room temperature even after three months (Supplementary Figure 1). Optimized nanosuspension stored at 4 ℃ showed a mean particle size of 323.8 nm with PDI and zeta potential values of 0.494 and −48.9 mV, respectively (Figure 6B) which indicated a small variation in evaluated parameters as compared to nanosuspension stored at room temperature. The nanosuspension stored at 4 ℃ was found physically stable after three months (Supplementary Figure 1). Present results revealed that nanosuspension stored at 4 ℃ showed smaller variation in evaluated parameters (physical stability, particle size, PDI, and zeta potential values) as compared to nanosuspension stored at room temperature and therefore, refrigerated conditions were considered optimum to store prepared optimized nanosuspension.

Although coarse aqueous suspension of *A. cepa* was unstable when freshly prepared and showed a mean particle size of 691.0 nm with PDI and zeta potential values of 0.633 and −9.50 mV, respectively. It was also evaluated under both storage conditions (room temperature and 4 ℃) to check the effect of temperature on the formulation. When the storage stability of the coarse suspension was determined, it showed comparatively fair results at refrigerated conditions as compared to that at room temperature (Figures 6C and 6D). At room temperature, a significantly large difference in particle size, PDI, and zeta potential values was observed as compared to freshly prepared coarse suspension (Figure 3B). The coarse suspension became

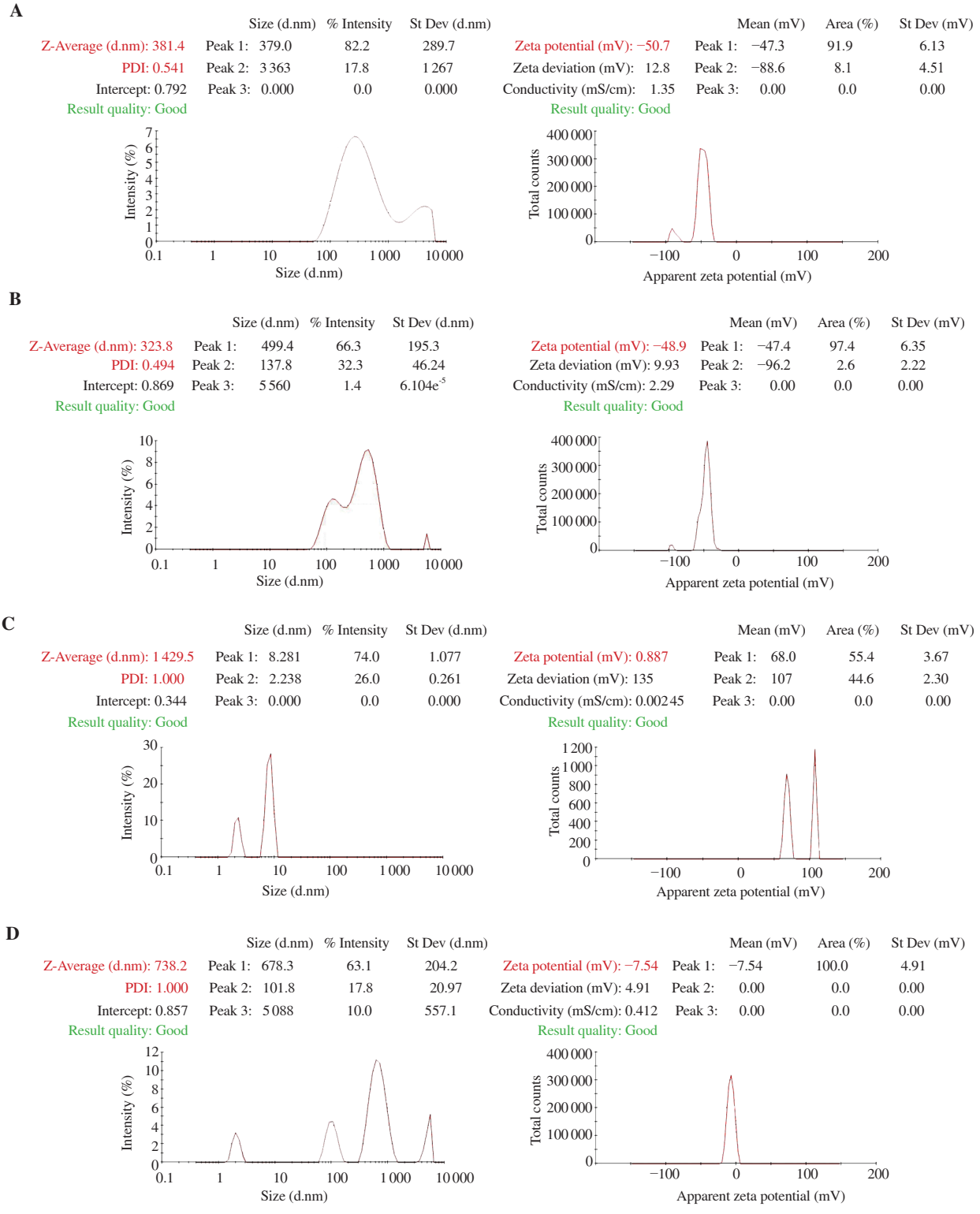

Figure 6. Particle size, PDI, and zeta potential results of optimized *Allium cepa* nanosuspension and *Allium cepa* coarse suspension stored at room temperature, and at 4℃. (A) *Allium cepa* nanosuspension at room temperature. (B) *Allium cepa* nanosuspension at 4℃. (C) *Allium cepa* coarse suspension at room temperature. (D) *Allium cepa* coarse suspension at 4℃.

highly coagulated after three months under both storage conditions.

3.4. Comparative antimicrobial activity of coarse suspension and nanosuspension

Comparative evaluation of the antibacterial activity illustrated significantly greater inhibition zone for *A. cepa* nanosuspension and standard quercetin than coarse suspension against both bacterial strains (P<0.05) (Supplementary Table 4). However, regarding antifungal activity, greater inhibition potential was noted for nanosuspension and standard quercetin and no inhibition zone was observed for coarse suspension.

3.5. Comparative antioxidant activity of coarse suspension and nanosuspension

The antioxidant results confirmed the enhanced DPPH radical scavenging potential of nanosuspension than coarse suspension (Figure 7). However, a slight difference was observed between the IC_{50} values of nanosuspension, standard quercetin, and ascorbic acid.

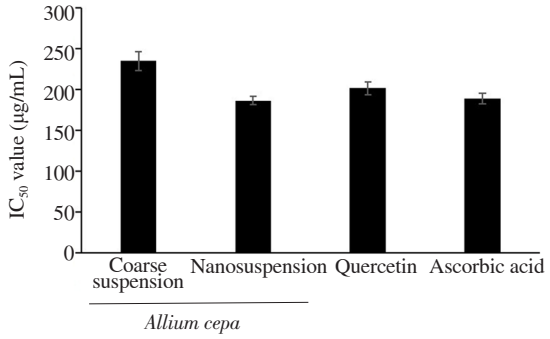

Figure 7. Antioxidant activity of coarse suspension, nanosuspension and standard quercetin.

3.6. Toxicity testing

A. cepa nanosuspension, coarse suspension, and standard quercetin were found non-mutagenic against the two tested strains of *S. typhimurium*, TA98, and TA100. Nanosuspension showed no positive well against TA98 and only six positive wells against TA100; however, the coarse suspension showed ten positive wells against TA98 and twelve positive wells against TA100 (Supplementary Table 5).

3.7. Dissolution studies

Results of dissolution study of *A. cepa* nanosuspension and coarse extract were expressed as quercetin (QT) equivalent. A greater concentration of quercetin was observed in dissolution medium for nanosuspension at all time intervals. After 120 min, nanosuspension showed a remarkably greater dissolution concentration (96.97%, QT equivalent) than *A. cepa* coarse extract (52.37%, QT equivalent) (Figure 8A).

3.8. Bioavailability studies

The plasma concentration-time profile of *A. cepa* nanosuspension and coarse suspension is presented in Figure 8B and pharmacokinetic parameters are given as Supplementary Table 6. As expected, a noteworthy increase was observed in the absorption of nanosuspension compared with coarse suspension at each time point and the rate of absorption augmented over time. The maximum concentration (C_{max}) was observed after two hours (T_{max}) of the dose administration indicating a 2.70-fold increase in the concentration of nanosuspension in the biological system. Furthermore, the greater area under the curve (AUC_{0-24h}) values for nanosuspension [(5702.66±15.25) μg•h/mL] than coarse suspension [(2666.05±11.57) μg•h/mL] illustrated a 2.14-fold increase in the relative bioavailability of nanosuspension.

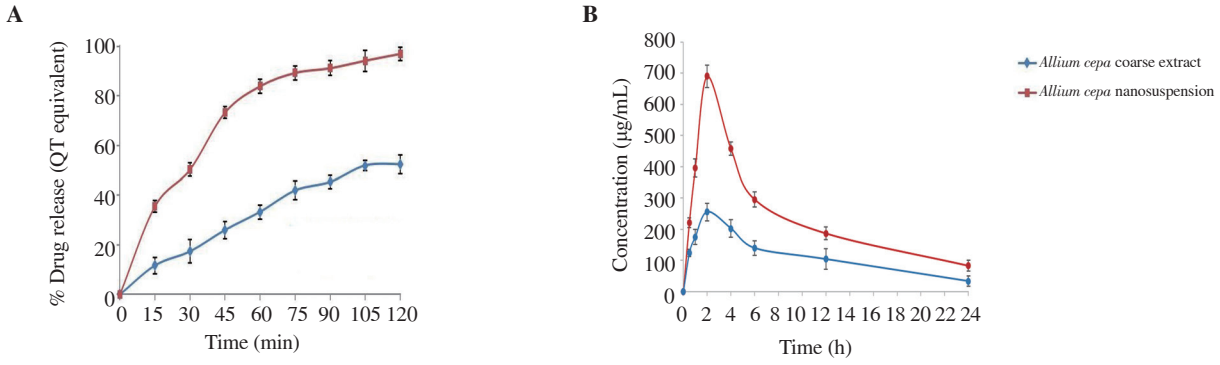

Figure 8. (A) Dissolution rate of *Allium cepa* nanosuspension and coarse extract. Results are presented as mean ± SD (n = 3); (B) Concentration of quercetin in rat plasma after oral administration of *Allium cepa* coarse suspension and nanosuspension. Results are presented as mean ± SD (n = 5).

4. Discussion

In the present study, the biopharmaceutical potential of quercetin contents of *A. cepa* peels was enhanced by using the most emerging nanoprecipitation approach. Quercetin which is a natural flavonoid and is present in *A. cepa* peels in good quantity has efficient pharmaceutical properties. However, its activity in the biological system is lower owing to its poor water solubility. Therefore, the present study was aimed to enhance the biopharmaceutical potential of quercetin using a surfactant stabilizer (SLS) that provides greater wetting to drug particles resulting in better dispersion and a greater extent of particle size reduction. To formulate stable nanosuspension with effectively smaller particle size and homogeneous distribution of particles, optimization of important formulation parameters was carried out by CCD of RSM. The benefit of using CCD is that it optimized the formulation parameters and reduced the experimental time and cost. Based on *P*-values of response parameters (R1 and R2), the CCD proposed the quadratic model as most suitable to elucidate the relationship between independent variables (A, B, and C) and response variables (R1 and R2). Significance of the selected quadratic model for both response parameters was also confirmed by ANOVA at a significance level of 0.05. The non-significant ($P>0.05$) lack-of-fit *F*-values, as well as the correlation between predicted R2 and adjusted R2 values, endorsed the better fitness of the model in experimental data for both response parameters. The coefficient of variation is the ratio between the standard error of estimate and the mean value and should have a value less than 10%. In the present study, the value of coefficient of variation less than 10% for both responses (R1 and R2) indicated that the model was reproducible and little variations in the mean values are acceptable.

The 3D response surfaces illustrated that all three parameters (A, B, and C) have a remarkable influence in reducing the particle size and maintaining the homogeneity of nanoparticles. Present results revealed that an optimum amount of drug (plant extract) can effectively help in the particle size reduction of nanosuspension[27]. Usually, the particles size reduction includes two stages: the crystal nucleus formation and the molecular growth. The rate of these two phenomena determines the particle size and its distribution[28]. According to these phenomena, when the amount of drug is small, a smaller number of particles are available for crystal nucleus formation and that's why the smaller amount of crystal nucleus is formed at the beginning of the nanoformulation which causes an increase in particle size and lowers the crystallization rate[22]. As the amount of drug increases, the number of nucleation also increases which lowers the particle size. With a further increase in the amount of drug, the rate of nucleation peaks and then plateaus. At this point, a further increase in the amount of drug causes the particle size to increase[27].

The concentration of the stabilizer also similarly affected the particle size as that of plant extract. The mean particle size of nanosuspensions was first decreased and then increased by increasing the concentration of the stabilizer. Normally the stabilizer paradoxically affects the particle size. At a shallow concentration, the stabilizer would fail to fully cover the surface of the formulated nanoparticles increasing in particle size. An increase in stabilizer concentration at this stage will provide better stabilization to the particles by properly adsorbing on the surface of newly formulated nanoparticles. However, at a higher concentration of stabilizer, adsorption would reach equilibrium due to the limited surface area[29]. Any further increase in stabilizer concentration would deplete the surface of the nanoparticles due to aggregation and generation of large molecules[27].

AFM analysis showed that all the particles were in nanometer size range with uneven particle distribution. This variability in particle size may be due to the slightly larger PDI value obtained by DLS, a technique where bigger particles scatter most of the incident light and the given intensity distributions are ascribed to a large population of particles even though smaller particles are present in solution. Comparison between DLS and AFM is justified when PDI values are less than 0.1[30]. SEM image of *A. cepa* nanosuspension revealed the presence of little bit spherical and non-uniform particles. This non-uniformity may also be due to the solidification of the surfactant on the surface of the particles during lyophilization[31]. Almost similar FTIR spectra of nanosuspension and SLS indicated that during formulation of nanosuspension, stabilizer interacted with plant extract by physical as well as by chemical means.

Stability testing showed a lesser increase in the particle size, PDI, and zeta potential values when stored at refrigerated conditions as compared to that at room temperature for three months. The results of storage stability of coarse suspension also showed smaller variation in particle size and zeta potential at refrigerated conditions.

Zeta potential, also known as electrokinetic potential, is a potential difference between the dispersion medium and the stationary layer of the liquid attached to the particles. It measures the magnitude of electrostatic attraction and repulsion between particles and hence is a fundamental parameter to determine the stability of colloidal particles. The zeta potential of suspensions and emulsions also helps to evaluate their stability at different temperature conditions. It is used to characterize nanoparticle surface charge, providing information regarding their stability and interaction with other molecules[32]. Zeta potential of a disperse system quantifies the electrostatic barriers and prevents the nanoparticles from agglomeration and aggregation[33]. Its value should be at least ± 30 mV for electrostatically stabilized systems or ± 20 mV for sterically stabilized nanosuspension systems[25]. Presently nanosuspension (kept under both storage conditions) showed zeta potential within the required range

and hence confirmed the stability of stored nanosuspension. Some zeta potential graphs showed two peaks which may be due to particle-particle interaction as described previously[34].

Although the particle size of stored nanosuspension (under both storage conditions) was within the range of pharmaceutical nanosuspension (200-600 nm)[35,36], the refrigerated conditions were considered optimum for better storage stability[25]. The larger, but acceptable increase in the particle size at room temperature may be due to the aggregation of nanoparticles. Another reason might be the Ostwald ripening, resulting from flocculation at room temperature[37].

Enhanced antibacterial and antifungal activity of nanosuspension can be attributed to a greater dissolution rate and subsequently improved diffusion of nanosuspension in culture media during the experimental growth of microbes[38]. Another reason for better antimicrobial potential may be nanosuspension's unique physicochemical properties (large surface to mass ratio, ultra-small size) which leads to its high reactivity and unique interactions with biological systems[39]. Present results are in line with the previous finding[6] in which nanoformulation exhibited better inhibitory potential than the coarse formulation.

Superior antioxidant activity of *A. cepa* nanosuspension confirmed the enhanced DPPH radical scavenging potential compared with coarse suspension. However, no major difference was observed between the IC_{50} values of nanosuspension, ascorbic acid, and standard quercetin. The IC_{50} value is the concentration of the sample that causes 50% loss of DPPH activity and is inversely related to the antioxidant activity. A smaller IC_{50} value means greater antioxidant activity[24]. Moreover, the DPPH radical scavenging activity of coarse plant extract, nanosuspension, standard quercetin, and ascorbic acid increases with the rise in concentration, as found previously[24,40].

The mutagenic activity of coarse plant suspension and nanosuspension was evaluated by Ames test against two strains of *S. typhimurium*, TA98 and TA100. The mutagenic potential was compared with the background plate. The test substance was considered mutagenic if the number of positive (yellow) wells were two folds higher than the background plate. Comparison of mutagenic activity revealed that nanosuspension and quercetin standard showed less or no mutagenic activity as compared to coarse suspension of *A. cepa*. It was interesting to note that the plant that was non-mutagenic in its coarse form became toxic to bacterial strains when formulated into nanosuspensions which revealed the potential of nanosuspension over coarse suspension. Anti-mutagenic activity of *A. cepa* is mainly due to the presence of quercetin which is present in excess amount in *A. cepa* peels and nanoformulation enhanced this potential due to reduced particle size and greater surface area.

Results of dissolution study illustrated a 1.85-fold increase in the dissolution rate of nanosuspension. The increased dissolution rate may be due to greater surface area and smaller particle size of nanosuspension as compared to coarse plant extract according to Noyes Whitney equation[26]. This might be because hydrophobicity of required constituent might decrease due to enhanced wettability and solubility of nanosuspension which is attributed to the surface stabilizers used for the formulation of nanosuspension[41]. Similar findings were observed in previous studies where nanosuspensions showed more significant dissolution than their respective coarse extracts[23,25].

To evaluate the beneficial effect of nanosuspension technology in enhancing the pharmaceutical potential of bioactive phytoconstituents, an *in vivo* study was carried out in male albino rats and the pharmacokinetic parameters were compared. Due to better absorption of nanosuspension in a biological system, a 2.70-fold increase in the concentration of nanosuspension was observed in the biological system as compared to coarse extract. Furthermore, the greater $AUC_{0\text{-}24h}$ value illustrated a 2.14-fold increase in the relative bioavailability of nanosuspension in comparison with coarse suspension. This improved bioavailability of nanosuspension may be due to the direct uptake of the drug through the gastrointestinal tract, better permeability by surfactants, and lesser degradation and clearance. In the gastrointestinal tract, particle size plays a prominent role in absorption rate. The mechanisms involved in such uptake include the diffusion of particles. Drugs having particle size up to 600 nm allow for efficient uptake in the intestine, especially in the lymphoid section of the tissue, and consequently bypasses the first-pass metabolism in the liver[42]. Moreover, the gastrointestinal absorption of drugs, having reduced aqueous solubility, may be improved by increasing the surface area. Furthermore, the surfactant used in the preparation of nanosuspension may also affect the solubility and permeability of drugs across the membranes of the gastrointestinal tract[43]. Comparable results were also reported by Yadav *et al.*[21] in which glimepiride nanosuspension showed about a two-fold increase in absolute bioavailability compared with coarse suspension.

In the present study, pharmaceutical potential and bioavailability of quercetin-rich *A. cepa* peel extract were enhanced by using nanosuspension technology. RSM approach, which is the most emerging statistical approach in the optimization study was successfully used to obtain optimized nanosuspension. The optimized nanosuspension showed mean particle size in the nanometer size range that was further confirmed by AFM and SEM analysis. A notable increase in the dissolution rate and oral bioavailability of quercetin contents of *A. cepa* peels was observed for nanosuspension. The formulated nanosuspension also showed enhanced biopharmaceutical potential as compared to coarse

suspension. Furthermore, the developed nanosuspension was found non-toxic and stable. In the present study, a single stabilizer was used to formulate nanosuspension. However, to obtain better particle size with good surface structures, a combination of different stabilizers can be used in future studies. Moreover, dissolution studies can be carried out under different pH conditions that will further help to understand the optimum conditions for the dissolution of nanosuspension.

Conflict of interest statement

The authors report no conflict of interest.

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Authors' contributions

FZ and NJ designed the study. FZ performed the experiments and wrote the manuscript. NJ was responsible for supervision and critical revision of the article. S. Ali and SJ provided research facilities. S. Aslam helped in experimental work. RH helped in data analysis.

References

[1] Vasava SS, Chotai NP, Patel HK. Formulation and evaluation of nanosuspension drug delivery system of etoricoxib. *Pharma Sci Moni* 2015; **6**(1): 10-27.

[2] Dineshkumar B. Nanosuspension technology in drug delivery system. *Nanosci Nanotech An Int J* 2013; **3**(1): 1-3.

[3] Lakshmi P, Kumar GA. Nanosuspension technology: A review. *Int J Pharm Pharma Sci* 2010; **2**(4): 35-40.

[4] Nimya AM, Krishnakumar K, John A, Kumar DB. A review on herbal drug nanosuspension. *World J Pharm Pharm Sci* 2015; **4**(7): 538-546.

[5] Karadag A, Ozcelik B, Huang Q. Quercetin nanosuspensions produced by high-pressure homogenization. *J Agric Food Chem* 2014; **62**(8): 1852-1859.

[6] Zafar F, Jahan N, Bhatti HN. Increased oral bioavailability of piperine from an optimized *Piper nigrum* nanosuspension. *Planta Med* 2019; **85**(3): 249-257.

[7] Pawar SS, Dahifale BR, Nagargoje SP, Shendge RS. Nanosuspension technologies for delivery of drugs. *J Nanosci Nanotechnol* 2017; **4**(2): 59-66.

[8] Sarrai AE, Hanini S, Merzouk NK, Tassalit D, Szabó T, Hernádi K, et al. Using central composite experimental design to optimize the degradation of tylosin from aqueous solution by photo-fenton reaction. *Materials* 2016; **9**(6): 1-11.

[9] Singh KP, Singh AK, Gupta S, Sinha S. Optimization of Cr (VI) reduction by zero-valent bimetallic nanoparticles using the response surface modeling approach. *Desalination* 2011; **270**(1-3): 275-284.

[10]Olayeriju OS, Olaleye MT, Crown OO, Komolafe K, Boligon AA, Athayde ML, et al. Ethylacetate extract of red onion (*Allium cepa* L.) tunic affects hemodynamic parameters in rats. *Food Sci Human Wellness* 2015; **4**(3): 115-122.

[11]Arshad MS, Sohaib M, Nadeem M, Amjad Z, Batool SM. Status and trends of nutraceuticals from onion and onion by products: A critical review. *Cogent Food Agric* 2017; **3**(1): 1-14.

[12]Prakash D, Singh, BN, Upadhyay G. Antioxidant and free radical scavenging activities of phenols from onion (*Allium cepa*). *Food Chem* 2007; **102**(4): 1389-1393.

[13]Ro JY, Ryu JH, Park HJ, Cho HJ. Onion (*Allium cepa* L.) peel extract has anti-platelet effects in rat platelets. *Springerplus* 2015; **4**(1): 17.

[14]Upadhyay RK. Nutraceutical, pharmaceutical and therapeutic uses of *Allium cepa*: A review. *Int J Green Pharm* 2016; **10**(1): 46-64.

[15]Zeng Y, Li Y, Yang J, Pu X, Du J, Yang X. et al. Therapeutic role of functional components in Alliums for preventive chronic disease in human being. *Evid Based Complement Alternat Med* 2017; **2017**: 9402849.

[16]Saxena A, Tripathi RM, Singh RP. Biological synthesis of silver nanoparticles by using onion (*Allium cepa*) extract and their antibacterial activity. *Dig J Nanomater Bios* 2010; **5**(2): 427-432.

[17]Sahni G, Panwar A, Kaur B. Controlled green synthesis of silver nanoparticles by *Allium cepa* and *Musa acuminata* with strong antimicrobial activity. *Int Nano Lett* 2015; **5**(2): 93-100.

[18]Gomaa EZ. Antimicrobial, antioxidant and antitumor activities of silver nanoparticles synthesized by *Allium cepa* extract: A green approach. *J Genet Eng Biotechnol* 2017; **15**(1): 49-57.

[19]Bouqellah NA, Mohamed MM, Ibrahim Y. Synthesis of eco-friendly silver nanoparticles using *Allium* sp. and their antimicrobial potential on selected vaginal bacteria. *Saudi J Biol Sci* 2019; **26**(7): 1789-1794.

[20]Aslam S, Jahan N, Rehman KU, Ali S. Formulation, optimisation and *in–vitro*, *in–vivo* evaluation of surfactant stabilised nanosuspension of *Ginkgo biloba*. *J Microencapsul* 2019; **36**(6): 576-590.

[21]Yadav SK, Mishra S, Mishra B. Eudragit-based nanosuspension of poorly water-soluble drug: Formulation and *in vitro–in vivo* evaluation. *AAPS Pharm Sci Tech* 2012; **13**(4): 1031-1044.

[22]Van Eerdenbrugh B, Vermant J, Martens JA, Froyen L, Van Humbeeck J, Augustijns P, et al. A screening study of surface stabilization during the production of drug nanocrystals. *J Pharm Sci* 2009; **98**(6): 2091-2103.

[23]Zafar F, Jahan N, Rahman KU, Asi MR, Ali S. Comparative evaluation of biological activities of native and nanosuspension of *Terminalia arjuna*. *Int J Agric Biol* 2019; **21**(4): 775-785.

[24]Zafar F, Jahan N, Rahman KU, Aslam S. Synergistic free radical scavenging potential of polyphenolic phytotherapeutics in various plants combinations. *Oxid Commun* 2016; **39**(3): 2213-2221.

[25]Zafar F, Jahan N, Rahman KU, Asi MR, Zafar WUI. Nanosuspension enhances dissolution rate and oral bioavailability of *Terminalia arjuna* bark extract *in vivo* and *in vitro*. *Asian Pac J Trop Biomed* 2020; **10**(4): 164-171.

[26]Gera S, Talluri S, Rangaraj N, Sampathi S. Formulation and evaluation of naringenin nanosuspensions for bioavailability enhancement. *AAPS Pharm Sci Tech* 2017; **18**(8): 3151-3162.

[27]Du J, Zhou Y, Wang L, Wang Y. Effect of PE gylated chitosan as multifunctional stabilizer for deacetyl mycoepoxydience nanosuspension design and stability evaluation. *Carbohydr Polym* 2016; **153**: 471-481.

[28]Xia D, Quan P, Piao H, Piao H, Sun S, Yin Y, et al. Preparation of stable nitrendipine nanosuspensions using the precipitation–ultrasonication method for enhancement of dissolution and oral bioavailability. *Eur J Pharm Sci* 2010; **40**(4): 325-334.

[29]Shen G, Cheng L, Wang LQ, Zhang LH, Shen BD, Liao WB, et al. Formulation of dried lignans nanosuspension with high redispersibility to enhance stability, dissolution, and oral bioavailability. *Chin J Nat Med* 2016; **14**(10): 757-768.

[30]Fritzen-Garcia MB, Zanetti-Ramos BG, de Oliveira CS, Soldi V, Pasa AA, Creczynski-Pasa TB. Atomic force microscopy imaging of polyurethane nanoparticles onto different solid substrates. *Mat Sci Eng C* 2009; **29**(2): 405-409.

[31]Xiong R, Lu W, Li J, Wang P, Xu R, Chen TT. Preparation and characterization of intravenously injectable nimodipine nanosuspension. *Int J Pharm* 2008; **350**(1-2): 338-343.

[32]Carvalho PM, Felício MR, Santos NC, Gonçalves S, Domingues MM. Application of light scattering techniques to nanoparticle characterization and development. *Front Chem* 2018; **6**: 237.

[33]Rachmawati H, Al–Shaal L, Müller RH, Keck CM. Development of curcumin nanocrystal: Physical aspects. *J Pharm Sci* 2013; **102**(1): 204-214.

[34]Wu C, Wang L, Harbottle D, Masliyah J, Xu Z. Studying bubble–particle interactions by zeta potential distribution analysis. *J Colloid Interface Sci* 2015; **449**: 399-408.

[35]Sutradhar KB, Khatun S, Luna IP. Increasing possibilities of nanosuspension. *J Nanotechnol* 2013; **2013**: 346581.

[36]Afifi SA, Hassan MA, Abdelhameed AS, Elkhodairy KA. Nanosuspension: An emerging trend for bioavailability enhancement of etodolac. *Int J Polym Sci* 2015; **2015**: 938594.

[37]Thakkar HP, Patel BV, Thakkar SP. Development and characterization of nanosuspensions of olmesartan medoxomil for bioavailability enhancement. *J Pharm Bioall Sci* 2011; **3**(3): 426-434.

[38]Esfandi E, Ramezani V, Vatanara A. Clarithromycin dissolution enhancement by preparation of aqueous anosuspensions using sonoprecipitation technique. *Iran J Pharm Res* 2014; **13**(3): 809-818.

[39]Shah SM, Ullah F, Khan S, Shah SM, Isreb M. Fabrication and evaluation of smart nanocrystals of artemisinin for antimalarial and antibacterial efficacy. *Afr J Tradit Complement Altern Med* 2017; **14**(1): 251-262.

[40]Bamidele A, Bamidele AP, Nnate DA. Evaluation of antioxidant potentials of the methanolic leaf extracts of vegetables, fruits and medicinal plants commonly consumed in Kaduna State, Nigeria. *J Med Plants Stud* 2017; **5**(1): 388-393.

[41]Kilor V, Sapkal N, Daud A, Humne S, Gupta T. Development of stable nanosuspension loaded oral films of glimepiride with improved bioavailability. *Int J App Pharm* 2017; **9**(2): 28-33.

[42]Yuan H, Chen J, Du YZ, Hu FQ, Zeng S, Zhao HL. Studies on oral absorption of stearic acid SLN by a novel fluorometric method. *Colloids Surf B Biointerfaces* 2007; **58**(2): 157-164.

[43]Ravichandran R. Studies on dissolution behaviour of nanoparticulate curcumin formulation. *Adv Nanopart* 2013; **2**: 51-59.