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RESEARCH ARTICLE

Antibacterial effects of impregnated scaffolds with solid lipid nanoparticles gels containing three essential oils against standard and clinical strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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ABSTRACT

Wound healing is a complicated process that is affected by physiological and environmental conditions. An antibacterial agent and a physical supporter as wound dressing improve wound healing time and process. In this study, solid lipid nanoparticles (SLNs) containing *Mentha longifolia*, *Mentha pulegium,* and *Zataria multiflora* essential oils with a particle size of 103±5, 195±4, and 130±7 nm were first prepared, and they were then transformed to gels. After that, scaffolds of electrospun polycaprolactone-alginate nanofibers (165±37 nm) were prepared. Chemical properties, surface hydrophilicity, and size and morphology of scaffolds were investigated using ATR-FTIR, contact angle, and SEM analyses. The gels were physically impregnated at two different amounts on the scaffolds, and antibacterial effects at 2, 6, and 24 h of incubation times were determined using the ATCC100 method. Impregnated scaffold with SLNs gel containing *Z. multiflora* essential oil showed higher antibacterial activity against all standard and clinical bacterial strains at incubation times. The growth of *Staphylococcus aureus* decreased by more than 90% in all incubation times. Besides, more than 60% reduction in the growth of *Pseudomonas aeruginosa* was observed after 24 h incubation time. As high potency and green constituents of the prepared prototype, it could be considered for clinical trials

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INTRODUCTION

Traumatic damages are a great healthcare issue that usually requires long and expensive medical treatment. Two subjects are important in the wound healing process: 1) bacterial or fungal infection and 2) chemical or physical supporter for improving the wound healing process. Infection of wounds by bacterial or fungal species led to the delayed wound healing process [1]. Among bacterial species, *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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are the most common hospital pathogens that can delay the wound healing process [2]. *S. aureus,* as a gram-positive bacterial species, is a global problem in clinical medicine that can cause skin and soft tissue infections [3]. It is cause for different skin infections such as cellulitis, impetigo, scaled skin syndrome, pimples, erysipelas, and carbuncles [4]. Also, *P. aeruginosa*, as a gram-positive bacterial species, can infect the skin and skeletal tissues, burn wounds, endocarditis surgical wounds [5, 6].

Antibiotic therapy is the first treatment line for bacterial infection. However, there is concern about antibiotic resistance of bacterial species such as *S. aureus* and *P. aeruginosa* [7]. So, the development of novel antibacterial agents is required for alternative antibiotics. Currently, metal nanoparticles such as silver, zinc oxide, and cadmium oxide nanoparticles [8, 9] and essential oil-based nanoformulations such as nanoemulsions, polymeric nanoparticles, and nanogels have received more attention [10-12]. However, essential oils (EOs) are preferred due to wide usage in medicine such as antibacterial activity, aromatherapy, cosmetic, wound healing, and skincare [13, 14]. Among common nanocarriers for stability improvement of EOs, solid lipid nanoparticles (SLNs) with higher capacity have been recently more considered. SLNs were introduced as nano-carriers composed of solid lipids (as core matrix), surfactants and/or co-surfactants (as stabilizers) that could deliver active hydrophobic drug molecules [15]. The ability to incorporate EOs into the hydrophobic core of SLN gives an opportunity to protect EOs from harsh environmental conditions [16, 17]. Moreover, by incorporating SLNs into gels, their topical usage is facilitated.

The second parameter in the wound healing process is chemical or physical supporters as wound dressing. There are various types of formulations and structures for supporting wound site as dressing, such as ointments [18, 19], creams [20, 21], hydrogels [22, 23], gels [24, 25], and scaffolds [26, 27]. Scaffolds have special applications for wound healing due to their strong physical support from wound site cells and the carrier role as therapeutic agents. The scaffolds could be prepared with biocompatible and biodegradable natural or synthetic polymers such as chitosan [28], polylactic acid [29], polyglycolic acid [30], poly lactic-co-glycolic acid [31], and polycaprolactone [32, 33]. Also, different methods could be applied for the preparation of scaffolds such as spinning (electro-, melt-, and wet-spinning) [31], freeze-drying [34], phase separation [35], and 3d-printing method [29]. However, the single fluid electrospinning method is still widely used to prepare micro-and nanofibers as a flexible, low-cost, and straightforward technique [36, 37].

In this study, SLNs gels containing *Mentha longifolia*, *Mentha pulegium,* and *Zataria multiflora* EOs were first prepared. Electrospun polycaprolactone-alginate (PCL/Alg) scaffolds were then prepared as a wound dressing. Finally, the antibacterial activity of impregnated scaffolds with gels was investigated against standard and clinical strains of *S. aureus* and *P. aeruginosa*.

MATERIALS AND METHODS

Bacteria and reagents

P. aeruginosa (ATCC853) and *S. aureus* (ATCC25923) standard strains were provided by Pasteur Institute (Tehran, Iran). The clinical strains used in the current study were provided in the previous study of this team from hospital specimens [38]. Briefly, *P. aeruginosa* Metallo-beta-lactamase (MBL)-Producing (Imipenemase and meropenem) and Methicillin-resistant *S. aureus* (MRSA) were collected from the urinary tract and wound infections at Valiasr hospital Fasa (Fasa, Fars Province, Iran). The routine culture media (the disk-diffusion method and MIC test) and biochemical (genetic-PCR test) tests were used for identified phenotypically and genotypically MRSA and MBL-producing *P. aeruginosa* [39, 40]. *M. longifolia* and *M. pulegium* were bought from Tabib Daru (Iran). *Z. multiflora* EO was purchased from Zardband Pharmaceuticals Company (Iran). Sodium alginate, carboxymethylcellulose, and polycaprolactone were supplied by Sigma-Aldrich (USA). Stearic acid, surfactants (span 60 and tween 80), Mueller-Hinton broth, and agar were bought from Merck Chemicals (Germany).

Preparation characterization of PCL/Alg scaffolds

The electrospinning method was applied to prepare PCL/Alg scaffolds as described in our previous report [38]. Briefly, polycaprolactone granules and alginate powder (10% and 3% w/v) were dissolved (2000 rpm, 24 h, room temperature) in hexafluoroisopropanol. The prepared polymer solution was then filled in a 10 mL syringe (as polymer reservoir) connected to a blunted stainless steel needle (22 gauge). They were placed on the syringe pump into an electrospinning device (FNM

Co. Ltd., Iran), and instrumental parameters were set as follows:

1. The acceleration potential was 15.6 Kv
2. The injection rate was 0.4 mL/h
3. The distance between the needle and rotating collector (120 rpm) was 90 mm
4. The drum rotation speed was 130 RPM

Morphology and diameter of the prepared PCL/Alg scaffold were analyzed by Scanning Electron Microscope (SEM, TESCAN Vega3, Czech Republic). Before the SEM observations, 1 × 1 cm square sliced of PCL/Alg scaffold was coated with gold vapors (Quorum Technologies, Q150R- ES), and then, the sample was put on the stage the device. The PCL/Alg scaffold size was determined by the free version of Digimizer Image Analysis Software (MedCalc Software Ltd., Ostend, Belgium).

To identify functional groups to confirm PCL/Alg scaffold preparation, an analysis of attenuated total reflection- Fourier Transform Infra-Red (ATR-FTIR) (Bruker Company, Model Tensor II, USA) was utilized. The scan range spectra of PCL/Alg NF were recorded in 400–4000 cm^{-1}. The contact angle measurement machine was applied to determine the hydrophilicity of the PCL/Alg scaffold (Sharif Solar, Tehran, Iran). Briefly, about 5 mL deionized water was injected on PCL/Alg NF scaffold, and the pictures were taken, and the contact angle was then measured.

Preparation of SLNs gel containing EOs

As described in our previous research, the high-pressure homogenizer method was used to prepare SLNs containing EOs of *M. longifolia*, *M. pulegium*, and *Z. multiflora* [41]. Briefly, 4% v/v of stearic acid (as hydrophobic lipid core of SLN) was melted at 85 °C, and then, the EO (1% v/v) was dissolved in it. Then, 2% v/v of span 60 (as lipophilic surfactant) and 4% v/v of tween 80 (as aqueous surfactant) was added. The high-shear homogenizer (D-91126 Schwabach, Heidolph, Germany) was applied to homogenize the final solution for 1 min at 8000 rpm. To reduce the size of particles at the obtained solution, a high-pressure homogenizer (APV Micron Lab 40, APV Systems, Unna, Germany) was utilized (3 cycles, 500 bar, thermostated at 90°C). The prepared SLNs were transformed to gel by adding 3% CMC to each solution (180 rpm, stirred overnight). Moreover, SLN gel without EO was also prepared using the same manner; only no EO was used.

Impregnation of PCL/Alg scaffolds with gels

Circular pieces of diameter 5 cm of PCL/Alg scaffold were cut and impregnated with 250 and 500 mg of the prepared gels of *M. longifolia*, *M. pulegium*, and *Z. multiflora* EOs, and SLN gel without EO; the prepared samples were abbreviated as ML-SLN, MP-SLN, ZM-SLN, and SLN(-oil).

Investigation antibacterial properties of impregnated scaffolds with gels

The antibacterial properties of the prepared PCL/Alg scaffolds and impregnated scaffolds with gels (i.e., ML-SLN, MP-SLN, ZM-SLN, and SLN(-oil)) were investigated according to the textures standard method (ATCC100) with a slight modification. Briefly, about 2 mL of each fresh bacterial suspension (2×10^5 CFU/mL) was filled separately in 5 cm plate plates. By adding ML-SLN, MP-SLN, and ZM-SLN to each plate, final concentrations of EOs were fixed at 1.25 and 2.5 mg/mL. Then, the treated plates were incubated at 37 °C for 2, 6, and 24 h. After each exposure period, 10 µL of suspensions were inoculated on a Muller-Hinton agar culture plate and incubated for 24 h [37]. The number of colonies that grew in the culture medium was counted and compared with the control group. The growth reduction was calculated using equation 2.

% of growth reduction= (CFU control – CFU sample/ CFU control) ×100 (2)

Statistical Analysis

All the tests were performed in triplicates, and the obtained results were presented as mean ± SD. In addition, the antibacterial activities of samples were compared together using paired-samples T-Test with at least significance level (α) of 0.05 (SPSS software v. 22, USA).

RESULTS

SLNs characterization

As shown in Fig. 1, the size of SLNs containing *M. longifolia, M. pulegium,* and *Z. multiflora* EOs were obtained as 103±5, 195±4, and 130±7 nm, respectively.

Chemical confirmation of PCL/Alg scaffolds

ATR-FTIR method was applied for confirmation of PCL/Alg scaffolds preparation (Fig. 2). The spectrum of polycaprolactone granules exhibits absorption band characteristics at around 2943 cm^{-1} (asymmetric) and 2865 (symmetric) cm^{-1}

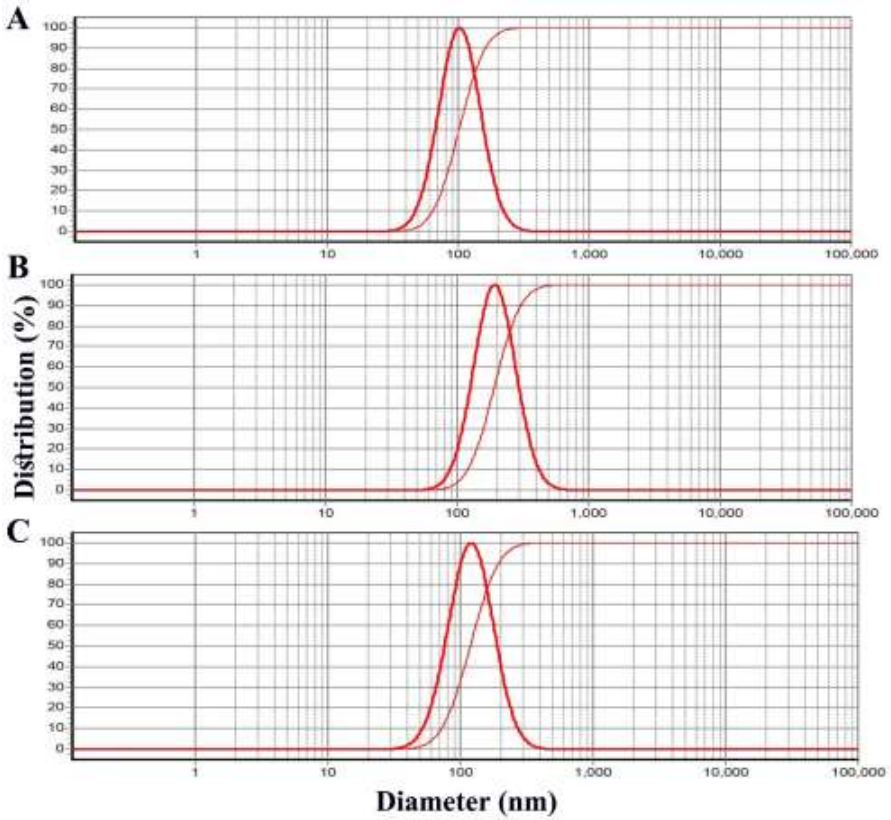

Fig. 1. DLS analysis of solid-lipid nanoparticles containing essential oils of A: *M. longifolia* (103±5 nm), B: *M. pulegium* (195±4 nm), and C: *Z. multiflora* (130±7 nm)

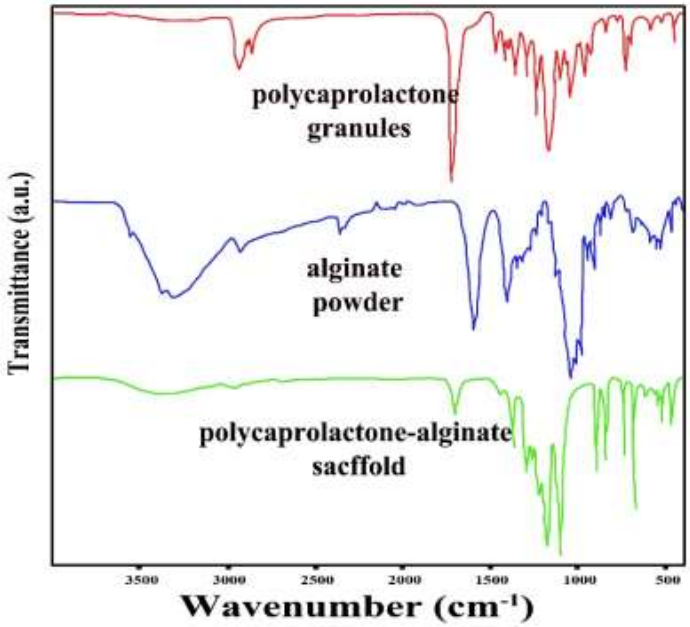

Fig. 2. ATR-FTIR spectra of the prepared electrospun scaffolds

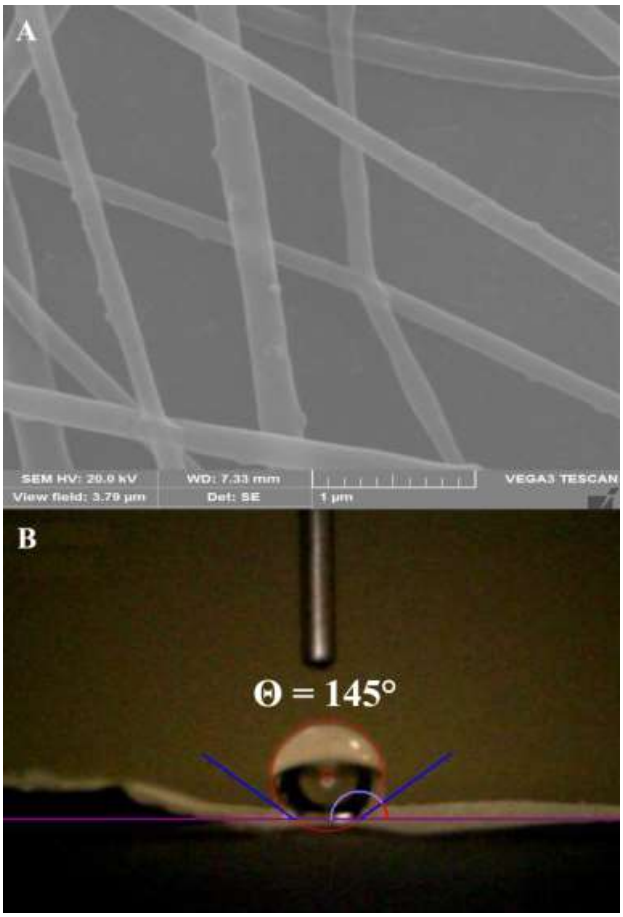

Fig. 3. A: Polycaprolactone-alginate scaffolds SEM image, and B: contact angle of water with surface of the scaffolds

correspond to -C-H. Also, the sharp band at 1721 cm^{-1} corresponds to the stretching band of the C=O, and the bands at 1470 and 1396 cm^{-1} were related to CH_2 groups. The band at about 1292 cm^{-1} could be related to asymmetric C-O-C stretching, and the peaks at about 1237 and 1167 cm^{-1} correspond to symmetric C-O-C stretching [42].

Pure sodium alginate (ATR-FTIR spectra) components showed the broad bands at about 3200-3600 cm^{-1} related to -OH is stretching vibration. The bands at 2971, 2940, and 2913 cm^{-1} correspond to –CH stretching vibration. The stretching vibration (asymmetric) of the carboxylate group (-COO) appears at 1597 cm^{-1} and peaks at 1218, and 1100 cm^{-1} relate to C-O-C stretching bonds.

PCL/Alg scaffolds ATR-FTIR spectrum exhibit an abroad band at 3391 cm^{-1} attributes to -OH stretching vibration. Also, a remarkable reduction of O-H stretching band intensity could be attributed to decreased water-polymer interactions through alginate cross-linking. It may enhance polymer intermolecular hydrogen bonding and therefore reduce its ability to hydrate. The peak at 1701 cm^{-1} is related to the carbonyl group, and a reduction in the peak intensities is associated with the free carboxylate groups of alginate in the scaffold. Also, the strong peak at 1100 cm^{-1} is related to C-O.

Scaffolds physical and biological properties

The average size of PCL/Alg nanofibers in scaffolds was 165±37 nm, and their size distribution was visually narrow (Fig. 3A). In addition, results showed that the contact angle of the water with the PCL/Alg scaffold was 145° which means it has a hydrophobic surface (Fig. 3B).

Antibacterial activity of scaffold and SLN gel without EO

Antibacterial effects of PCL/Alg scaffold and impregnated scaffold with 500 mg of SLN gel

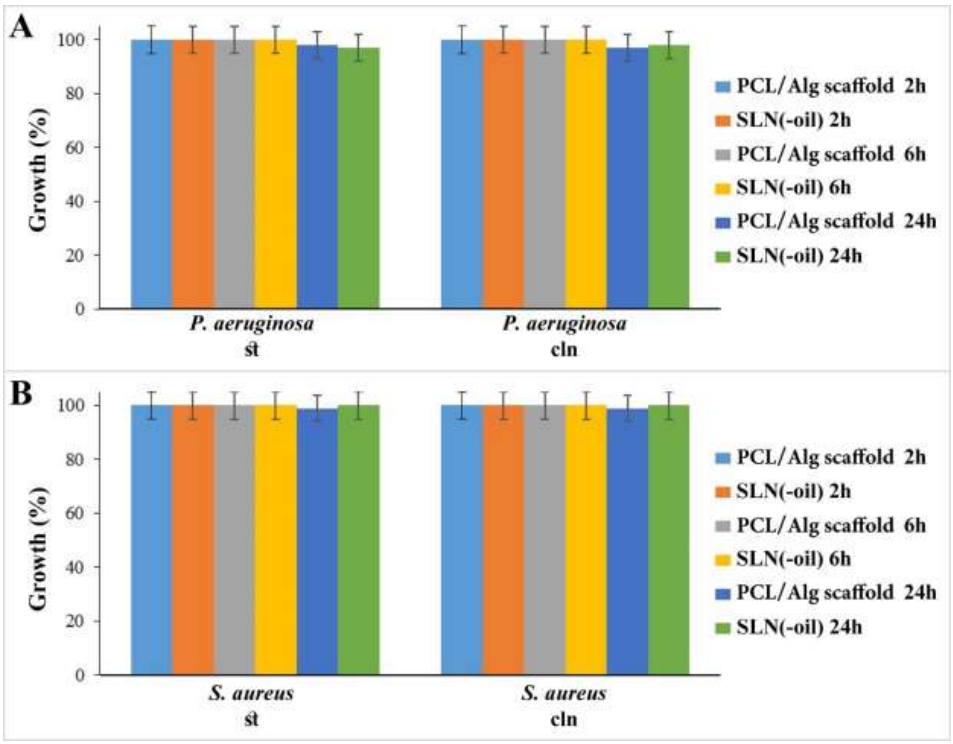

Fig. 4. The antibacterial effect of polycaprolactone-alginate (PCL/Alg) scaffolds and impregnated PCL/Alg scaffolds with 500 mg solid lipid nanoparticles gels without essential oil (SLN(-oil)) against standard (st) and clinical (cln) strains of A: *P. aeruginosa* and B: *S. aureus*

without EO (SLN(-oil)) are shown in Fig. 4. They did not show inhibitory effects on the growth of standard and clinical strains of *P. aeruginosa* and *S. aureus*. The growth of all strains at all incubation times (2, 6, and 24 h) was ~ 100%; they did not show significant differences with the control group ($P > 0.05$). Because the results of impregnating the scaffold with 250 mg SLN(-oil) were the same as 500 mg, the results are not shown.

Antibacterial effects of impregnated scaffolds with gels

Antibacterial effects of impregnated PCL/Alg scaffold with 1.25 and 2.5 mg/mL gels (i.e., ML-SLN, MP-SLN, and ZM-SLN) against standard and clinical strains of *P. aeruginosa* and *S. aureus* at incubation times of 2, 6, and 24 h are summarized in Fig. 5(A, B, and C). Moreover, details of statistical analysis amongst antibacterial effects of all samples at all incubation times are given in supplementary docs (Table S1, S2, and S3). The results indicated that *S. aureus* was more sensitive than *P. aeruginosa* at standard and clinical bacterial strains.

At 2 h incubation time

As shown in Fig. 5A, ZM-SLN 2.5 mg/mL was significantly more potent than other samples ($P<0.001$) with 72 and 40% growth reduction in the standard and clinical strains of *P. aeruginosa*. Moreover, ~ 100% reduction in the growth of *S. aureus* (standard and clinical strains) was observed after treatment with ZM-SLN 2.5 mg/mL; however, its potency compared with ZM-SLN 1.25 mg/mL had no significant differences ($P < 0.05$).

At 6 h incubation time

Antibacterial effects of samples with 6 h incubation times are depicted in Fig. 5B. The growth of standard and clinical strains of *P. aeruginosa* after treatment with ZM-SLN 2.5 mg/mL was reduced to 72 and 64%; this effect was significantly more potent than other samples ($P<0.001$). Interestingly, growth of standard and clinical strains of *S. aureus* were completely inhibited after treatment with ZM-SLN 1.25 and 2.5 mg/mL; their effect was significantly more potent than ML-SLN 1.25 and 2.5 mg/mL and MP-SLN 1.25 and 2.5 mg/mL ($P<0.001$).

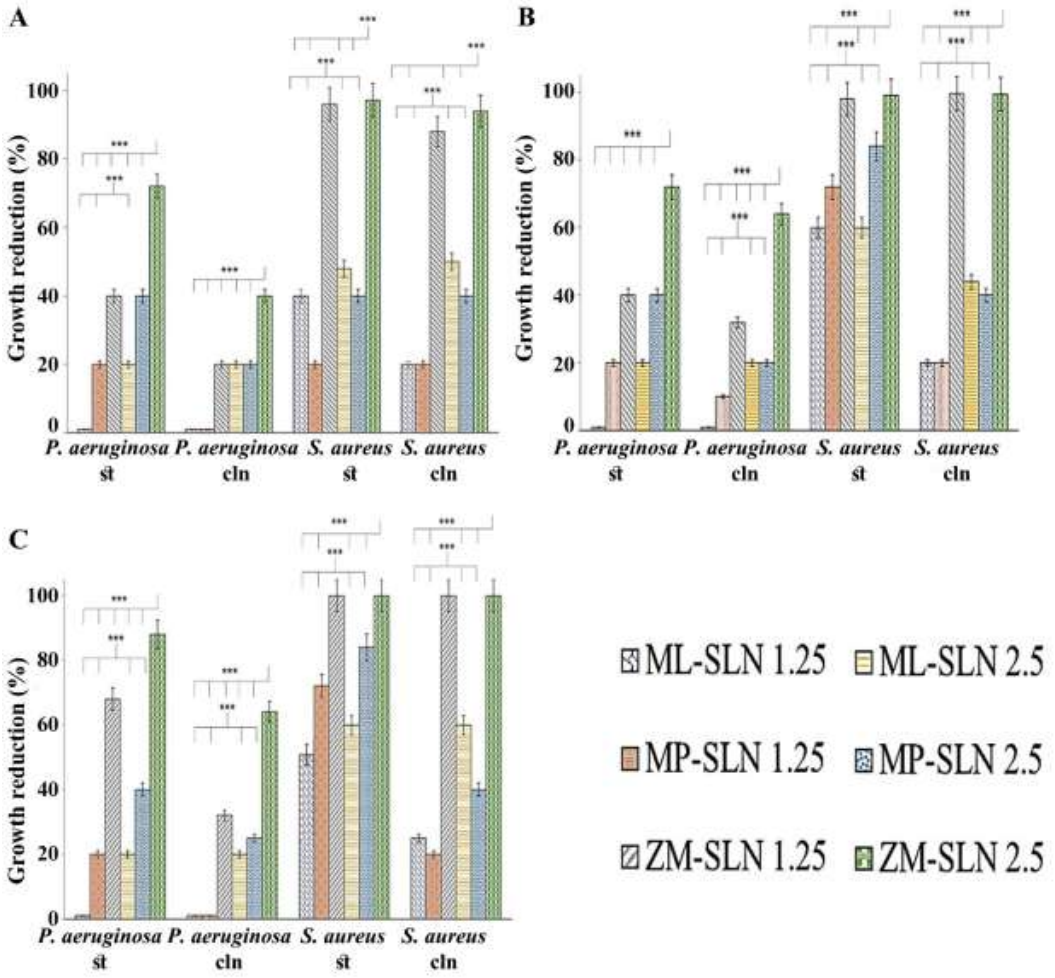

Fig. 5. The antibacterial effect of impregnated scaffolds with solid lipid nanoparticles gels containing EOs of *M. longifolia* (ML-SLN 1.25 and 2.5 mg/mL), *M. pulegium* (MP-SLN 1.25 and 2.5 mg/mL), and *Z. multiflora* (ZM-SLN 1.25 and 2.5 mg/mL) against standard (st) and clinical (cln) strains of *P. aeruginosa* and *S. aureus*. Exposure times were A: 2 h, B: 6 h, and C: 24 h.

At 24 h incubation time

Results of antibacterial effects of samples with 24 h incubation are shown in Fig. 5C. Around 88% and 64% growth reduction in standard and clinical strains of *P. aeruginosa* were observed after treatment with ZM-SLN 2.5 mg/mL; its potency was significantly more than other samples (P<0.001). Growth of both standard and clinical strains of *S. aureus* was fully inhibited after treatment with ZM-SLN 1.25 and 2.5 mg/mL; their potency was significantly more than other samples (P<0.001).

DISCUSSIONS

The chemical composition of the used *M. longifolia*, *M. pulegium,* and *Z. multiflora* EOs in the current study was identified by GC-MS analysis and reported in our previous studies [15, 37]. The five major identified ingredients of *M. longifolia* consist of pulegone (47.7%), 1,8-Cineole (12.6%), piperitenone (5.7%), menthofuran (4.6%), and carvone (4.1%). More, the five major identified ingredients of *M. pulegium* consist of pulegone (69.1%), *trans*-cyclohexanone, 5-methyl-2-(1-methylethyl) (9.4%), limonene (4.1%), (1RS,4SR)-8-hydroxy-p-menthan-3-one (3.7%), and iso-pulegone (3.4%). Also, the five major identified ingredients of *Z. multiflora* EO consist of carvacrol (30.2%), thymol (25.2%), o-cymene (10.7%), ɣ-terpinene (6.1%), and α-pinene (3.6%) [15, 37].

Obtained size of the prepared SLNs was comparable with the literature. For example, Yuxingcao essential oil was loaded in SLNs with

a particle size of 171 nm [43]. Frankincense and Myrrh EOs in another work were loaded in SLNs with particles sizes of 113 and 150 nm, respectively [44]. SLNs containing linalool and citral with 203 and 97.7 nm particle sizes, respectively, were also reported [45, 46].

For the determination of the hydrophilicity of the scaffold, the contact angle method was performed. The hydrophilicity of the scaffold surface is dependent on surface chemistry and roughness. The hydrophilic surfaces have a water contact angle (θ) of less than 90°, while the hydrophobic surface vice versa [47]. Previous studies showed that polycaprolactone scaffolds have an inherently hydrophobic surface [48]. Dodero *et al.* showed that the alginate layer of the scaffold had strong hydrophilic behavior with a θ=30°, while the polycaprolactone layer appeared to be highly hydrophobic with θ=160° [49]. The current study results show that contact angle 145° water with the PCL/Alg; scaffold has a hydrophobic surface. These results suggested that maybe there are more constituents of polycaprolactone (with hydrophobic property) at the scaffold structure and surface than alginate constituents (with hydrophilic property).

Substances that could not reduce bacterial growth by more than 50% are considered ineffective from the literature. A 50-90% reduction indicates acceptable efficacy, and more growth reduction than 90% is considered a potent antibacterial efficacy [37, 50]. As results show, at incubation times of 6 and 24 h, the efficacy of only ZM-SLN 2.5 mg/mL against all examined bacteria strains is considered acceptable or potent (growth reduction > 64%). Efficacy of all samples, including ML-SLN 1.25 and 2.5 mg/mL, MP-SLN 1.25 and 2.5 mg/mL, and ZM-SLN 1.25 and 2.5 mg/mL with 6 and 24 h incubation time against the standard strain of *S. aureus* are acceptable. Moreover, the efficacy of ML-SLN 1.25 and 2.5 mg/mL, MP-SLN 1.25 and 2.5 mg/mL at all incubation times (3, 6, and 24 h) against both standard and clinical strains of *P. aeruginosa* are not acceptable.

In the current study, *Z. multiflora* EO nanoformulations' antibacterial effects were obtained more potent than two other EOs (*M. longifolia* and *M. pulegium*); results were consistent with the literature. For instance, *Z. multiflora* EO demonstrated a greater zone of microbial growth inhibition than *M. longifolia* [51]. Also, Saeidi *et al.* showed that a hydroalcoholic extract of *Z. multiflora* had higher antibacterial activity against human pathogenic bacterial species, including *Bacillus cereus*, *Staphylococcus saprophyticus*, *Klebsiella pneumonia*, *Salmonella typhi*, *Shigella dysenteriae*, *Proteus mirabilis,* and *P. aeruginosa,* than the aqueous extract of *M. longifolia* [52]. In addition, *Z. multiflora* compared with *M. longifolia* showed a higher preventive effect on growth *S. aureus* ATCC 29213 [53]. Moreover, *Z. multiflora* had higher antibacterial activity compared with *M. pulegium* [54]. The minimum inhibitory concentrations of *Z. multiflora* and *M. pulegium* were 2 and 42 mg/mL, respectively [54].

CONCLUSION

This study aimed to prepare impregnated scaffolds with solid lipid nanoparticle gels containing essential oils of *M. longifolia*, *M. pulegium*, and *Z. multiflora* as antibacterial agents. The results showed that polycaprolactone-alginate (PCL/Alg) scaffold did not affect the growth of standard and clinical strains of *P. aeruginosa* and *S. aureus* at all incubation times (2, 6, and 24 h). In addition, impregnated scaffold with solid lipid nanoparticle gel (without essential oil) did not affect the growth of all examined bacteria. Moreover, impregnated scaffold with solid lipid nanoparticle gel of *Z. multiflora* EO at both concentrations of 1.25 and 2.5 mg/mL had a significantly higher antibacterial activity against the standard and clinical strains of *P. aeruginosa* and *S. aureus* at all incubation times of 2, 6, and 24 h. Thus, the prepared prototype could be introduced for future preclinical study for determining pharmacokinetics, cytotoxicity, and wound healing properties.

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COMPETING INTERESTS

There was no conflict of interest among the authors.

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