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Nanoformulation of *Curcuma longa* Root Extract and Evaluation of Its Dissolution Potential

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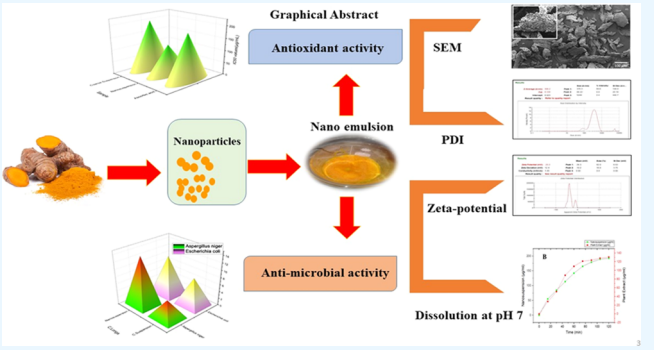
ABSTRACT: Medicinal plants have been widely used for therapeutic purposes for a long time, but they have been found to have some major issues such as low water solubility and bioavailability. In the present study, the nanoformulation of *Curcuma longa* L. plant extract was prepared to enhance its dissolution potential and biological activities. For the formulation of the nanosuspension, an ethanolic extract of *C. longa* was prepared through Soxhlet extraction using the nanoformulation technique. The nanosuspensions were formulated using four different stabilizers, namely sodium lauryl sulfate (SLS), hydroxy propyl methyl cellulose (HPMC), poly(vinyl alcohol) (PVA), and polysorbate-80 (P-80). The scanning electron microscopy (SEM), polydispersity index, and ζ potential were used for characterization of the nanoformulation. Among all of these, the surfactant stabilizer SLS was found to be the best. The average particle size of the selected optimized nanosuspension was found to be 308.2 nm with a polydispersity index (PDI) value of 0.330. The ζ potential value of the optimized nanosuspension was recorded at −33.3 mV. The SEM image indicated that the particles were slightly agglomerated, which may have occurred during lyophilization of the nanosuspension. The highest dissolution rate recorded at pH = 7 was 192.32 μg/mL, which indicates pH = 7 as the most appropriate condition for the dissolution of the *C. longa* nanosuspension. The antioxidant, antimicrobial, and antifungal activities of the optimized nanosuspension were also determined with regard to the coarse plant extract. The study findings suggested that the nanoprecipitation approach helps in enhancing the dissolution potential and biological activities of *C. longa* root extract.



1. INTRODUCTION

Nanotechnology is a newly emerging field with versatile applications in cosmetics, drug delivery, packaging of food ingredients, and many other applications in the field of medical science.[1] The biosynthesis of nanoparticles is facing some serious challenges. Nanoparticles are applied as the best antioxidants and antimicrobial agents for accelerating reactions.[2] The synthesis of nanoparticles involves many techniques, including the wet chemical process, decomposition, and microwave-assisted method.[3,4] The chemical agents utilized in these methods pose health issues as these are toxic and flammable.[5] Therefore, nontoxic and environment-friendly methods for the preparation of nanoparticles are now under consideration.[6]

The use of herbal products for the treatment of various human ailments has a very long history. Plants produce a large number of bioactive natural products with interesting chemical diversity and biological effects. Poor water solubility, poor permeability, limited systemic availability, instability, and intensive first-pass metabolism of phytomedicines and their distribution are the major challenges of phytochemicals.[7] Moreover, nanotechnology has opened many ways for the development and formulation of drugs with enhanced therapeutic potentials, pharmacokinetics, and pharmacodynamics. Solid lipid nanoparticles (SLNPs), liposomes, nanoemulsions, and polymeric nanoparticles are the various nano-based formulations consisting of herbs that have been described with amazing properties.[8−10]

Green synthesis of nanoparticles is a safe alternative to the trivial synthetic methods, and it has gained enormous attention from researchers since the 2010s. Green pathways for

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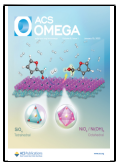

synthesizing nanoparticles have recently engrossed a lot of interest due to their cost effectiveness, simplicity of synthesis, environmental friendliness, sustainable supplies, and so on.[11,12] With the green synthesis of nanoparticles, there has been a considerable reduction in the toxic/harmful discard.[13] Green synthesis of nanoparticles can be used to boost clinical practice because of its potential to reframe the anticancer,[14] antibacterial,[15,16] bioimaging,[14,17,18] biosensing,[19] and drug delivery activities.[20,21] The most important part of green synthesis is the use of nontoxic chemicals and reusable constituents. Many biomolecules, including microbes, vitamins, herbs, fungi, biodegradable polymers, plant extracts, and enzymes, are used in synthesis of nanomaterials.[22] On a commercial scale, using plant extracts to produce nanoparticles results in reduced costs. A previous study illustrated that the plant extracts used for nanomaterial preparation in the process of green synthesis play crucial roles as capping and reducing agents.[13]

Turmeric (*Curcuma longa* L.) is a Zingiberaceae-family rhizomatous, flowering, herbaceous, and perennial plant. It is found all over South and Southeast Asia, while some of its species can be found in Australia, the South Pacific, and Australia.[23] Its roots are used as a flavor in food and it has gained great fame in culinary, medical, and scientific fields. It is the source of curcumin that has been used in therapeutics for several years.[24] Curcumin has various pharmacological properties, such as immunomodulatory, chemoprotective, antihyperlipidemic, antineoplastic, antiulcer, and neuroprotective.[25−30] Literature has revealed that curcumin has some potential against the coronavirus 2019.[31−34] Curcumin must be accessible at the infectious position for the best therapeutic efficacy. Despite having therapeutic activities, it has limited benefits because of its low bioavailability. Drugs with low solubility could be formulated via different techniques, including emulsions, cosolvents, dissolution in a surfactant, liposomes, polymer solution, solid dispersion, and pH adjustment.[35−38] Nanotechnology has offered a credible drug delivery system for increasing the efficiency of the drugs by enhancing their flow in the blood and also decreasing their harmful effects. For enhancing the solubility and absorption of curcumin, its size is reduced to a nanometer scale via biological membranes.[39] Though various strategies have been used for the size reduction and dissolution potential of curcumin, still it is challenging.

Medicinal plants have been widely used for therapeutic purposes for a long time, but phytochemicals have the major issues of low water solubility, poor permeability, limited systemic availability, instability, and intensive first-pass metabolism of the phytomedicine and its distribution. To overcome these challenges, nanosuspensions can be used as the best alternative. Therefore, the current study emphasized the green synthesis of curcumin nanoparticles to enhance effectively their biological activities and dissolution potential. These synthesized nanoparticles were characterized physiochemically to observe their pre-pharmacological parameters and pharmacological activities.

2. RESULTS AND DISCUSSION

2.1. Preparation and Optimization Studies. In the present study, the nanoprecipitation method was selected and applied for the preparation of nanosuspensions because of its simplicity, reproducibility, rapidity, and cost effectiveness. For the preparation of the stable nanoformulation, important process parameters like the stabilizer, extract amount, and its ratios with the stabilizer and amount of stabilizer were optimized.

2.1.1. Selection of Stabilizer. The goal of the stabilizer screening was to choose an appropriate stabilizer for the formation of a stable nanosuspension. The importance of the selection of stabilizer in the formulation of nanosuspensions is that it maintains the physical stability, enhances the activation energy, and suppresses the agglomeration of the whole nanosystem. Another significant function of the stabilizer is to make available a significant mechanical and thermodynamic fence at the boundary that slows down the amalgamation of formulated nanoparticles and hence prevents Ostwald ripening.[40] Furthermore, the type of stabilizer may have a remarkable effect on the size of nanoparticles. To select an appropriate stabilizer, different stabilizers (PVA, SLS, P-80, and HPMC) were used for the formulation of nanosuspensions (Table 1).

Table 1. Screening of Stabilizers for the Formulation of *C. longa* Nanosuspensions

nanosuspension code	stabilizer used	physical stability	stability after 3 months (room temp)
C.L 1	HPMC	stable	unstable
C.L 2	PVA	stable	unstable
C.L 3	SLS	**stable**	**stable**
C.L 4	P.80	stable	unstable

During the stabilizer selection, a constant concentration of each stabilizer (1%) was used, with a predefined amount of plant extract (0.25 g) and antisolvent to solvent ratio (1:10). The stabilizer that provided a physically stable nanosuspension was selected for further studies. Table 2 shows the results for

Table 2. Optimized Values of the Selected Stabilizer

sr., no	name of stabilizer	amount of plant extract (g)	amount of stabilizer (g)	plant extract to stabilizer ratio
1	SLS	0.25	0.25	1:1

the selection of the suitable stabilizer for *C. Longa* nanoformulation. SLS was found to be the best stabilizer for *C. Longa* nanoformulation because it provides the most stable nanoformulation when prepared freshly and remains stable for three months after preparation of the nanoformulation.

The remaining three (HPMC, PVA, and P.80) also give physical stability, but after our desired time, their nanosuspension does not remain stable for further use, which is why they were not suitable for the formation of a stable nanoformulation.

2.1.2. Optimization of Stabilizer Amount. In this step, we have to find the appropriate amount of the selected stabilizer and its ratio with regard to the plant extract for the formation of a physically stable nanosuspension. In this regard, the following results were found, and the given amount and ratio of stabilizer to plant extract were found to be the most appropriate for the formation of a stable nanosuspension (Table 2).

2.2. Scanning Electron Microscopy (SEM) Analysis of *C. longa* Nanosuspension. To investigate the shape and size, the synthesized nanosuspension was characterized through SEM. The obtained images show irregular flakes in the shape of the *C. longa* nanosuspension (Figure 1). The SEM

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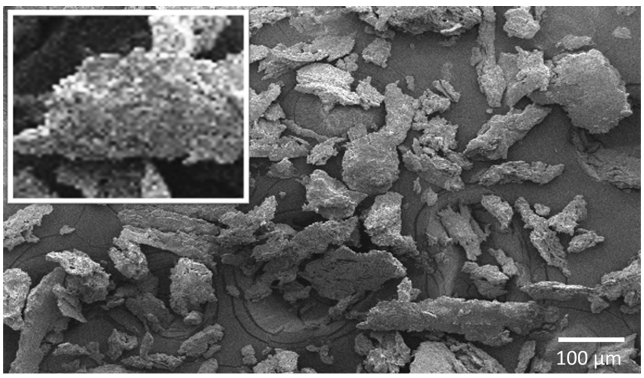

Figure 1. SEM image of the optimized *C. longa* nanosuspension.

image shows agglomerated flakes, which might occur during the drying or lyophilization of the sample, and demonstrates the good surface properties of the *C. longa* nanosuspension. The appearance of bigger and nonuniform particles at certain locations may be attributed to individual particle adhesion and aggregation during drying.

2.3. ζ Size and Potential Analysis. Further, the obtained nanosuspension was analyzed through a Zetasizer. ζ potential analysis is usually performed to investigate the morphology and stability of a nanosuspension. The stability of the nanosuspension is maintained by the electrostatic repulsion requiring a minimum ±30 mV ζ potential.[41] For stable nanoparticles, the ζ potential value must be greater or lesser than +25 or −25 mV.[42] The recorded results for the *C. longa* nanosuspension show good stability and interaction with the plasma membrane at a ζ potential of −33.3 mV (Figure 2).

2.4. Particle Size and Polydispersity Index. The polydispersity index (PDI) determines the heterogeneity of the sample based on the size distribution. The value of PDI set by the International Organization for Standardization (ISO) is less than 0.05, similar to mono-dispersed samples, and values > 0.7 show a large-magnitude distribution. Figure 3 shows the optimized value (0.330) of PDI for *C. longa* nanosuspension and a particle size diameter of 308.2 nm.

2.5. Physical Stability Studies of Nanosuspensions. The physical appearance of the suspension at various time intervals indicates its stability. Table 3 shows the stability of the synthesized nanosuspension, that stored at room temperature, and a refrigerated sample. The results indicate good physical stability at room temperature, but after refrigerating a surface layer was developed; however, the PDI and particle size remained intact. The layer can partially dissolve after shaking the sample.

2.6. *In Vitro* Dissolution Profile of *C. longa* Nanosuspension and the Coarse Extract. Because plants contain a huge number of bioactive phytoconstituents and it is extremely difficult to assess the quantities of all of these components, only one essential bioactive component was employed as a reference chemical to evaluate the findings in the current research. For this purpose, quercetin, which is the major constituent of *C. longa* root extract, was used as a standard compound to evaluate the dissolution study. The concentration of active constituents was evaluated from the calibration curve of quercetin. The dissolution profiles of the coarse plant extract and nanosuspensions are shown in Figure 4. The dissolving media was a phosphate buffer medium with a pH of 7.4, and sink conditions were maintained throughout the dissolution rate tests. It is seen in Figure 4 that the dissolution rates of the plant extract and nanosuspension increase with time, creating a huge difference between their dissolution rates. A drug's dissolving rate can be boosted by reducing the particle size and increasing the surface area, according to the equation. Some of the physical characteristics that influence a drug's solubility and dissolution rate in physiological parameters include the particle size, shape, state (amorphous or crystalline), and habit (needle or spherical).[43]

The dissolution rate was evaluated at pH = 7.2, which showed a remarkable change with change in pH. From Figure 4C, it may be noted that the dissolution rate of the plant extract and nanosuspension at the 120 min time point was 120.49 and 177.5 μg/mL, respectively.

The dissolution enhancement by nanosuspensions might be related to particle conversion to amorphous nature, decrease in

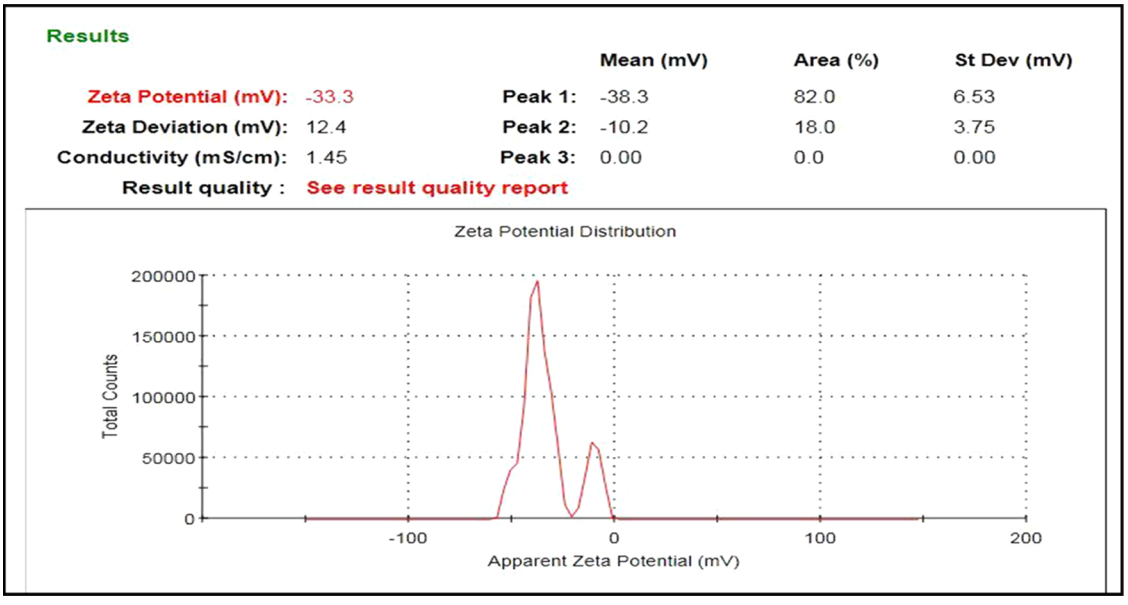

Figure 2. ζ potential graph of optimized *C. longa* nanosuspension.

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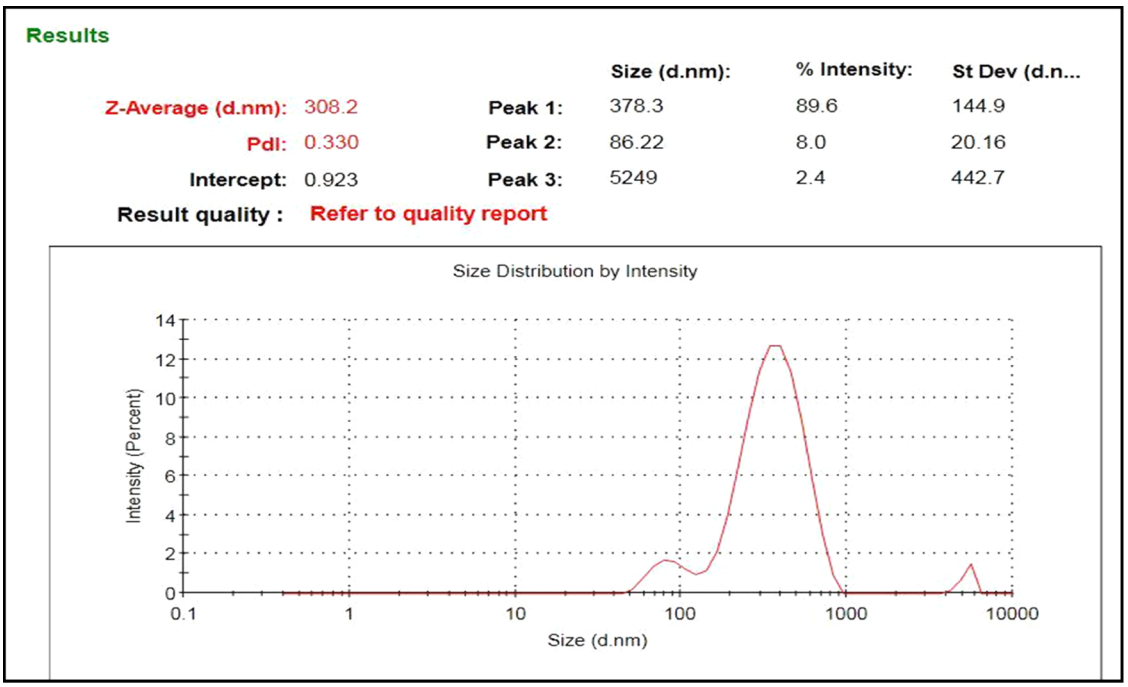

Figure 3. Particle size and PDI value of the optimized *C. longa* nanosuspension.

Table 3. Physical Stability Studies of *C. longa* Nanosuspensions

stability	parameters	results
freshly prepared nanosuspension	size (*z*-average nm)	308.2
	PDI	0.330
	physical appearance	clear and stable
room temperature (25−30 °C)	size (*z*-average nm)	353.4
	PDI	0.361
	physical appearance	clear and stable
refrigerated condition (2−8 °C)	size (*z*-average nm)	327.9
	PDI	0.40
	physical appearance	stable

the particle size from micron to nanometer range (size measurements), and particle shape (SEM determination). The graphical presentation (Figure 4) indicates that the dissolution rate increases steadily with passage of time. At 0, 30, 45, 60, 75, 90, and 120 min, the dissolution rate of the coarse plant extract is 0, 51.43, 79.11, 88.12, 101.21, 111.38, and 120.49 μg/mL, respectively, and for the nanosuspension, it is 0, 81.69, 112.86, 141.58, 159.68, 168.79, and 177.54 μg/mL, respectively.

From the above results, we can conclude that for different pH values the dissolution rates are different. This is due to the difference in dissolution velocity of the component, which varies with the pH because of the anionic and cationic interaction of component particles in the medium or due to some van der waal interactions.[44] In conclusion, we observed that among all the three pH values (6.8, 7.0, and 7.2), at pH = 7.0 the plant extract and nanosuspension showed the highest dissolution rate, which shows that it is the most appropriate medium for the absorption in any solvent and medium.

2.7. DPPH Radical Scavenging Activity. To investigate the antioxidant effects of curcumin, the DPPH test is a quick, straightforward, and commonly used method for determining a compound's free radical scavenging activity, as described in the literature.[45] The DPPH method (2.2-diphenyl-1-picryl hydrazyl) is based on the capture of DPPH free radicals by antioxidants, producing a decrease in absorbance at 515 nm wavelength.[46] At room temperature, DPPH is stable and creates a violet solution in organic solvents such as methanol, ethanol, and so on, which is decreased in the presence of curcumin and shows reduction in the color.[47] The radical scavenging activity of the coarse plant extract and nanosuspension was found in the range of approximately 65−80% at a curcumin concentration of 0.02−0.10 mg/mL. The ascorbic acid (AA), a common antioxidant in foods, was used as a reference and presented a higher antioxidant activity than the curcumin.[48] The IC_{50} value is inversely proportional to the free radical scavenging activity/antioxidant property of the sample. The nanosuspension, coarse suspension, and ascorbic acid have IC_{50} values of 123.8, 205.2, and 189.06 μg/mL, respectively, as shown in Table 4.

As shown in Figure 5, the IC_{50} value varies inversely to antioxidant activity; in this sense, it is found that the coarse suspension has least antioxidant activity due to the highest value of IC_{50} and the nanosuspension has the highest activity, even greater than ascorbic acid, which is used as the standard. This is due to the presence of phenolic components because as their concentrations increase, there is an increase in the antioxidant activity rate.[49]

2.8. Antibacterial and Antifungal Activity. Antibacterial and antifungal activities were assessed against *Escherichia coli* and *Aspergillus Niger*, respectively. Different strains were used for evaluating the antimicrobial activity, which include *C. longa* coarse suspension and nanosuspension, fluconazole, rifampicin, and methanol. The antimicrobial activity mostly depends upon the phenolic components: the higher the concentration of phenolic components, the higher the activity. In Table 5, the inhibition diameter for the strains of coarse suspension and nanosuspension of *C. longa* is 7.75 ± 0.15 and 11 ± 0.06 mm, respectively. The higher the inhibition diameter, the higher the antimicrobial activity. The antimicrobial activity is basically due to the concentration of the hydrophobic compounds

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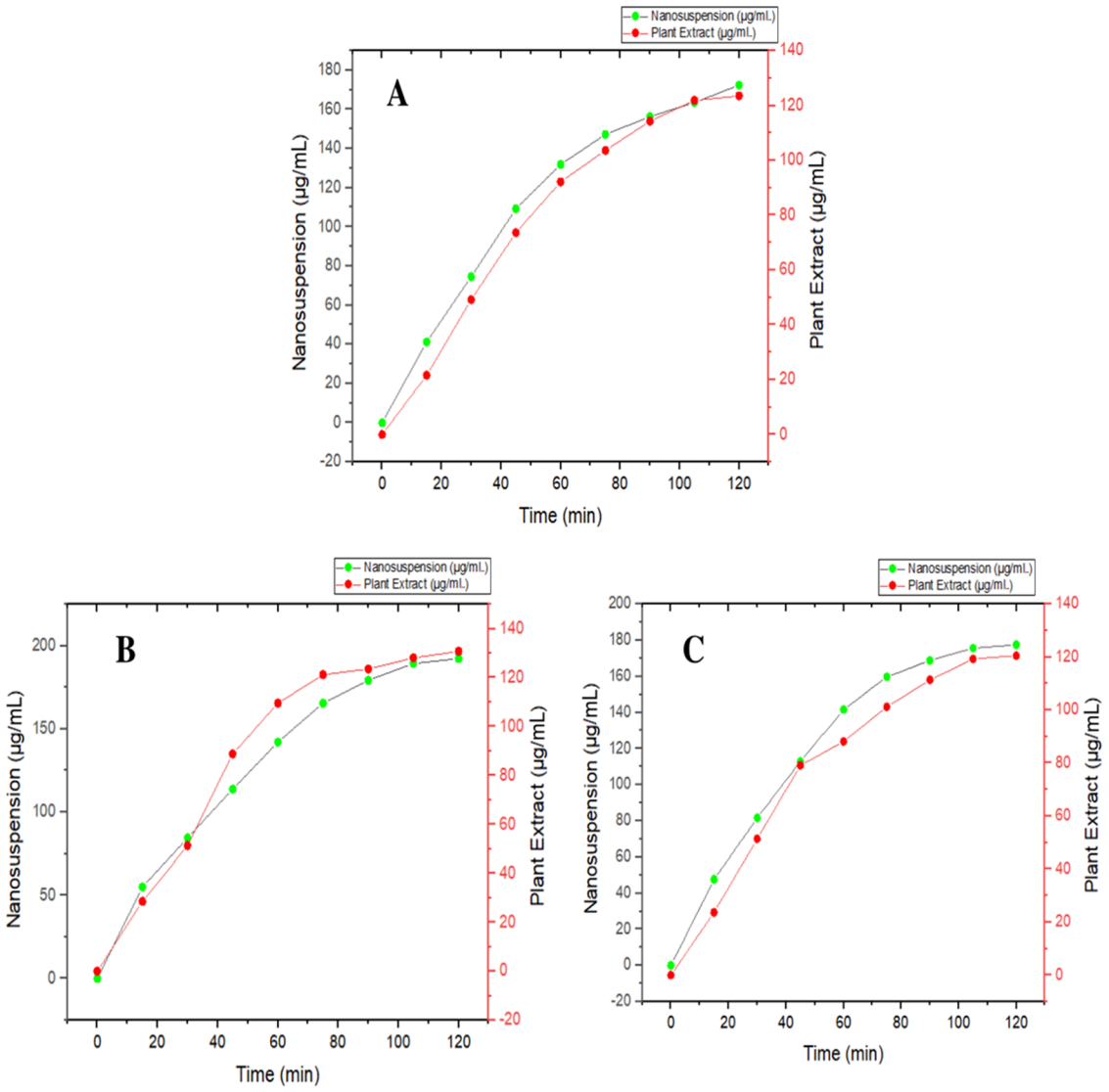

Figure 4. (A) Dissolution graph of the plant extract and nanosuspension at pH = 6.8; (B) dissolution graph of the plant extract and nanosuspension at pH = 7.0; and (C) dissolution graph of the plant extract and nanosuspension at pH = 7.2.

Table 4. Antioxidant Potential of *C. longa* Coarse Plant Suspension and Nanosuspension

sample	IC_{50} values (μg/mL)
ascorbic acid	189.06
nanosuspension	123.8
coarse suspension	205.2

present in turmeric. As the concentration varies, their antimicrobial activity also varies.[48] It is observed that the nanosuspension of *C. longa* was more effective against the fungus and bacterial strain than the *C. longa* coarse suspension, as indicated in Figure 6. Methanol does not affect the antimicrobial activity of any microorganism in the absence of any hydrophobic component. The strains fluconazole and rifampicin have maximum values of inhibition region diameter: 43.5 ± 0.23 mm for antifungal activity and 37.5 ± 0.13 mm for antibacterial activity. This is due to their higher rates of antifungal and antibacterial activity in the respective order.

According to all of these investigations, the polycationic character of turmeric extracts is the key to their antifungal effects, and the length of the polymeric chain boosts this

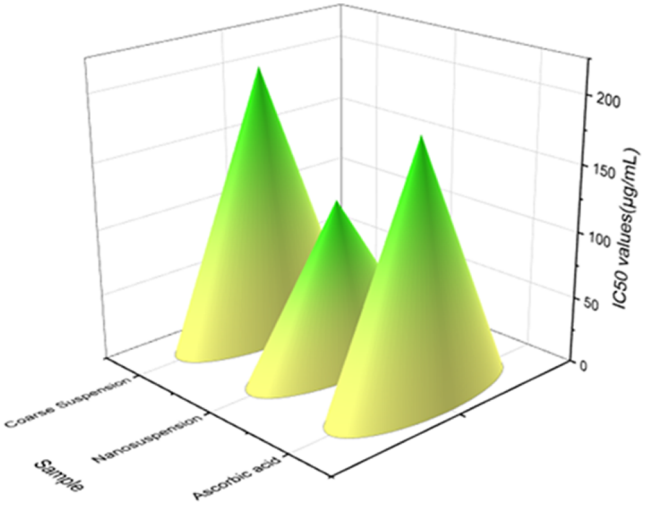

Figure 5. Graph of the antioxidant potential of *C. longa* coarse plant suspension and nanosuspension.

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Table 5. Antimicrobial Activities of *C. longa* Coarse Suspension and Nanosuspension

plant/ standard	treatment	antifungal activity (mm)	antibacterial activity (mm)
strain used		*A. niger*	*E. coli*
C. longa	C. suspension	5.0 ± 0.02	7.75 ± 0.15
	nanosuspension	13.7 ± 0.16	11 ± 0.06
fluconazole		43.5 ± 0.23	
rifampicin			37.5 ± 0.13
methanol			0

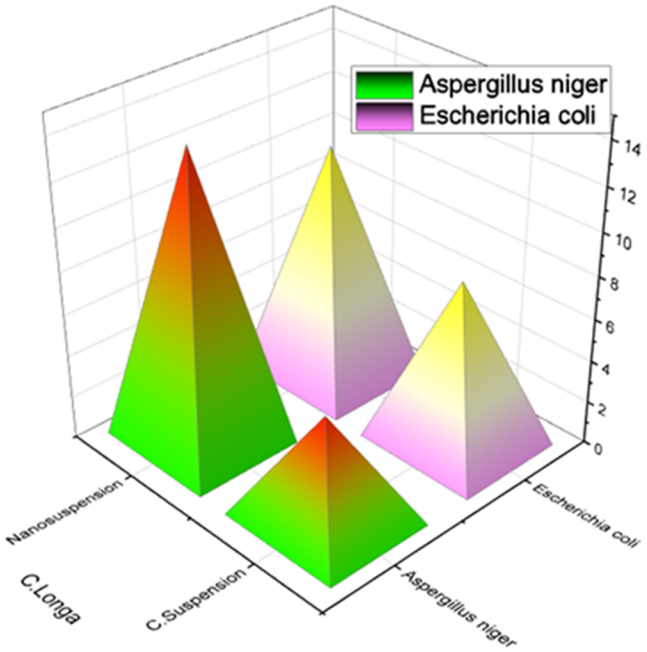

Figure 6. Graph of the antimicrobial activities of *C. longa* coarse suspension and nanosuspension.

activity. This study concluded that the nanoformulation has increased levels of antimicrobial activities, many times greater than those of the normal coarse extract of turmeric. This is due to the enhancement of the bioavailability of particles upon formation of their nanoformulation as it increases the activity ratio compared to coarse extracts. Due to these antimicrobial and antioxidant activities, turmeric ascorbic acid and many other plants are used for the preservation of food and also for antiseptic purposes as they retard microorganisms' activity (Figure 7).

Figure 7. Formation of nanosuspension.

3. CONCLUSIONS

C. longa nanosuspension had considerably higher antioxidant and antimicrobial capability when compared to its original suspension in the current investigation, demonstrating the usefulness of innovative nanosizing techniques in boosting the biological activities of herbal extracts. Sodium lauryl sulfate was proved the best stabilizer for preparing the nanosuspension of *C. longa* among different stabilizers. The nanosuspension had a good dissolving rate with the maximum dissolution recorded at pH-7, which is predicted as the most suitable condition for *C. longa* to be absorbed in any solvent, medium, or body fluids. Moreover, antioxidant and antimicrobial activities were enhanced in the nanosuspension as compared to the coarse extract, which showed the high efficiency of the nanosuspension. The *C. longa* nanosuspension showed significant antibacterial activity against *E. coli* and *A. niger* with zone of inhibition values of 11 ± 0.06 and 13.7 ± 0.16, respectively. The nanosuspension had the highest antioxidant activity (IC_{50} = 123.8 μg/mL), even greater than that of ascorbic acid. According to the current findings, nanosuspensions of the selected herbal extract can be employed as a better option to treat various disorders with enhanced therapeutic efficacy when compared to coarse suspensions because of their biological potentials.

4. EXPERIMENTAL SECTION

4.1. Reagents. This study's chemicals were all of analytical grade. The *n*-hexane and ethanol (EtOH) were procured from Sigma-Aldrich. Stabilizers SLS, PVA, HPMC, and P-80 were purchased from Caledon (Canada).

4.2. Plant Material and Sample Preparation. The roots of *C. longa* (turmeric) were collected from the north of Okara, Punjab, and Pakistan during Nov 2020. The plant was placed in the dark for 15 days in order to dry it out at room temperature. At the end of the 15th day, it was crushed into powder using a mortar and pestle and passed on to a set of standard mesh sieves. Finally, a fine powder of *C. longa* was collected for the onward extraction. The ethanolic extract of the plants was prepared using the Soxhlet apparatus. Excessive fat contents of the plant were removed by defatting with *n*-hexane. A 30 g fine powder of *C. longa* was placed in the thimble of a Soxhlet extractor for defatting, and 200 mL of *n*-hexane was poured into it. The entire system was put on standby for 8 h. The flavonoids were extracted from the predefatted plant extract using 200 mL of ethanol. The resulting ethanolic extract was filtered and concentrated under reduced pressure and was stored for the onward steps.

4.3. Preparation of Nanoformulation. The nanosuspension was made using the nanoprecipitation method suggested in a previous study.[41] Plant extract 0.25 g was dissolved in 10 mL of ethanol (organic phase) and 0.25 g of the stabilizer was dissolved in 100 mL of distilled water (aqueous phase). The ensuing organic layer was gradually (1 mL/min) poured with the help of a syringe into the aqueous phase with constant stirring at 1000 rpm for 6 h at room temperature. The entire formulation was stored at room temperature.

4.4. Optimization of Formulation Parameters. Several preparative factors, such as the stabilizer, concentration of the stabilizer, and the amount of plant extract, were enhanced in the current study for the preparation of the stable nanoformulation with the lowest particle size. Initially, screening of

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stabilizers was done to determine the best possible stabilizer for each plant extract keeping all other parameters constant. After deciding on the stabilizer, the remaining parameters (concentration of the stabilizer and amount of plant extract) were adjusted.

For the formulation of stable nanosuspensions, four different stabilizers (PVA, SLS, HPMC, and P-80) were used. In the present study, all of the four stabilizers (PVA, SLS, HPMC, and P-80) were screened by using 0.25 g of plant extract and 0.25 g of stabilizer, and the solvent to antisolvent ratio was fixed at 1:10. The concentration of the stabilizer is an important parameter for the formulation of stable nanosuspensions. In this study, the amounts of stabilizer used were 0.125, 0.25, 0.5, and 1.0 g.

The amount of the plant extract was kept constant, i.e., 0.25 g. The experimental conditions used for the preparation of the *C. longa* nanoformulation are given in Table 6.

Table 6. Optimization Conditions for the Preparation of the Stable Nanoformulation of *C. longa*

sr., no	amount of plant extract (g)	amount of stabilizer (g)	plant to stabilizer ratio
1	0.25	0.125	1:0.5
2	0.25	0.25	1:1
3	0.25	0.5	1:2
4	0.25	1.0	1:4

4.5. Characterization of the Nanoformulation. *4.5.1. SEM.* Physical evaluation of nanoformulations is based on stability; the one with higher stability is taken as the ideal nanoformulation. Scanning electron microscopy (SEM) (JEOL, JSM-6400, Japan) with a secondary electron detector was used to obtain the digital pictures of the surface morphology at an accelerating voltage of 15 kV. The solid nanoformulation (obtained after evaporating the excess amount of solvent) was used for this purpose.

4.5.2. Particle Size and Polydispersity Index. The mean particle size (*z*-average, nm) and polydispersity index (PDI) of the prepared nanosuspensions were measured by the dynamic light scattering (DLS) technique using Malvern Zetasizer (Nano ZS). For measuring particle size and PDI, freshly prepared nanoformulations were added to the glass cuvette and placed in a sample holder unit, and measurement was carried out using software. ζ potential was also measured similarly by using a quartz cuvette.[50]

4.6. *In Vitro* Dissolution Studies of the Optimized Nanoformulation. The *in vitro* dissolution behavior of the optimized nanoformulation as compared to the coarse plant extract was determined by adopting the literature method as described in previous literature.[51] For *in vitro* dissolution testing of the coarse herbal extract and nanoformulation, a semipermeable membrane was utilized. For dissolution testing, the nanoformulation was added to a semipermeable (egg) membrane and this membrane was placed in different phosphate buffer systems (pH = 7.2, pH = 7.0, and pH = 6.8) as dissolution media, followed by magnetic stirring. Throughout the whole experiment, the dissolving medium's temperature was set constant at 370.5 °C, and the stirring speed was set to 50 rpm. An aliquot (5 mL) was withdrawn from the dissolution media at predetermined time intervals (0, 15, 30, 45, 60, 75, 90, 120 min) and the same volume of the prewarmed (37 °C) dissolution medium (phosphate buffer) was added to the dissolution vessel immediately to maintain the sink conditions. The concentration of dissolved drugs was determined spectrophotometrically. Pure quercetin (QT) was used as a standard compound for the *C. longa* coarse plant extract and its nanoformulation to compare their dissolution rates. Samples were analyzed at 373 nm wavelength (λ_{max} of quercetin) spectrophotometrically. The percentage release of coarse plant extracts and that of the optimized nanosuspension were compared. The regression equation produced by the properly designed calibration curve of quercetin was used to estimate the concentration of active components. Results for the coarse plant extract and nanosuspension experiments were provided as the percentage of drug dissolved. Experiments were carried out in triplicate.

4.7. Antimicrobial Activity. The antimicrobial potential of the plant extracts and their respective nanosuspensions was determined with the disc diffusion method as described in previous literature[52] by employing one fungal strain (*A. niger*) and a bacterial strain (*E. coli*).

4.8. Antibacterial Activity (Assay Protocol). Nutrient agar (28.08 g/L) medium was poured into Petri dishes and injected with the bacterial cultures. Tiny filter paper discs were saturated with 30 μL (20 mg/mL) of the plant suspension and nanosuspension samples. Methanol and rifampicin were employed as negative and positive control, respectively. The discs were placed flatly on the growth media and the Petri dishes were incubated at 37 °C for 24 h. Inhibiting the development of bacteria, herbal extracts with antibacterial activity resulted in the formation of clear zones. By using a zone reader, we were able to measure the inhibited zone (zone of inhibition) in millimeters (mm). The antibiotic's active site is surrounded by a zone of inhibition where bacterial colonies cannot grow. To assess the bacteria's susceptibility to the antibiotic sample, the zone of inhibition was assessed.

4.9. Antifungal Activity (Assay Protocol). Potato Dextrose Agar (PDA) (39.06 gm/L) was added in Petri dishes and inoculated with the fungal species. Appropriately cut discs of filter paper were impregnated with 30 μL samples (20 mg/mL) of plant extracts and the nanoformulation. Fluconazole (5 μL, 15 mg/250 μL) was used as a positive control. The plates were incubated at 2 °C for 48 h, and the antifungal activity was measured using a zone reader to determine the inhibited zones.

4.10. DPPH Radical Scavenging Activity. The antioxidant activities of the native plant suspension and optimized nanosuspension were assessed by DPPH assay by following the previous literature method.[53] Five different concentrations of coarse plant suspensions and the respective nanoformulation in the range of 0.02−0.1 mg/mL were made. An aliquot (3 mL) of this concentration was taken and a newly prepared DPPH solution (0.1 mM, 1.0 mL) was added to it. At room temperature, these solutions were incubated for 30 min. A ultraviolet−visible (UV−Vis) spectrophotometer (Shimadzu, Japan) was used to measure the solution's absorbance at 517 nm. The significant free radical scavenging activity was shown by the decrease in absorbance with increasing concentrations. To analyze the data, ascorbic acid was employed as a standard chemical. The same procedure was applied to the blank solution. The following formula was applied to calculate the % age inhibition of the DPPH radical.

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%age inhibition of DPPH = $[1 - A_1/A_0] \times 100$

$\therefore A_1$ = absorbance of samples

$\therefore A_0$ = absorbance of control

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Notes

The authors declare no competing financial interest.

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