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RESEARCH PAPER

Nanoniosome-encapsulated levoflaxicin as an antibacterial agent against *Brucella*

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Abstract

A study was conducted to examine the prevalence of brucellosis (in animal farms) in the vicinity of Islamabad and Rawalpindi. A total of 170 milk samples were collected randomly from several farmhouses. The collected milk samples were initially screened by a *Brucella* selective medium. The bacterial isolates grown on the selective medium were subjected to biochemical identification for further confirmation of *Brucella* species. Among the tested samples, 28 (16.4%) were found positive for selective medium and 14 (8.2%) were found positive after biochemical confirmation. The antimicrobial susceptibility of several antibiotics performed by the disc-diffusion method did not yield any significant findings. Encapsulating antimicrobial drugs in unilamellar niosomes is an effective approach to treat the endemic infection. In this study, the antimicrobial activity of niosome-encapsulated levofloxacin is compared with free drug. The drug-encapsulating and empty niosomes were synthesized by using two surfactants Tween 80 and Span 40. Niosomal characterization included electron microscopy, dynamic light scattering, and zeta potential. The encapsulation efficiency was found to be 78% and 74% for Span 40 and Tween 80 niosomes, respectively. The antibacterial activity of niosomal levofloxacin was evaluated against the identified *Brucella* species and the antimicrobial activity of the free drug was increased many folds after encapsulation. In this study, levofloxacin niosomes were successfully synthesized against Brucellosis.

KEYWORDS

antibacterial activity, brucellosis, levofloxacin, niosomes

1 | INTRODUCTION

Brucella is a nonmotile and fastidious Gram-negative coccobacillus of length 0.5–1.5 μm and width 0.5–0.7 μm [1]. Inside the human body, the *Brucella* species live in the cytoplasm of cells in the form of clusters and do not make spores or capsules [2]. The bacterium belongs to the α2-proteobacteriacea family and genus *Brucella*. The genus *Brucella* is divided into six species named *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella canis*, *Brucella canis ovis*, and *Brucella neotomae* [3]. All *Brucella* species are zoonotic in nature and could cause infections in human beings. *B. melitensis*, *B. suis,* and *B. abortus* are known to be more pathogenic to humans and could also infect cattles, pigs, and other small ruminants, respectively [4]. *B. canis* is a pathogen of dogs and has low zoonotic potential as compared to other *Brucella* species. Two *Brucella* species, *B. neotomae* and *B. ovis*, that infect rats and sheep,

respectively, have not yet been reported to cause infections in human beings [5].

Brucellosis is a term used for the bacterial infection when infection due to *Brucella* is caused in living organisms. It can transmit from animal to animal and from animal to human by infected tissues, blood, urine, vaginal discards, aborted fetuses, close contact with infected animals, infected raw milk, and infected dairy products such as soft cheese, yogurt, and ice cream [6]. Aerosols may also contain an infectious dose of the bacteria, ranging from 10 to 100 organisms. These bacterial pathogens can colonize in the digestive and respiratory system from where they can spread through blood and the lymphatic system to the whole body [7]. Infections can also be transmitted sexually in humans and animals because the bacterium exists in both vaginal and semen secretions [8].

Brucellosis is globally prevalent. According to the World Health Organization, more than 500,000 new cases are reported each year [9]. Brucellosis is endemic in many Asian countries such as Saudi Arabia, Iran, Iraq, India, and China. But only snippet seroprevalence studies have been previously conducted in Pakistan. Arain and Saeed [10] reported the outbreak of brucellosis in Central Punjab, where 63% and 57% seropravalence was found with Rose Bengal Plate test and iELISA, respectively. In the Peshawar district, 19.02% prevalence in cattle was reported [11].

The current treatment of brucellosis does not assist in eradication of disease, as the relapse rate is 5–10% even after medication. Moreover, (multidrug) resistance emergence in the bacteria also affects the treatment. There are other drawbacks of antibiotic treatment as well, such as side effects in children, (need for rather) long-term treatment, trouble in parental administration of aminoglycosides, and low therapeutic efficacy [12].

Niosomes are nonionic surfactant vesicles that have the ability to encapsulate both hydrophilic and hydrophobic drugs in their unique structure. They can be synthesized in the nanoscale with easy chemical methods. Niosomes have attractive characteristics. For example, they are stable, less toxic, cheap, and do not require special conditions for storage. The permeability of small ions in niosomes is higher, which makes them attractive drug carriers [13]. They interact with bacterial cell membranes by fusion and contact release to unload their encapsulated drug(s) directly on or inside bacterial cell [14]. As nanocarriers, niosomes have many advantages. For example, they are biocompatible, biodegradable, nonimmunogenic, and structurally stable, and subsequently improve the antimicrobial activity and therapeutic index of chemotherapautics [15]. Encapsulation of drugs in niosomes has several advantages as compared with nonencapsulated drugs, such as enhanced drug bioavailability, metabolic stability, and absorption, which would prolong its circulation in blood, causing sustained release at the site of administration, increasing the potency, and reducing the dose as well as toxicity [14]. Niosomes could pave the way toward a novel approach to treat brucellosis as they are promising drug carriers. Levofloxacin is an antibiotic with a broad spectrum of applications, which belongs to the fluoroquinolones family. It is one of the recommended treatments for brucellosis, because it reduces the risk of nephrotoxicity, showing good pharmacokinetics characteristics, and is also effective in eliminating disease relapse [16].

In this investigation, *Brucella* has been isolated, purified, and identified from raw milk samples of animals. We also report a (novel) step to address the eradication of brucellosis through the synthesis and formulation of levofloxacin-loaded nanometer-sized niosomes and evaluate their (in vitro) antibacterial activity against *Brucella* species. To the best of our knowledge, this is the first time that niosome-loaded levofloxacin is used against *Brucella*.

2 | MATERIALS AND METHODS

2.1 | Sample collection, culture, and preservation

A total of 170 raw milk samples were collected from different farmhouses in the vicinity of Islamabad and Rawalpindi, Pakistan. Samples were collected after investigation of the animal symptoms and disease history like abortion, birth of a weak calf, retention of fetal membrane, and signs of infection in the membrane. Samples were transported on ice and stored at 4°C in the lab. They were (then) cultured on nutrient agar (OXOID Ltd., England) plates and subsequently purified. The pure colonies were stored as glycerol stocks at below freezing temperature.

2.2 | Microbiological and biochemical identification of *Brucella* species

The purified bacterial isolates were identified as *Brucella* species through selective growth medium. All samples were cultured on *Brucella* selective medium having a composition of *Brucella* broth base (OXOID Ltd.), aseptically inactivated horse serum (Caisson Laboratories) and *Brucella* selective supplement SR003A (OXOID Ltd.) reconstituted in 1:1 water and methanol. The prepared solution was incubated for 15 min at 37°C. All ingredients were mixed and homogenized gently. Bacterial isolates cultured on *Brucella* selective medium were examined for colony morphology through gram staining and identified by biochemical characterization, including tests for urease, oxidase, and catalase. Some more investigations,

including motility test and growth of the isolates on blood and MacConkey agar, were also performed to confirm the identification of *Brucella* species.

2.3 | Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the disc-diffusion method with recommended standards of the National Committee for Clinical Laboratory Standards to evaluate whether the bacterial strains are susceptible or resistant [17]. Sterile swabs were dipped in the adjusted suspension inoculum and swabbed on Mueller Hinton agar (OXOID Ltd.) plates. Inoculated plates were allowed to stand for at least 3 min before applying antimicrobial disks, including levofloxacin, imipenem, netimicin, cephradine, clathromycin, amikacin, norfloxacin, tobramycin, linezolid, nitrofurantoin, followed by incubating them overnight at 37°C.

2.4 | Niosomes synthesis

Niosomes were synthesized by the sonication method as reported by Ruckmani et al. [18]. The synthesis was achieved using two surfactants, Span 40 and Tween 80 (Sigma-Aldrich); 60 mg of surfactant (Span 40 and Tween 80) or 25 mg cholesterol was suspended in an aqueous phase of 7 ml of phosphate buffer saline (PBS). Solutions were probe sonicated (Cole Parmer Model CV18) for 30 min, which resulted in the formation of nisome vesicles. The solutions were further ultrasonicated by bath sonication (ELMA Velp Scientifica) for 10 min at 60°C to get unilamellar vesicles and spun at 20,500*g* (Sigma 3-18K) for 30 min. The pellet of niosomes was resuspended in PBS and stored at 4°C for further analysis.

To prepare drug-encapsulated niosomes, 7 ml levofloxacin was suspended in 7 ml of PBS, 60 mg surfactant (Span 40 and Tween 80), and 25 mg cholesterol, and a homogenous solution was formed. This homogenous solution was probe sonicated for 30 min and ultrasonicated for 10 min to get the drug-encapsulated unilamellar vesicles. The sonicated solution was centrifuged to get the pellet of drug encapsulated niosomes, and the pellet was resuspended in PBS. The prepared drug encapsulated noisomes were stored at 4°C for further analysis.

2.5 | Characterization of niosomes

A scanning electron microscope (SEM; JEOL JSMS910, Japan) and a transmission electron microscope (TEM; JEM 2100 TEM) were used to determine the morphology and size (diameter) of nonencapsulated and drug-encapsulated niosomes, respectively. While the particle sizing and zeta potential of drug-encapsulated niosomes were examined through ZetaSizer (ZS Nano, Malvern, UK).

2.5.1 | Encapsulation efficiency of niosomes

The entrapment efficiency of niosomes was measured according to Ruckmani and Sankar [18]. To measure the encapsulation efficiency of drug-encapsulated niosomes, the absorption spectrum of the drug by making dilutions of the drug from 1,000 to 100 μg/ml and coefficient of determination (R^2) was measured by a linear regression curve of drug (levofloxacin) dilutions. Drug-encapsulated niosomal formulation was centrifuged at 15,700*g* for 30 min at 4°C to separate niosomes from the nonencapsulated drug. The concentration of the free drug in the supernatant was measured by an ultraviolet spectrophotometer (Thermo Scientific Scientific). The process was repeated many times to ensure that free drug was completely removed. The percentage of encapsulated drug in niosomes was calculated by using the following formula:

$$\%\ \text{of drug encapsulation} = \frac{\text{total drug} - \text{free drug}}{\text{total drug}} \times 100. \quad (1)$$

2.6 | Antimicrobial activity of niosomes

The antimicrobial activity of niosomes was evaluated by a well-diffusion assay [17] and the broth microdilution method. Bacterial inoculum was spread uniformly on MH agar plate, wells (6 mm diameter) were bored onto the agar plates, and 30 and 60 μl of niosomes solution was added into them and incubated overnight at 37°C. The zones of inhibition were observed/measured and recorded. The broth microdilution assay was performed [19]. Inocula were added with 100 μl of niosomes solution and incubated at 37°C on a shaking incubator at 100 rpm. The optical density was measured at a wavelength of 600 nm.

3 | RESULTS

3.1 | Identification of *Brucella* species

The culture of samples on *Brucella* selective medium did not lead to the growth of microorganisms after 24 h of incubation at 37°C; however, 27 bacterial colones were seen thriving when the incubation was further extended till 48 h. Finally, a total of 28 (16.47%) isolates were found thriving on the *Brucella* selective medium after 72 h of

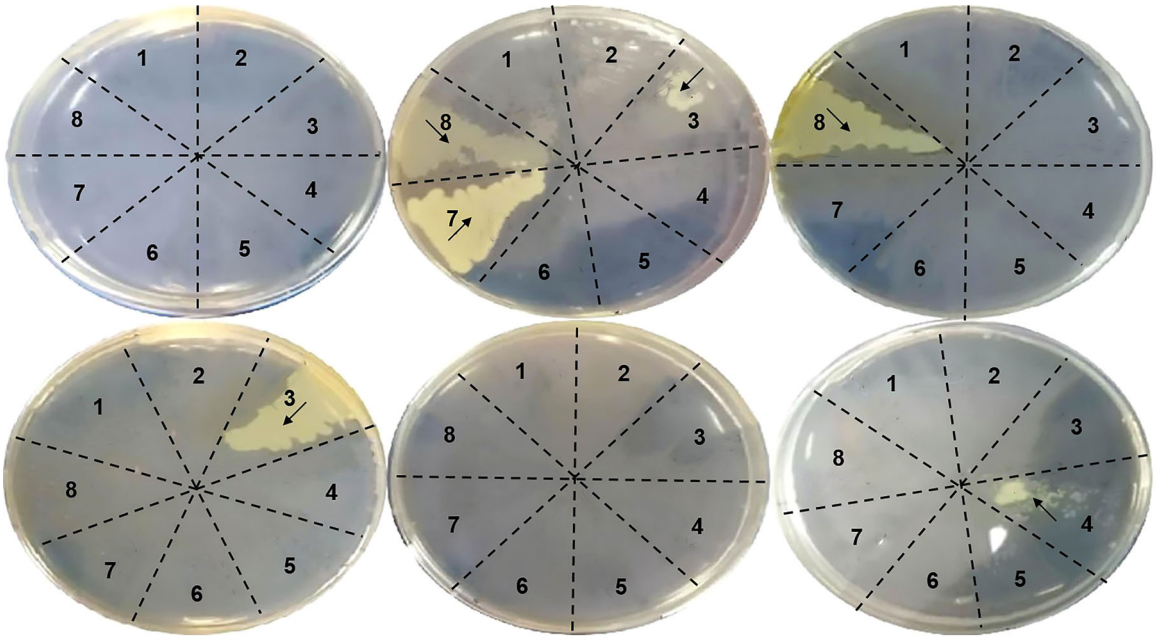

FIGURE 1 Culture of *Brucella* species on selective medium after 72 h of incubation at 37°C. Petri plates had been divided into eight zones where each zone was used for a *Brucella* isolate. Growth was not observed in all of the isolates and black arrows are inserted, which indicate the sample where bacteria thrived while transparent area represents the counterpart microorganisms that did not thrive

incubation but later on, no growth was observed afterward, as shown in Figure 1.

The colony morphology of all of the isolates that were found thriving on selective medium was evaluated via Gram staining and further scrutinized by biochemical tests. A total of 14 (8.23%) out of 28 positive samples were confirmed as *Brucella* species (see Table 1) and stored in glycerol for further analysis.

3.2 | Antimicrobial susceptibility testing

Drug susceptibility assay of all bacterial isolates was evaluated against different broad-spectrum antibiotics and zones of inhibition were measured but an explicit perspective was not established due to insufficient data of drug susceptibility criterion for *Brucella* species. The findings had been narrowed down to the observation that most of *Brucella* isolates were resistant against nitofurantoin, linezolid, and cephradine because no zones of inhibition were observed against these antibiotics.

3.3 | Niosomes synthesis and characterization

The (drug-encapsulated and nonencapsulated) niosomes synthesized by the surfactant Span 40 were named as AAK-SK-NS and by Tween 80 as AAK-SK-NT.

3.3.1 | Electron microscopy

The morphology and size (diameter) of the empty encapsulated niosomes were determined via SEM imaging. The niosomes appeared spherical in shape with diameter ranging from 75 to 85 nm, as shown in Figure 2a. The drug/levofloxacin-encapsulated niosomes were imaged in a TEM; the niosomes were observed as black spheres (70–80 nm in diameter), as illustrated in Figure 2b.

3.3.2 | Particle sizing and zeta potential

The analysis of dynamic light scattering (DLS) results exhibited the hydrodynamic diameter of 80 ± 3.4 nm, which is clearly in agreement with electron microscopy data.

TABLE 1 Samples statistics of collection, isolation, and cultivation on *Brucella* selective medium

No. of samples collected	Culture on *Brucella* selective growth medium after 48 h; 37°C	Cultivation on *Brucella* selective medium after 72 h; 37°C	Identification *Brucella* species after biochemical tests
170	27	28	14

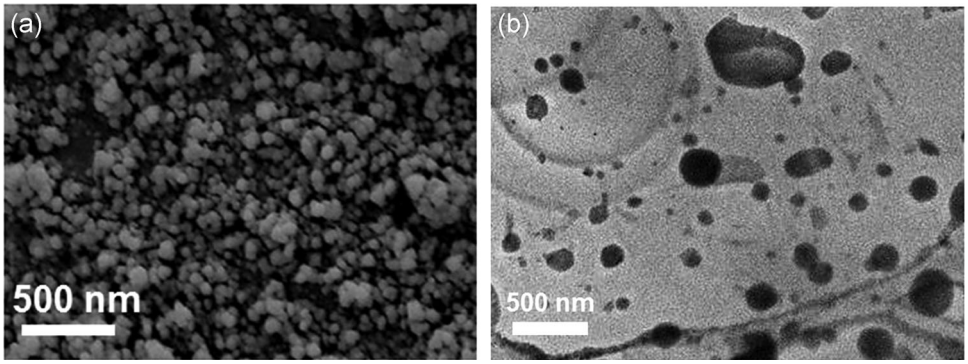

FIGURE 2 Electron microscopy image of niosomes; (a) a scanning electron microscope image of empty niosomes and (b) a transmission electron microscope image of niosomes filled with levofloxacin. The drug-niosmes appeared as dark spheres because of negative staining with phosphotungstic acid (PTA)

The polydispersity index (PDI) value was recorded as 0.2 and signal decay was rapid and smooth, as seen in the raw correlation data (Figure 3a), clearly supporting that the samples were fairly monodispersed. The zeta potential values recorded for encapsulated niosomes were -42 ± 0.35 mV, which indicated that the nanocarrier particles had good stability, [20] as shown in Figure 3b. PDI and a single (major) peak in the DLS analysis supported this fact. This was also supported by the SEM/TEM images, as no aggregation could be seen in these images.

3.3.3 | Encapsulation efficiency

The value of lambda maximum (λ_{max}) was analyzed at 376 nm through the absorption spectrum of dilutions of the drug (see Figure 4a). The optical density of all dilutions was measured at 376 nm and a linear regression curve was plotted between concentration on the *x*-axis and absorbance on the *y*-axis. The calculated encapsulation efficiency of the drug inside the nanovesicles was 78% for AAK-SK-NS and 74% for AAK-SK-NT niosomes, respectively (see Figure 4b).

3.4 | Antimicrobial activity of niosomes

The antimicrobial activity of the empty and drug-encapsulated niosomes was investigated by well diffusion and microdilution assays.

The empty niosomes did not exhibit any antibacterial activity, while clear zones of inhibition (of varying degrees) were recorded for the levofloxacin-encapsulated

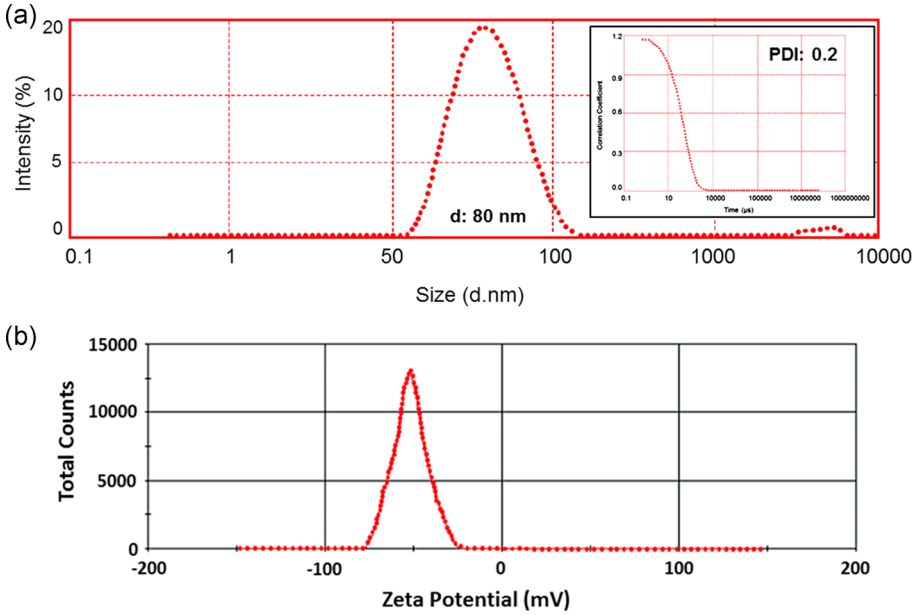

FIGURE 3 (a) Dynamic light scattering analysis (b) zeta potential value of niosomes filled with levofloxacin

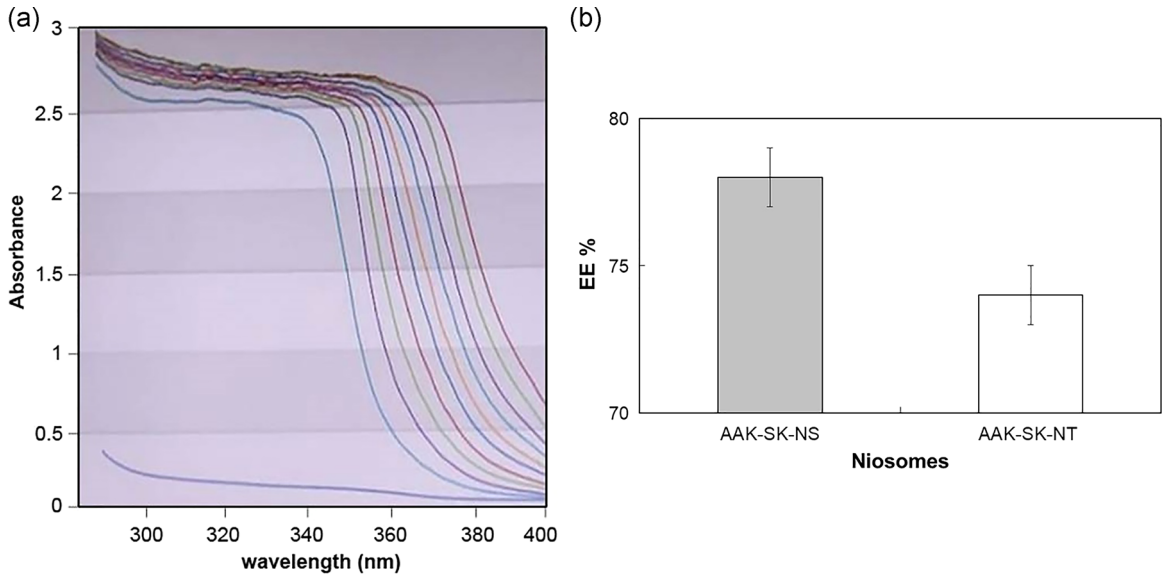

FIGURE 4 (a) Absorption spectrum of drug dilutions from 1,000 to 100 μg/ml in PBS. Pure PBS was taken as reference. The lambda maximum was measured as 376 nm. (b) Entrapment efficiency of levofloxacin encapsulated niosomes named AAK-SK-NS and AAK-SK-NT

niosomes against all bacterial isolates, as given in Figure 5. The antibacterial potential of drug-encapsulated niosomes synthesized with Span 40 and Tween 80 showed variable results at 30 and 60 μl; the zones of inhibition AAK-SK-NS and AAK-SK-NT measured for all bacterial samples are presented graphically in Figure 6a,b.

Since determining antibacterial assay through only well diffusion was not enough to establish facts, broth microdilution assay was also considered. The optical density of broth cultures was recorded at the wavelength 600 nm, as it is directly related to the viable bacterial cell count. The recorded optical densities of nonencapsulated niosomes were similar and/or close to the values measured for the positive control, that is, bacteria thrived well, proving the fact that empty niosomes were not antimicrobial. On the other hand, the optical densities of drug-encapsulated niosomes were close to the values observed for the negative control, meaning that no (or very low) cell growth occurred. Moreover, both of the niosomes generations were put to this test and the results are illustrated in Figure 6c,d.

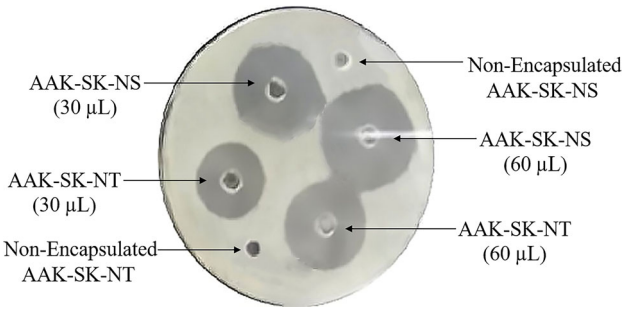

FIGURE 5 Antimicrobial activity of nonencapsulated and levofloxacin encapsulated niosomes (AAK-SK-NT and AAK-SK-NS) by well diffusion assay

4 | DISCUSSION

In developing countries, brucellosis is an endemic disease that causes catastrophic damages to the livestock industry. Disease symptoms in infected animals are economically significant, including reduced fertility, abortions, poor weight gain, and decline in milk production [21]. Previous reports have indicated that brucellosis is highly prevalent throughout the Middle East and Asian countries such as Iraq, Jordan, Saudi Arabia, Kyrgyzstan, and Azerbaijan [22]. The current study is a quantitative analysis of prevalence of brucellosis in the livestock of Islamabad and Rawalpindi territories. The prevalence was measured to be 8.23% by growth on *Brucella*-selective media. The growth of the bacteria isolated was observed after 72 h of incubation. The growth pattern indicated the slow-growing and fastidious nature of *Brucella* species [23].

The study also aimed to determine the drug susceptibility profile of identified *Brucella* species against different antibiotics. Antimicrobial susceptibility by the disc diffusion method has yet not been standardized and antibiotics breakpoint values have not been described yet by CLSI and EUST to analyze the susceptibility of these pathogens [24,25]. Since breakpoints for *Brucella* species have not yet been established, these strains cannot be confidently characterized [20]. It is reported that *Brucella* species are getting resistant against rifampicin and

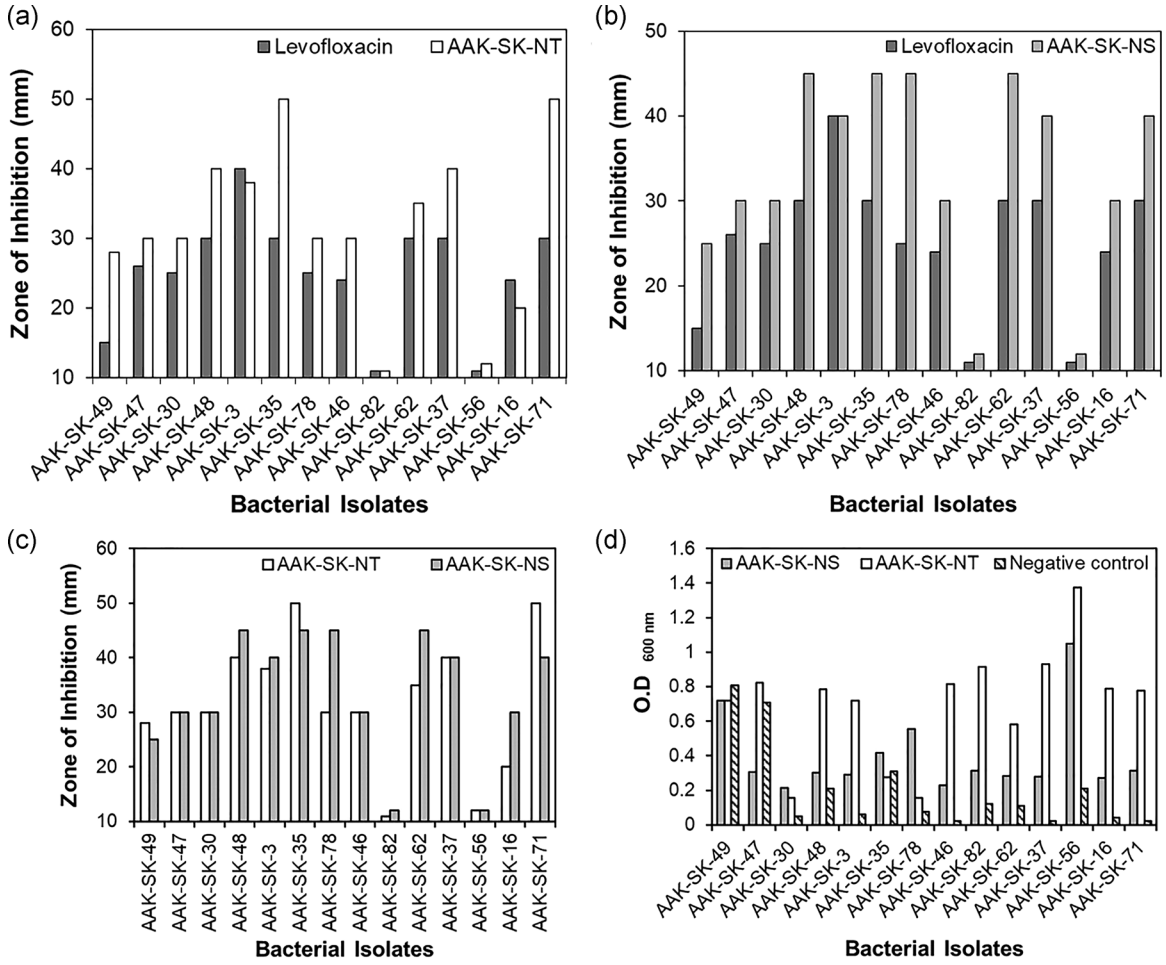

FIGURE 6 (a) Comparison of antibacterial activity of levofloxacin and AAK-SK-NS against all bacterial isolates by well diffusion assay; (b) comparison of the antibacterial activity of levofloxacin and AAK-SK-NT against all bacterial isolates by well-diffusion method; (c) comparison of the antibacterial activity of AAK-SK-NT and AAK-SK-NS against all bacterial isolates by the well-diffusion method; (d) comparison of the antibacterial activity of AAK-SK-NT and AAK-SK-NS against all bacterial isolates by broth microdilution assay

cotrimoxazole and antibiotics from the quinolones family [26]. Since the mid-1980s, laboratory researchers have found antibiotics from the fluoroquinolones family, such as levofloxacin active against intracellular bacteria, reduced risk of nephrotoxicity, and good pharmacokinetics characteristics that made this drug an attractive alternative drug choice for brucellosis treatment [16]. This had fascinated us and was the reason we had opted for this drug.

In the context of the emerging mulitdrug resistance against endemic diseases, antibiotic-encapsulated niosomes is a novel approach for (targeted) drug delivery. It has been established that encapsulation of the drug in niosomes can enhance its antimicrobial activity and reduce drug-associated toxic side effects [27].

In the current study, the diameter of nonencapsulated and encapsulated niosomes measured under SEM and TEM, respectively, showed that niosomes were perfectly spherical in shape, and did not show any charging effect that indicated the absence of organic impurities and/or unreacted chemicals. Electron microscopy imaging proved that both empty as well as drug-encapsulated nanoniosomes were of the same shape and size. The hydrodynamic diameter measured by DLS was in agreement with electron microscopy data. The measured diameter was slightly greater than the diameter obtained through electron microscopy. The greater diameter was due to the fact that a thin electric dipole layer of solvent adhered in the former case while the latter would give an estimation of projected area [28]. The obtained PDI value and the signal decay in the raw correlation data strongly supported the monodispersity of noisomes. The measured surface potential value of niosomes indicate that the nanocarrier particles had good stability [29]. The filled niosomes were negatively charged and repelled each other due to electrostatics, which made the suspension stable [28].

The encapsulation efficiency of niosomes named AAK-SK-NS was slightly higher as compared to AAK-SK-NT at

both concentrations of 30 and 60 µl. The type of surfactants affects the physical properties of niosomes such as size, stability, toxicity, and encapsulation efficiency. Span 40 and Tween 80 both belong to the alkyl ester family of surfactants [14]. Span 40 contains a longer saturated alkyl chain and a small head region that enhanced its encapsulation efficiency, stability, and phase transition properties [30] whereas Tween 80 has an unsaturated alkyl chain and a larger head region, which reduced its entrapment efficiency, stability, and phase transition [31]. The encapsulation of levofloxacin in niosomes is in agreement with the results presented by Imran et al. [32]. The data here represents the higher antibacterial activity of antibiotic-encapsulated niosomes, when compared with free levofloxacin. There are several mechanisms through which niosomes would interact with the bacterial cells such as contact release, fusion, and adsorption [33]. These mechanisms help to release drug adjacents to and inside the bacterial cell, increase concentration of drug, and protect antibiotics form enzymatic degradation [34]. Low susceptibility of several antibiotics is due to the low permeability of the outer membrane of bacteria and the efflux mechanism. Niosomal carrier is an interesting approach to enhance the antibacterial activity of several drugs. The lipid bilayer of niosomes fuse with outer membrane of bacteria, thus altering the therapeutic index of a drug, reducing drug toxicity, prolonging circulation of the drug, and enhancing the accumulation of the drug in the target site (see in Figure 7) [34]. The antibacterial activity of drug-encapsulated niosomes was studied by many researchers. The outcomes of the present study are in agreement with Begum et al. [35] and Avoka et al. [36].

From the results of our current research, it can be concluded that the encapsulation of levofloxacin enhances its antibacterial activity by many folds. It is indicated that niosomal levofloxacin can be an effective and valid approach to treat the endemic disease and can be an efficient novel way to address the developing drug-resistance in bacteria.

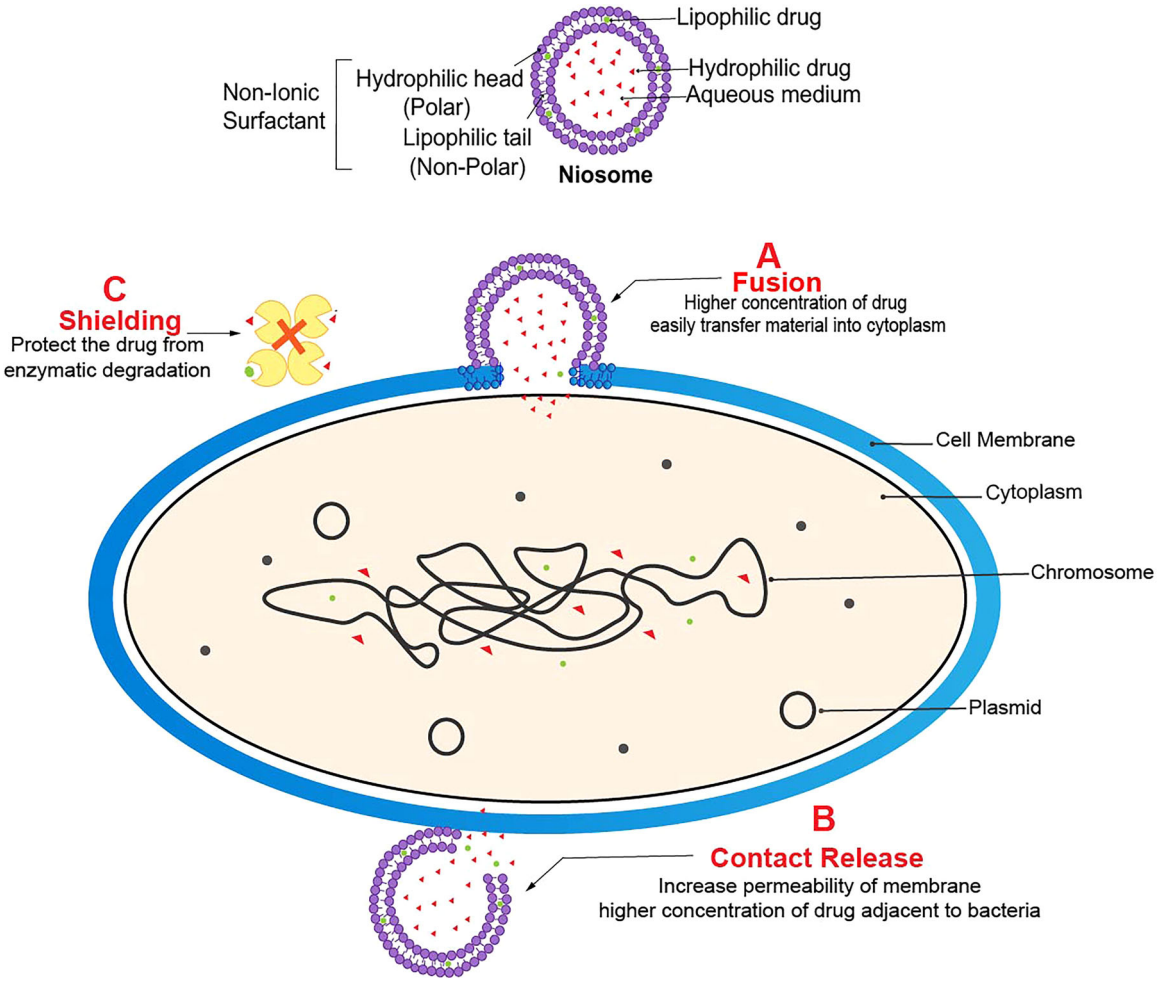

FIGURE 7 Possible modes for the mechanism of action of niosomes entering gram-positive *Brucella* bacterial cells. The niosomes can deliver the encapsulated drug inside them by a fusion or b contact release. Niosomes can also perform c shielding to protect the drug from degradation

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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