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Formulation of Piperine-Loaded Nanoemulsion: In Vitro Characterization, Ex Vivo Evaluation, and Cell Viability Assessment

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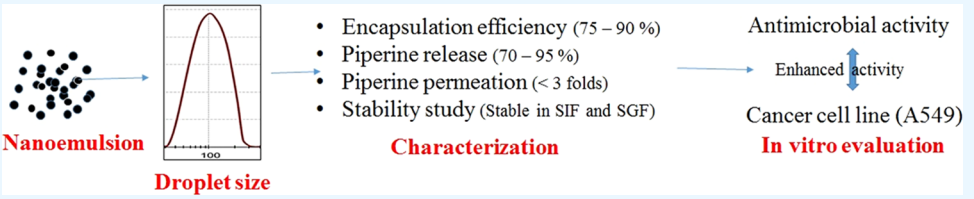

ABSTRACT: Piperine is an alkaloid, but its therapeutic efficacy is limited due to poor aqueous solubility. In this study, piperine nanoemulsions were prepared using oleic acid (oil), Cremophore EL (surfactant), and Tween 80 (co-surfactant) using the high-energy ultrasonication approach. The optimal nanoemulsion (N2) was further evaluated using transmission electron microscopy, release, permeation, antibacterial, and cell viability studies based on minimal droplet size and maximum encapsulation efficiency. The prepared nanoemulsions (N1−N6) showed a transmittance of more than 95%, a mean droplet size between 105 ± 4.11 and 250 ± 7.4 nm, a polydispersity index of 0.19 to 0.36, and a ζ potential of −19 to −39 mV. The optimized nanoemulsion (N2) showed significantly improved drug release and permeation compared with pure piperine dispersion. The nanoemulsions were stable in the tested media. The transmission electron microscopy image showed a spherical and dispersed nanoemulsion droplet. The antibacterial and cell line results of piperine nanoemulsions were significantly better than the pure piperine dispersion. The findings suggested that piperine nanoemulsions may be a more advanced nanodrug delivery system than conventional ones.

1. INTRODUCTION

With a predicted rise in cancer incidence and death rates, 1.9 million cancer-related deaths were reported in 2018.[1,2] Cancer-related deaths significantly increased in 2020, reaching approximately 10 million (World Health Organization). Among the different types of cancer, cervical cancer accounts for the highest death rate. Breast, lung, and prostate cancers are the most common types in new cases. Approximately 2.2 million new lung cancer cases are reported annually.[3,4] Chemotherapy, radiation therapy, and surgery are the three basic forms of cancer treatment. However, chemotherapy damages healthy cells and promotes the growth of malignancies that are resistant to treatment.[5] Therefore, the active targeting of cancer cells with anticancer medications has been investigated. Approximately 60% of the anticancer drugs clinically used are natural medicines.[2] Natural drugs are safe, affordable, and have versatile biological-medicinal activities offered by natural prodrugs.[6,7] The bitter alkaloid piperine is found in various Piper species and is the main bioactive component in black pepper. Its pharmacological activities reported in different diseases like anti-inflammatory,[8] antioxidants,[9] neuroprotective activities,[10] and various malignancies.[11−14] It has also shown its in vitro and in vivo activities against different cancers, including breast, ovarian, colon, lung, liver, and prostate.[2,9,15−17] It revealed a higher Bax/Bcl-2 ratio caused by a lower amount of Bcl-2 proteins and a higher level of Bax proteins.[18] Piperine can decrease lung cancer cell proliferation and differentiation and trigger death through some of these signaling pathways. Moreover, its restricted use as a delivery system is due to its poor water solubility.[13,19] Different nanodelivery systems like nanovesicles,[20] solid lipid nanoparticles,[21] and nanoparticles[13] were prepared to overcome the solubility-related issues.

Oral administration is the most recommended route among the different delivery systems.[22] However, most of the drugs are water-insoluble, which typically results in limited oral bioavailability and creates serious difficulties in developing new delivery systems.[23] To overcome the drawbacks associated with piperine, a formulation strategy needs to be designed to enhance the aqueous solubility and avoid using organic solvents to ensure better therapeutic efficacy.

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The use of nanoemulsion was reported as a delivery system to enhance drug solubility and therapeutic efficacy.[24−26] It is a single-phase, isotropic, and kinetically stable nanocolloidal dispersion system. Due to the nanodroplet size with a greater surface area to volume ratio, the solubility and permeability of drugs increased.[25,26] Compared with other delivery systems, it has numerous advantages such as enhanced drug solubilization, rapid onset of action, less variation in gastrointestinal fluid volume, a longer shelf life, toxicological safety, and the potential for large-scale production.[27,28] Nanoemulsions may be of two types: oil-in-water (oil dispersed in water) or water-in-oil (water dispersed in oil). It can encapsulate hydrophilic and hydrophobic drugs to enhance solubility, permeability, and systemic absorption.[29] The nanosize range and low interfacial tension due to the presence of surfactant lead to lesser agglomeration and better stability.[30]

The study was designed to formulate piperine nanoemulsions using oleic acid, Cremophore EL, and Tween 80 using ultracentrifugation. The prepared nanoemulsions were evaluated to select the optimum composition and then further evaluated for other parameters. Finally, antimicrobial and cell viability studies were performed to check the in vitro efficacy of the piperine nanoemulsions.

2. MATERIALS AND METHODS

2.1. Materials. Oleic acid was procured from Avonchem (Cheshire, England). Cremophore EL was procured from BASF (Ludwigshafen, Germany). Transcutol and labrafil were provided by Gattefosse. CAPMUL was supplied by ABITEC. PEG 400 and eucalyptus oil were procured from BDH. Ethyl oleate, Tween 80, Poloxamer 188, and Poloxamer 127 were procured from Sigma Aldrich. The drug (piperine) was purchased from Beijing Mesochem Technology Co. Pvt. Ltd (Beijing, China).

2.2. Screening of Components. Each component (oil, surfactant, and co-surfactant) was selected to prepare the nanoemulsion based on maximum solubility. Different oils (oleic acid, eucalyptus oil, ethyl oleate, capmul, and labrafil), surfactants (poloxamer 188, poloxamer 127, and Cremophore EL), and co-surfactants (Tween 80, transcutol, PEG 400) were used for the solubility test. Each vial contained oil, surfactant, and a co-surfactant, with excess piperine. The samples were vortexed to homogenized and then shaken in a water bath for 72 h. To separate the supernatant, the samples were centrifuged at 5000 rpm for 1 h and the supernatant was separated. The supernatant was diluted with methanol, and the piperine concentration in each component was measured using an ultraviolet (UV) spectrophotometer.

2.3. Formulation of Nanoemulsion. Based on the maximum solubility of piperine, oleic acid, Cremophore EL, and Tween 80 were chosen as the oil, surfactant, and co-surfactant for nanoemulsions, respectively. The emulsification efficiency determined the selection of the Smix phase (surfactant and co-surfactant mixture). The nanoemulsion was prepared using the high-energy ultrasonication approach with slight modifications.[31] The coarse emulsion was prepared by adding piperine to the selected oil and Smix mixture. The aqueous phase was added to the oil phase mixture with continuous vortexing for 1 min using a vortex mixture.[32] Then, the prepared coarse emulsion phase underwent additional ultrasonication (30 s/cycle) in a water bath at a sonication amplitude of 40%.[33] The prepared nanoemulsions were transferred to a glass vial and evaluated to select the optimum composition. Finally, the stable nanoemulsions were further characterized for different parameters.

2.4. Droplet Evaluation. The nanoemulsions were assessed for droplet diameter, polydispersibility index (PDI), and zeta potential (ZP) using a size analyzer (Malvern, ZS90, Malvern, U.K.). The nanoemulsions were diluted with double-distilled water, and the mean diameter and PDI were measured. Using a specific cuvette, the same sample was used to evaluate the ZP. The PDI and ZP values must be in the standard range for a stable formulation.[34]

2.5. Thermodynamic Stability. The transparent nanoemulsion is thermodynamically stable if the globule diameter is between 100 and 200 nm. Therefore, the thermodynamic stability was assessed by analyzing the samples under various stress conditions (heating and cooling, centrifugation, and freeze-thaw cycles).[35] The prepared piperine nanoemulsions were evaluated for a heating and cooling cycle by keeping the sample at a high temperature (45 °C) and then transferring it to a cold temperature (4 °C). The sample was exposed to centrifugation at 3500 rpm for 30 min and then assessed for homogeneous distribution. Furthermore, the samples were evaluated for the freeze/thaw cycles. The nanoemulsion was stored at −21 °C and then thawed at room temperature for three cycles. The sample without turbidity and the single phase was selected as a stabilized formulation.

2.6. Encapsulation Efficiency. The nanoemulsion was estimated for the piperine encapsulation using the ultracentrifugation process.[21] The sample was diluted in methanol and sonicated for 15 min. Then, samples were centrifuged (Centurion Scientific, West Sussex, U.K.) at 10,000 rpm for 30 min, and the supernatant was collected. The amount of drug entrapped was analyzed using a spectrophotometer at 342 nm.

2.7. pH and Transmittance. The prepared nanoemulsion was used to evaluate the transmittance (%) using the spectrophotometer at 540 nm. Before analysis, the nanoemulsion was diluted in double-distilled water (1:100), and then, the diluted sample was transferred to a cuvette to analyze the percentage transmittance using double-distilled water as a blank.[36] The nanoemulsions were also evaluated for pH using a pH meter.

2.8. Piperine Release Study. The piperine release was evaluated to check the release pattern from the nanoemulsions. The dialysis bag (12−14 kDa) was filled with piperine nanoemulsion (N2) (equivalent to 5 mg of piperine).[37] The bag was dipped into phosphate buffer saline (PBS; pH 6.8) as release media, and the temperature was fixed at 37 ± 0.5 °C with continuous stirring. The released content (3 mL) was collected at a fixed time, and an equal volume of blank PBS was replenished to maintain the uniform study condition. The collected released content was filtered and diluted to measure the piperine concentration using a UV spectrophotometer. The release study was also performed for pure piperine dispersion with the same dose (equivalent to 5 mg) in the same condition to compare the release pattern.

2.9. Piperine Permeation Study. The study protocol was approved by the IAEC, College of Pharmacy, King Saud University (Ethical Reference No. KSU-SE-21-09). The rats were sacrificed, and the intestine was excised and rinsed with cold Krebs bicarbonate buffer (4 °C) to remove the food residue. Then, the intestine was cut into small pieces and mounted in the diffusion cell. The mucosal side of the membrane faced the donor phase, and the serosal side faced the receiver phase. The receiver phase was filled with Krebs−

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Ringer bicarbonate buffer, and a continuous supply of oxygen (95%) and CO_2 (5%) was applied. The donor cell was filled with pure piperine dispersion and piperine nanoemulsions (N2), which had a dose of 2 mg of piperine. 1 mL of permeated content was withdrawn from the receptor chamber and replenished with the fresh buffer. The collected sample at each time point was filtered and diluted to measure the piperine concentration using a UV spectrophotometer. Different parameters, such as flux and enhancement ratio, were calculated from the permeated piperine concentration.

2.10. Transmission Electron Microscopy (TEM). TEM examination of the prepared piperine nanoemulsions (N2) was conducted to assess their shape. One drop of nanoemulsions was applied to a carbon-coated grid, and then, the staining agent uranyl acetate (2% w/v) was added. It was set aside for 5 min to finish the staining procedure. Finally, the sample was visualized under a microscope (Joel JEM1010, Japan).

2.11. Stability Study. The stability study was performed in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) to evaluate the piperine nanoemulsion. SGF was prepared by adding HCl (0.35 mL) and NaCl (100 mg) to double-distilled water (50 mL). Pepsin (100 mg) was then added, and the pH was adjusted to 1.2 using HCl. SIF was prepared by adding KH_2PO_4 (340 mg) to double-distilled water (50 mL). Pancreatin (500 mg) and 0.2 M NaOH (3.85 mL) were mixed uniformly. The pH of the medium was adjusted to 6.8 using NaOH.[38] The prepared sample (2 mL) was mixed with SGF (10 mL) and SIF (10 mL) and incubated for 2 h. After incubation, the samples were collected, and their size, PDI, and encapsulation efficiency (EE) were measured. The initial values of these parameters were compared with the final values.[39]

2.12. Antimicrobial Study. Pure piperine, piperine nanoemulsion (N2), imipenem, ceftazidime, and nystatin were tested for their antibacterial effects against various pathogens. *Candida albicans*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* microorganisms were obtained from the microbiology lab (American Type Culture Collection). Each sample was completely dissolved in distilled water, and each bacterial strain was grown to an optical density of 600 with a bacterial load of 5×10^{-4} CFU/mL. The bacterial cultures were grown in a nutrient broth medium. After 24 h of incubation, the media were thoroughly mixed with 1 mL of the culture suspension. The liquid was poured into a sterile Petri plate and set aside to solidify. The wells were prepared aseptically using a sterile stainless steel borer. Each test sample was added to the well, and the plate was covered. The samples were incubated, and the zone of inhibition (ZOI) was measured and reported as mean ± SD.

2.13. Cell Lines and Culture. Cell viability was evaluated using a tetrazolium salt-based (MTT) cell viability assay following the manufacturer's instructions. Cell line A549 (lung cancer) was plated in 24-well plates (Nunc) with a density of 1.5×10^5 cells/well. Subsequently, 1 mL media was added at 37 °C with 5% CO_2. The cells were incubated for 48 h before the experiment with Dulbecco's Modified Eagle's Medium, supplemented with 10% FBS, L-glutamine (4500 mg/L), 1% nonessential amino acids (100×), and penicillin/streptomycin. After removing the media, 1 mL of piperine and piperine nanoemulsion (N2) was added to the culture media. The cells in each well were diluted to 1:100, 2:100, 3:100, 4:100, and 5:100. The media containing piperine and piperine nanoemulsion (N2) were removed from the wells. Cells were collected after treatment with trypsin-EDTA and resuspended in 1 mL of PBS and centrifuged at 1500 rpm for 3 min. The treated cells were incubated with an MTT solution at 37°C for 4 h. The MTT solution was prepared with a 5 mg/mL concentration in PBS. The formazan crystals were synthesized in wells having cells. After that, the formazan crystals were suspended and dissolved in DMSO (100 μL in each well). The viable cells were measured by measuring absorbance at 570 nm using a microplate reader (Bio-Rad, Richmond, CA). The measuring was done in triplicate, and the data were calculated using a previously described formula.[40]

3. RESULTS AND DISCUSSION

3.1. Screening of Components. The selection of oil, surfactant, and co-surfactant is a very important criterion for determining the solubilizing capacity of a drug. A higher-solubilizing capacity leads to a higher drug loading. The solubility data for different oils (Oleic acid, Eucalyptus oil, Ethyl oleate, Capmul, and Labrafil), surfactants (Poloxamer 188, Poloxamer 127, and Tween 80), and co-surfactants (Transcutol, PEG 400, and Cremophore EL) are displayed in Figure 1. The solubility of the oils was found to be oleic acid > ethyl oleate > labrafil > capmul > eucalyptus oil. The maximum solubility was shown using oleic acid (77.11 ± 4.7 mg/g). The selection of surfactant and co-surfactant was also determined from the solubility result. The maximum solubility was found in Tween 80 (38.54 ± 4.5 mg/g) and Cremophore EL (29.8 ± 2.9 mg/g). Finally, oleic acid, tween 80, and cremophore EL were selected as formulation components based on the high solubility of piperine.

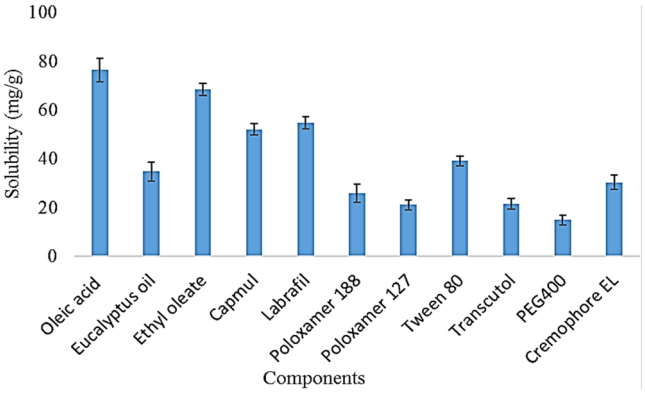

Figure 1. Solubility data of piperine in different nanoemulsions components. Data shown as mean ± SD (n = 3).

3.2. Formulation of Nanoemulsion. The ultrasonication process developed various piperine nanoemulsions by varying the ingredients (Table 1). Six formulations (N1−N6) have been selected from the different nanoemulsions for further characterization. The mean droplet size of the prepared nanoemulsions was significantly ($p < 0.05$) influenced by the Smix ratio. As the Smix ratio increases, the droplet size of nanoemulsions decreases. Smix at a ratio of 1:1 generates nanoemulsions with a higher droplet size (121 ± 3.8 nm) than Smix (2:1; 105 ± 4.11 nm). The droplet size gradually increased when the Smix ratio increased (1:2, 1:3, and 3:2).

3.3. Droplet Characterization. All of the prepared nanoemulsions exhibited a mean diameter in the nanorange, as shown in Table 2. The size of the nanoemulsion was found

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Table 1. Formulation Composition of Piperine Nanoemulsion

code	oil (%)	Smix	water (%)	piperine (mg)
N1	20	1:1	65	25
N2	20	2:1	58	25
N3	20	3:1	48	25
N4	20	1:2	53	25
N5	20	1:3	57	25
N6	20	3:2	61	25

in the range of 105 ± 4.1 (N2) to 250 ± 7.4 nm (F7). A significant ($p < 0.05$) variation in the mean diameter was found in the prepared nanoemulsions. The difference in size can be attributed to the variation in nanoemulsion composition. Moreover, the nanoemulsions (N2) exhibited the lowest droplet size due to the optimum Smix ratio present, and the co-surfactant concentration was lesser than the surfactant (Figure 2). Smix helped to reduce the droplet size by forming a barrier between small droplets. In addition, the formulations were assessed for PDI, and the results showed a low value (<0.7). The low PDI value shows a greater homogeneity of formulations.[34] The ζ potential of the piperine nanoemulsion is presented in Table 2. The formulations showed negative ζ potential (−19 to −39) due to an anionic group in the co-surfactant. The selected piperine nanoemulsion (N2) displayed a ζ potential value of −32 mV. The higher negative or positive value (±30 mV) of ζ potential gives greater stability.[41]

3.4. Thermodynamic Stability. The piperine nanoemulsions were evaluated to check their capability to withstand the stress test (Table 3). The samples were assessed at different temperatures (−21, 40 °C, and room temperature) to select a stable composition. The prepared nanoemulsions were stable under mechanical and thermal stress. Due to a potential metastable formulation, the samples that were determined to be unstable (phase separation, turbidity, nucleation, and precipitation) were discarded from further analysis. The formulations N4 and N6 failed as a result of high turbidity. The failed formulations lost their original transparency, isotropic behavior, and physical stability. This test shows that nanoemulsions have a longer shelf life than traditional emulsions.[42]

3.5. Encapsulation Efficiency. EE of piperine nanoemulsions was found in the range of 76.85 ± 2.2 to 89.32 ± 1.9% (Table 2). A significant ($p < 0.05$) variation in the EE was observed between the prepared nanoemulsions. The change in the results can be attributed to variations in the formulation composition. The lowest encapsulation occurred in the formulation (N3) prepared with oil 20%, Smix 3:1, and water 48%, whereas the highest encapsulation was shown in the composition (N2) prepared with oil 20%, Smix 2:1, and water 58%. The maximum encapsulation rate was observed when the Smix ratio was 2:1. In contrast, 1:1, 3:1, 1:2, 1:3, and 3:2 demonstrated lesser encapsulation. The optimum concentration of Smix helped to achieve increased solubility and stability of nanoemulsions.

3.6. pH and Transmittance. The piperine nanoemulsions showed a pH value within the normal range, as shown in Table 2. The formulations were also evaluated for % transmittance, and the values were found to be close to 100%. The transmittance result confirms the formation of a transparent and clear formulation. The change in the composition of nanoemulsions showed a nonsignificant variation in the % transmittance. The enhancement in the surfactant concentration leads to a slight decrease in the % transmittance.

3.7. Piperine Release Study. The release pattern from the prepared piperine nanoemulsions (N1−N6) was compared with pure piperine under the same conditions, as shown in Figure 3. The nanoemulsions displayed significantly enhanced piperine release (72.5 ± 2.9 to 96.2 ± 3.5%) than pure piperine. Piperine nanoemulsions exhibited a relatively rapid release. The drug release (%) from the prepared nanoemulsions was as follows: N2 > N3 > N1 > N6 > N4 > N5. The formulation (N2) demonstrated a maximum release of 96.2 ± 3.5% among the prepared nanoemulsions. This enhanced release of piperine may correlate with enhanced piperine solubility in the carriers. The nanodroplet size, low viscosity, and PDI greatly enhanced drug release. The nanosized droplet has an increased effective surface area for release, leading to better and faster release.[43] The poor solubility of piperine may lead to limited drug release. Nanoemulsions containing surfactants and co-surfactants enhance drug solubilization and subsequently promote drug release. The nanoemulsion droplets release the drug in the dissolution medium by partitioning the drug between the oil and water phase in the release medium.[28]

3.8. Piperine Permeation Study. The intestinal permeation study assessed the variation in the permeation data from the pure piperine and piperine nanoemulsions (N2). The results revealed a significant ($p < 0.001$) difference in the permeation flux. The permeation flux of pure piperine was 249.6 ± 11.4 $\mu g/cm^2/h$, whereas the piperine nanoemulsion (N2) depicted a significantly ($p < 0.001$) enhanced flux (856.4 ± 19.3 $\mu g/cm^2/h$). The nanoemulsion demonstrated an enhancement ratio of 3.4 fold compared with the pure piperine dispersion. The nanoemulsion can improve the intestinal permeation of drugs through various mechanisms, including improved drug solubility, protection from chemical and enzymatic degradation in the gastrointestinal fluid, and increased interaction with the intestinal membrane or mucus layer.[44,45] Moreover, these nanoemulsions provide extra drug transport mechanisms such as endocytosis. The combined

Table 2. Evaluation Parameters of Piperine Nanoemulsion[a]

code	mean diameter (nm)	polydispersibility index	ζ potential (mV)	pH	transmittance (%)	encapsulation efficiency (%)
N1	121 ± 3.8	0.34	−24	7.1	99.1	79.94 ± 3.3
N2	105 ± 4.1	0.21	−32	7.0	98.7	89.32 ± 1.9
N3	153 ± 3.8	0.19	−27	6.9	98.3	76.85 ± 2.2
N4	178 ± 5.7	0.27	−19	7.0	99.4	86.03 ± 1.4
N5	250 ±7.4	0.36	−22	7.6	99.1	87.88 ± 4.1
N6	236 ± 6.3	0.24	−28	7.5	98.6	79.1 ± 1.2

[a]Data shown as mean ± SD ($n = 3$)

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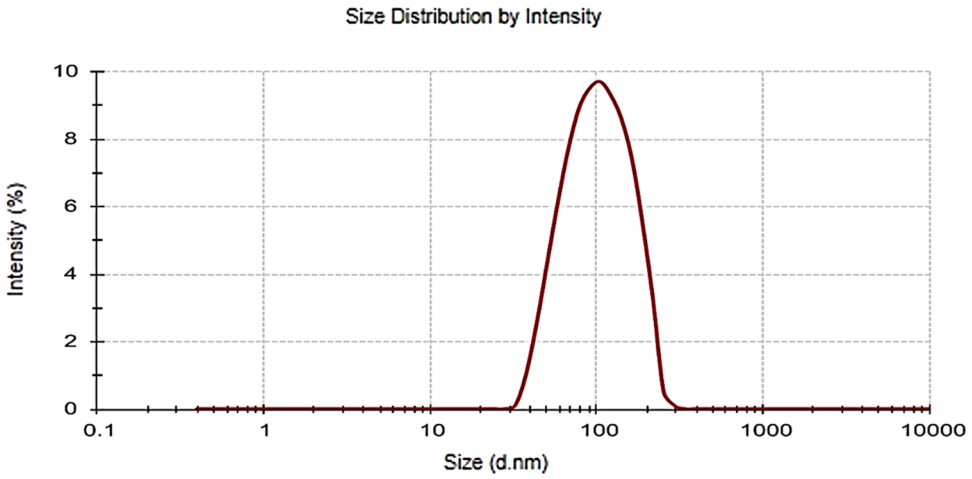

Figure 2. Droplet size of the optimized piperine nanoemulsion (N2).

Table 3. Thermodynamic Stability of the Prepared Piperine Nanoemulsions[a]

code	heating cooling cycle	centrifugation	freezing	inference
N1	+	+	+	pass
N2	+	+	+	pass
N3	+	+	+	pass
N4	+	−	+	fail
N5	+	+	+	pass
N6	−	+	−	fail

[a]Pass (+); fail (−).

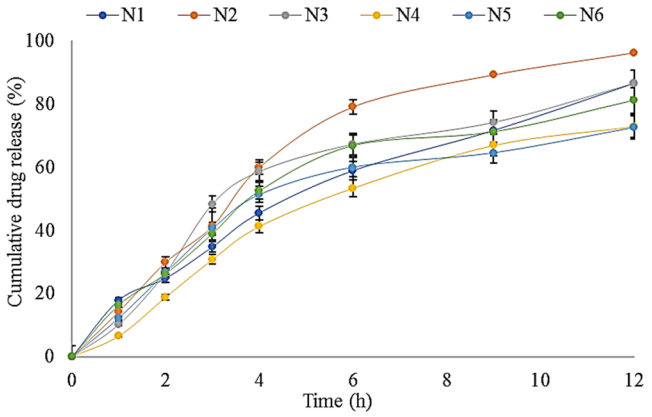

Figure 3. Drug release study data of piperine nanoemulsions (N1−N6). Data shown as mean ± SD (n = 3).

influence of formulation parameters such as nanosize, electrostatic interaction, and the presence of surfactants and lipids may have increased penetration. These components increase the solubility and permeability of drugs.

3.9. TEM Evaluation. To evaluate the morphological size, shape, and nature of the globular distribution, TEM was used to check the aggregation of droplets and heterogeneous globule distribution. The developed nanoemulsion was spherical, clearly dispersed, and highly stable (not shown).

3.10. Gastrointestinal Stability. The prepared piperine nanoemulsion (N2) for oral delivery was evaluated for gastrointestinal stability to evaluate the effect of acidic and enzymatic degradation. The sample was incubated in SGF and SIF fluid, and their size, PDI, and EE were evaluated (Table 4). The prepared samples were stable, and no noticeable difference

Table 4. Stability Study Data of the Prepared Piperine Nanoemulsion (N2)

parameters	initial	final (SGF)	final (SIF)
size (nm)	107 ± 2.1	111 ± 2.8	119 ± 2.6
PDI	0.18 ± 0.02	0.21± 0.01	0.29 ± 0.02
encapsulation efficiency (%)	88.14 ± 3.65%	84.33 ± 4.21	81.21 ± 3.38

in the results was observed. The initial size, PDI, and EE were found to be 107 ± 2.1 nm, 0.18 ± 0.02, and 88.14 ± 3.65%, respectively. As a result of the incubation in SGF, there was a slight change in size, PDI, and EE that were 111 ± 2.8 nm, 0.21 ± 0.01, and 84.33 ± 4.21%, respectively. Similarly, piperine nanoemulsions (N2) incubated with SIF showed a nonsignificant changed size (119 ± 2.6 nm), PDI (0.29 ± 0.02), and EE (81.21 ± 3.38%). The outcomes of the study revealed that the prepared nanoemulsions were stable in both tested fluids.

3.11. Antimicrobial Activity. Figure 4 shows the results of the antibacterial analysis of piperine nanoemulsions and pure piperine against several pathogens. Pure piperine showed a ZOI for *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, and *C. albicans* of 16 ±1.6, 19 ± 1.4, 17 ± 1.3, 15 ± 1.8, and 16 ± 1.7 mm, respectively. The limited solubility of piperine results in a diminished impact on the studied organisms due to poor permeability to the organism's cell wall. The activity of piperine nanoemulsion (N2) was significantly (p < 0.001) higher than that of pure piperine. It displayed ZOI against *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, and *C. albicans* of 22 ± 1.7, 21 ± 1.5, 19 ± 1.4, 20 ± 2.3, and 19 ± 1.9 mm, respectively. Compared with *E. coli*, *B. subtilis*, and *C. albicans*, the activity against *S. aureus* and *P. aeruginosa* was significantly higher (p < 0.05). According to Parekh and Chanda (2007) and Hanumantappa et al. (2014), the difference in activity against different organisms is caused by the permeability barrier created by either the multilayer cell walls present in gram-negative bacteria or the membrane accumulation mechanism or the presence of periplasmic space enzymes that can degrade externally presented foreign molecules.[46,47] Due to the high solubility of piperine in oil, piperine nanoemulsion (N2) has been proven to have better action. The effective surface area for permeation is higher for smaller particles. The standard drugs (imipenem, ceftazimide, and nystatin) were also tested for their antibacterial properties.

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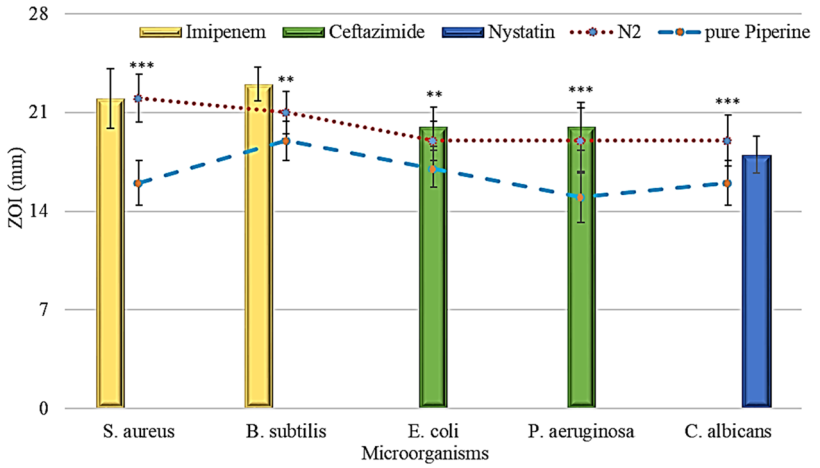

Figure 4. Antimicrobial profile of pure piperine, piperine nanoemulsion (N2), imipenem, ceftazimide, and nystatin. The study data shown as mean ± SD (n = 3). *** highly significant and ** significant to pure piperine.

Imipenem showed activity against *S. aureus* (22 ± 2.1 mm) and *B. subtilis* (23 ± 1.2 mm). Nystatin displayed a ZOI of 18 ± 0.9 mm against *C. albicans*, whereas ceftazidime displayed a ZOI of 20 ± 2.1 mm against *E. coli* and 19 ± 1.4 mm against *P. aeruginosa*. The findings revealed that nanoemulsion showed higher antibacterial activity than pure piperine and was closer to the standard drugs.

3.12. Cell Viability. The developed piperine nanoemulsion (N2) and pure piperine were tested for cell viability against lung cancer A549 cells (Figure 5). Concentration-dependent

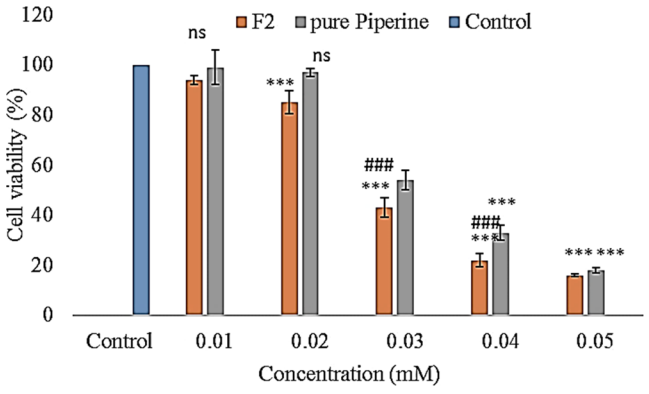

Figure 5. Cell viability study of pure piperine and piperine nanoemulsion (N2) against lung cancer cell line (A549). The study data shown as mean ± SD (n = 3). *** highly significant to control, ### highly significant to pure piperine, ns- nonsignificant.

effects were observed. In the 0.01−0.05 mM concentration range, study revealed a highly significant (p < 0.001) effect against the cell line compared with control. A substantial variation in the activity was observed between pure piperine and piperine nanoemulsion (N2). The pure piperine showed cell viability (%) of 97, 54, 33, and 20% at 0.02, 0.03, 0.04, and 0.05 mM. Moreover, the prepared nanoemulsion (N2) showed enhanced activity at the same concentration of 0.02 (85%), 0.03 (43%), 0.04 (22%), and 0.05 (16%) mM. A significant effect at each tested concentration was observed compared with the control. The most significant (p < 0.001) activity was observed at concentrations of 0.03 and 0.04 mM. A slight difference in the activity was observed at a concentration of 0.05 mM. There was no significant difference in the activity at lower concentrations of 0.01 and 0.02 mM. This study indicated that the produced formulation (N2) had significantly (p < 0.001) higher activity due to the increased solubility of piperine in the presence of the surfactants.

4. CONCLUSIONS

The piperine-loaded nanoemulsion was prepared using ultrasonication to improve solubility and physicochemical characteristics. The developed nanoemulsions displayed nanodroplet size, optimum ζ potential, greater stability, and transmittance (%). The drug release analysis demonstrated a considerable increase in the piperine release. The nanoemulsions also passed the stability assessment at the tested conditions. The antibacterial and cell viability tests revealed that the prepared nanoemulsion has a better effect than pure piperine. From this study, it can be concluded that the prepared nanoemulsion system is an ideal oral delivery system to treat bacterial infection and lung cancer.

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Notes

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■ REFERENCES

(1) Ferlay, J.; Colombet, M.; Soerjomataram, I.; Dyba, T.; Randi, G.; Bettio, M.; Gavin, A.; Visser, O.; Bray, F. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. *Eur. J. Cancer* **2018**, *103*, 356−387.

(2) AbouAitah, K.; Stefanek, A.; Higazy, I. M.; Janczewska, M.; Swiderska-Sroda, A.; Chodara, A.; Wojnarowicz, J.; Szałaj, U.; Shahein, S. A.; Aboul-Enein, A. M.; Abou-Elella, F.; Gierlotka, S.; Ciach, T.; Lojkowski, W. Effective Targeting of Colon Cancer Cells with Piperine Natural Anticancer Prodrug Using Functionalized Clusters of Hydroxyapatite Nanoparticles. *Pharmaceutics* **2020**, *12*, No. 70.

(3) https://www.who.int/news-room/fact-sheets/detail/cancer/ (Acessed on 19th Feb 2023).

(4) Ansari, A.; Hussain, A.; Wadekar, R.; Altamimi, M. A.; Malik, A.; Mujtaba, M. A.; Ansari, M. Y.; Siddique, MUM.; Goyal, S. N. Nanovesicles based drug targeting to control tumor growth and metastasis. *Adv. Cancer Biol.: Metastasis* **2023**, *7*, No. 100083.

(5) Wirsdörfer, F.; de Leve, S.; Jendrossek, V. Combining radiotherapy and immunotherapy in lung cancer: Can we expect limitations due to altered normal tissue toxicity? *Int. J. Mol. Sci.* **2019**, *20*, No. 24.

(6) Ji, H. F.; Li, X. J.; Zhang, H. Y. Natural products and drug discovery. Can thousands of years of ancient medical knowledge lead us to new and powerful drug combinations in the fight against cancer and dementia? *EMBO Rep.* **2009**, *10*, 194−200.

(7) Atanasov, A. G.; Waltenberger, B.; Pferschy-Wenzig, E. M.; Linder, T.; Wawrosch, C.; Uhrin, P.; Temml, V.; Wang, L.; Schwaiger, S.; Heiss, E. H.; et al. Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol. Adv.* **2015**, *33*, 1582−1614.

(8) Bang, J. S.; Oh, D. H.; Choi, H. M.; Sur, B.-J.; Lim, S.-J.; Kim, J. Y.; Yang, H.-I.; Yoo, M. C.; Hahm, D.-H.; Kim, K. S. Anti-inflammatory and antiarthritic effects of piperine in human interleukin 1beta-stimulated fibroblast-like synoviocytes and in rat arthritis models. *Arthritis Res. Ther.* **2009**, *11*, No. R49.

(9) Selvendiran, K.; Singh, J. P.; Krishnan, K. B.; Sakthisekaran, D. Cytoprotective effect of piperine against benzo[a]pyrene induced lung cancer with reference to lipid peroxidation and antioxidant system in Swiss albino mice. *Fitoterapia* **2003**, *74*, 109−115.

(10) Yang, W.; Chen, Y. H.; Liu, H.; Qu, H. D. Neuroprotective effects of piperine on the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease mouse model. *Int. J. Mol. Med.* **2015**, *36*, 1369−1376.

(11) Greenshields, A. L.; Doucette, C. D.; Sutton, K. M.; Madera, L.; Annan, H.; Yaffe, P. B.; Knickle, A. F.; Dong, Z.; Hoskin, D. W. Piperine inhibits the growth and motility of triple negative breast cancer cells. *Cancer Lett.* **2015**, *357*, 129−140.

(12) Yaffe, P. B.; Power Coombs, M. R.; Doucette, C. D.; Walsh, M.; Hoskin, D. W. Piperine, an alkaloid from black pepper, inhibits growth of human colon cancer cells via G1 arrest and apoptosis triggered by endoplasmic reticulum stress. *Mol. Carcinog.* **2015**, *54*, 1070−1085.

(13) Ren, T.; Hu, M.; Cheng, Y.; Shek, T. L.; Xiao, M.; Ho, N. J.; Zhang, C.; Leung, SSY.; Zuo, Z. Piperine-loaded nanoparticles with enhanced dissolution and oral bioavailability for epilepsy control. *Eur. J. Pharm. Sci.* **2019**, *137*, No. 104988.

(14) Vaibhav, K.; Shrivastava, P.; Javed, H.; Khan, A.; Ahmed, M. E.; Tabassum, R.; Khan, M. M.; Khuwaja, G.; Islam, F.; Siddiqui, M. S.; et al. Piperine suppresses cerebral ischemia-reperfusion-induced inflammation through the repression of COX-2, NOS-2, and NF-kappa B in middle cerebral artery occlusion rat model. *Mol. Cell. Biochem.* **2012**, *367*, 73−84.

(15) Gunasekaran, V.; Elangovan, K.; Niranjali Devaraj, S. Targeting hepatocellular carcinoma with piperine by radical-mediated mitochondrial pathway of apoptosis: An in vitro and in vivo study. *Food Chem. Toxicol.* **2017**, *105*, 106−118.

(16) Do, M. T.; Kim, H. G.; Choi, J. H.; Khanal, T.; Park, B. H.; Tran, T. P.; Jeong, T. C.; Jeong, H. G. Antitumor efficacy of piperine in the treatment of human HER2-overexpressing breast cancer cells. *Food Chem.* **2013**, *141*, 2591−2599.

(17) Yaffe, P. B.; Power Coombs, M. R.; Doucette, C. D.; Walsh, M.; Hoskin, D. W. Piperine, an alkaloid from black pepper, inhibits growth of human colon cancer cells via G1 arrest and apoptosis triggered by endoplasmic reticulum stress. *Mol. Carcinog.* **2015**, *54*, 1070−1085.

(18) Lin, M. T.; Lin, B. R.; Chang, C. C.; Chu, C. Y.; Su, H. J.; Chen, S. T.; et al. IL-6 Induces AGS Gastric Cancer Cell Invasion via Activation of the C-Src/RhoA/ROCK Signaling Pathway. *Int. J. Cancer* **2007**, *120*, 2600−2608.

(19) Ezawa, T.; Murata, I.; Takao, K.; Sugita, Y.; Kanamoto, I.; Inoue, Y. Characterization of the Dissolution Behavior of Piperine/Cyclodextrins Inclusion Complexes. *AAPS PharmSciTech.* **2018**, *19*, 923−933.

(20) Zakaria, M. Y.; Fayad, E.; Althobaiti, F.; Zaki, I.; Abu Almaaty, A. H. Statistical optimization of bile salt deployed nanovesicles as a potential platform for oral delivery of piperine: accentuated antiviral and anti-inflammatory activity in MERS-CoV challenged mice. *Drug Delivery* **2021**, *28*, 1150−1165.

(21) Bhalekar, M. R.; Madgulkar, A. R.; Desale, P. S.; Marium, G. Formulation of piperine solid lipid nanoparticles (SLN) for treatment of rheumatoid arthritis. *Drug Dev. Ind. Pharm.* **2017**, *43*, 1003−1010.

(22) Wang, C.; Wang, M.; Chen, P.; Wang, J.; Le, Y. Dasatinib Nanoemulsion and Nanocrystal for Enhanced Oral Drug Delivery. *Pharmaceutics* **2022**, *14*, 197.

(23) Bonifacio, B. V.; da Silva, P. B.; dos Santos Ramos, M. A.; Negri, K.M.S.; Bauab, T. M.; Chorilli, M. Nanotechnology-based drug delivery systems and herbal medicines: A review. *Int. J. Nanomed.* **2014**, *9*, 1−15.

(24) Algahtani, M. S.; Ahmad, M. Z.; Ahmad, J. Nanoemulgel for Improved Topical Delivery of Retinyl Palmitate: Formulation Design and Stability Evaluation. *Nanomaterials.* **2020**, *10*, 848.

(25) Rai, V. K.; Mishra, N.; Yadav, K. S.; Yadav, N. P. Nanoemulsion as pharmaceutical carrier for dermal and transdermal drug delivery: formulation development, stability issues, basic considerations and applications. *J. Controlled Release* **2018**, *270*, 203−225.

(26) Gul, U.; Khan, M. I.; Madni, A.; Sohail, M. F.; Rehman, M.; Rasul, A.; Peltonen, L. Olive oil and clove oil-based nanoemulsion for topical delivery of terbinafine hydrochloride: in vitro and ex vivo evaluation. *Drug Delivery,* **2022**, *29*, 600−612.

(27) Akhtar, J.; Siddiqui, H. H.; Fareed, S.; Badruddeen; Khalid, M.; Aqil, M. Nanoemulsion: for improved oral delivery of repaglinide. *Drug Delivery* **2016**, *23*, 2026−2034.

(28) Karami, Z.; Saghatchi Zanjani, M. R.; Nasihatsheno, N.; Hamidi, M. Improved oral bioavailability of repaglinide, a typical BCS

Class II drug, with a chitosan-coated nanoemulsion. *J. Biomed. Mater. Res., Part B* **2020**, *108*, 717−728.
(29) Salim, N.; Ahmad, N.; Musa, S. H.; Hashim, R.; Tadros, T. F.; Basri, M. Nanoemulsion as a topical delivery system of antipsoriatic drugs. *RSC Adv.* **2016**, *6*, 6234−6250.
(30) Elmataeeshy, M. E.; Sokar, M. S.; Bahey-El-Din, M.; Shaker, D. S. Enhanced transdermal permeability of terbinafine through novel nanoemulgel formulation: development, *in vitro* and *in vivo* characterization. *Future J. Pharm. Sci.* **2018**, *4*, 18−28.
(31) Kaur, K.; Kumar, R.; Arpita; Goel, S.; Uppal, S.; Bhatia, A.; Mehta, S. K. Physiochemical and cytotoxicity study of TPGS stabilized nanoemulsion designed by ultrasonication method. *Ultrason. Sonochem.* **2017**, *34*, 173−182.
(32) Alam, M. S.; Algahtani, M. S.; Ahmad, J.; Kohli, K.; Shafiq-Un-Nabi, S.; Warsi, M. H.; Ahmad, M. Z. Formulation design and evaluation of aceclofenac nanogel for topical application. *Ther. Delivery* **2020**, *11*, 767−778.
(33) Li, P. H.; Chiang, B. H. Process optimization and stability of D-limonene-in-water nanoemulsions prepared by ultrasonic emulsification using response surface methodology. *Ultrason. Sonochem.* **2012**, *19*, 192−197.
(34) Tabatabaeain, S. F.; Karimi, E.; Hashemi, M. Satureja khuzistanica Essential Oil-Loaded Solid Lipid Nanoparticles Modified With Chitosan-Folate: Evaluation of Encapsulation Efficiency, Cytotoxic and Pro-apoptotic Properties. *Front. Chem.* **2022**, *10*, No. 904973.
(35) Tarik Alhamdany, A.; Saeed, AMH.; Alaayedi, M. Nanoemulsion and Solid Nanoemulsion for Improving Oral Delivery of a Breast Cancer Drug: Formulation, Evaluation, and a Comparison Study. *Saudi Pharm J.* **2021**, *29*, 1278−1288.
(36) Laxmi, M.; Bhardwaj, A.; Mehta, S.; Mehta, A. Development and characterization of nanoemulsion as carrier for the enhancement of bioavailability of artemether. *Artif. Cells, Nanomed., Biotechnol.* **2015**, *43*, 334−344.
(37) AbouAitah, K.; Higazy, I. M.; Swiderska-Sroda, A.; Abdelhameed, R. M.; Gierlotka, S.; Mohamed, T. A.; Szałaj, U.; Lojkowski, W. Anti-inflammatory and antioxidant effects of nanoformulations composed of metal-organic frameworks delivering rutin and/or piperine natural agents. *Drug Delivery* **2021**, *28*, 1478−1495.
(38) Kaithwas, V.; Dora, C. P.; Kushwah, V.; Jain, S. Nanostructured lipid carriers of olmesartan medoxomil with enhanced oral bioavailability. *Colloids Surf., B* **2017**, *154*, 10−20.
(39) Ebrahimian, M.; Mahvelati, F.; Malaekeh-Nikouei, B.; Hashemi, E.; Oroojalian, F.; Hashemi, M. Bromelain loaded lipid-polymer hybrid nanoparticles for oral delivery: Formulation and characterization. *Appl. Biochem. Biotechnol.* **2022**, *194*, 3733−3748.
(40) Bashlouei, S. G.; Karimi, E.; Zareian, M.; Oskoueian, E.; Shakeri, M. Heracleum persicum Essential Oil Nanoemulsion: A Nanocarrier System for the Delivery of Promising Anticancer and Antioxidant Bioactive Agents. *Antioxidants* **2022**, *11*, 831.
(41) Gurpreet, K.; Singh, S. K. Review of Nanoemulsion Formulation and Characterization Techniques. *Indian J. Pharm. Sci.* **2018**, *80*, 781−789.
(42) Ali, M. S.; Alam, M. S.; Alam, N.; Siddiqui, M. R. Preparation, characterization and stability study of dutasteride loaded nanoemulsion for treatment of benign prostatic hypertrophy. *Iran. J. Pharm. Res.* **2014**, *13*, 1125−1140.
(43) Freag, M. S.; Elnaggar, Y. S.; Abdallah, O. Y. Development of novel polymer-stabilized diosmin nanosuspensions: in vitro appraisal and ex vivo permeation. *Int. J. Pharm.* **2013**, *454*, 462−471.
(44) Karamanidou, T.; Bourganis, V.; Kammona, O.; Kiparissides, C. Lipid-based nanocarriers for the oral administration of biopharmaceutics. *Nanomedicine* **2016**, *11*, 3009−3032.
(45) Meirelles, G. C.; Mendesa, C.; Caon, T.; Teixeira, H. F.; von Poser, G.; Ponchel, G. Intestinal permeability enhancement of benzopyran HP1-loaded nanoemulsions. *Eur. J. Pharm. Sci.* **2019**, *127*, 115−120.
(46) Parekh, J.; Chanda, S. Antibacterial and phytochemical studies on twelve species of Indian medicinal plants. *African J. Biomedical Res.* **2007**, *10*, 175−181.
(47) Nayaka, H. B.; Londonkar, R. L.; Umesh, M. K.; Tukappa, A. Antibacterial Attributes of Apigenin, Isolated from Portulaca oleracea L. *Int. J. Bacteriol.* **2014**, *2014*, No. 175851.

https://doi.org/10.1021/acsomega.2c08187
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