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ORIGINAL ARTICLE

Nanostructured lipid carriers loaded with *Melaleuca alternifolia* Oil preparation, physicochemical assessment, and evaluation of antimicrobial effects against *Staphylococcus epidermidis*

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Abstract

Thanks to their outstanding advantages, nanostructured lipid carriers (NLCs) have recognized in various fields these days. One way to discover extra useful products against typical bacteria (e.g., *Staphylococcus epidermidis*) is NLCs loaded with essential oils. This paper aims to provide NLCs to encapsulate *MA* oil, characterize, and survey the obtained *MA* oil-loaded NLCs on *S. epidermidis*. The combination has provided using the hot melt homogenization technique. Afterward, the particle size distribution (PSD) (particle size analyzer), morphology (SEM), zeta potential (surface charge of NLCs), and the stability (the effect of acidity) of the prepared NLCs were analyzed. This has followed by estimating the MIC of *MA* oil-loaded NLCs and comparing carriers and oil emulsion of *MA* against *S. epidermidis*. *MA* oil-loaded NLCs are spherical NPs with a mean size of 104.5 nm and narrow size distribution (PDI=0.22). The antibacterial evaluation results demonstrated that *MA* oil-loaded NLCs had a higher *in vitro* antimicrobial activity compared to the oil emulsion of *MA*. Consequently, NLCs could be a suitable carrier to enhance new antimicrobial agents.

Keywords: *Antimicrobial Activity; Melaleuca Alternifolia Oil; Nanostructured Lipid Carriers; Scanning Electron Microscope (SEM); Staphylococcus Epidermidis.*

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INTRODUCTION

In pharmaceutical research, nanostructured lipid carriers (NLCs) have been applied in different fields, especially for dermal and transdermal delivery [1-4]. Lipid carriers have several benefits, including active ingredient preservation, chemically-assisted degradation, controlled drug release, and physically-consistent colloidal systems [2, 5]. There are also noticeable advantages to lipid carriers, including biocompatibility and industrial scalability, and distinguishability. Thanks to their unique properties, NLCs are widely used in dermal and cosmetic products. Among the benefits of lipid nanoparticle formulations are hydrated skin, smoothness, and occlusion. Thus, they are preferred in producing cosmetic products containing different fatty acids and polypeptides [6, 7].

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A significant body of research has recently been conducted on the antimicrobial effects of products resulting from NLCs. According to recent reports, the antibacterial and antifungal activities of essential oils can be improved by encapsulating them in NLCs that provides a more active carrier system [8]. Additionally, essential oil-loaded NLCs exhibited high performance against methicillin-resistant bacteria, even though this property was not due to NLCs alone or free drugs [9].

One of the most common reasons for device-related infections in hospitals is *Staphylococcus epidermidis (S. epidermidis*), a well-known opportunistic pathogen [8]. It causes various problems, including biofilm formation around the medical prosthesis, intravenous catheters placed in the body, dialysis patients, and other plastic devices [10]. It has been known as one of the most prevalent bacteria related to dental implant installation [11]. It lives nearby human skin and can be isolated from normal and acne vulgaris-affected skin sites. According to other studies, *S. epidermidis* is the most usual (60.7 %) type of *staphylococcal* species isolated from dental plaques [12]. It is also the most significant factor that endangers alveolar epithelial cells by an infection that induces pro-inflammatory responses, necessitating further research [13]. Some efforts have been made recently to enhance more effective agents from certain natural products [14, 15].

Melaleuca alternifolia, commonly known as tea tree (TT), is a tree or tall shrub species in the myrtle family, *Myrtaceae*., local to Australia. It is originated from South East Queensland, North Coast, and adjacent ranges of New South Wales [16]. For example, TT can be used as a medical treatment for approximately a century in Australia. Indigenous Australians of eastern inland areas treat coughs and colds using the extracted oils from crushed leaves of TTs as medicine [17]. They also use these leaves to treat wounds, followed by a poultice. TT leaves can also treat sore throats or skin conditions. Thanks to its unique features, the myrtle family (*Myrtaceae*) has used to distill essential oils. Tea tree oil (TTO), a topical antibacterial, is a commercial product of this dominant species. Due to its antimicrobial properties, this oil is used as a topical antiseptic agent, especially in acne treatment [18]. Other medical benefits include its ability to reduce inflammation and treat fungal infections like athlete's foot (tinea pedis) [19].

Notably, nanoformulations from various materials have extensively used in nutrition and pharmaceutical fields. This study aims to provide and characterize *MA oil*-loaded NLCs and determine the antimicrobial effects of these nanoparticles (NPs) on *S. epidermidis* according to their benefits in encapsulating natural components.

MATERIALS AND METHODS

M. alternifolia oil was obtained from Royce Company, Tehran, Iran. Citral, Mueller-Hinton agar, Tween® 80, nutrient dextrose agar, and Mueller-Hinton Broth have bought from Merck Co. (Germany). Miglyol® 812 and Poloxamer® 407 have maintained from Sigma-Aldrich (USA) and Sasol Company (Germany). Glyceryl palmitostearate (Precirol® ATO-5) has purchased from Gattefossé Company (France).

Preparation of MA oil-loaded NLCs

MA oil-loaded *NLCs* were prepared using hot homogenization, Precirol (as triglyceride), and poloxamer. The mixture has provided using the following procedure: First, *M. alternifolia* oil (100 mg) has dissolved in melted Precirol (400 mg) at about 80 °C (oil phase). Second, mixed poloxamers (188mg poloxamer 407 and 188mg poloxamer 188) have dissolved in water (12 ml). The resulting mixture has then stirred in the oil phase at 20000 rpm (Heidolph Homogenizer DIAX 900, Germany) at 80 °C. Finally, the formulation was kept to cool down at room temperature after 15 min. Notably; Miglyol was used in blank NLC samples instead of *M. alternifolia* oil.

Characterization of MA oil-loaded NLCs

Size and surface charge distribution

A laser diffraction particle size analyzer (SALD-2101, Shimadzu, Japan) was used to analyze the size of the prepared NLCs. The formulation has diluted by double-distilled water. Each sample has evaluated three times. Zetasizer (Malvern, United Kingdom) has utilized to determine zeta potential (surface charge) of *MA* oil-loaded NLCs. The particle size distribution and the zeta potentials (ZP) of the prepared NLCs were performed in several times (1, 6, 12 and 24 days after production) in order to evaluate the stability of the emulsion.

Scanning Electron Microscopy (SEM)

Images of *MA* oil-loaded NLCs were obtained

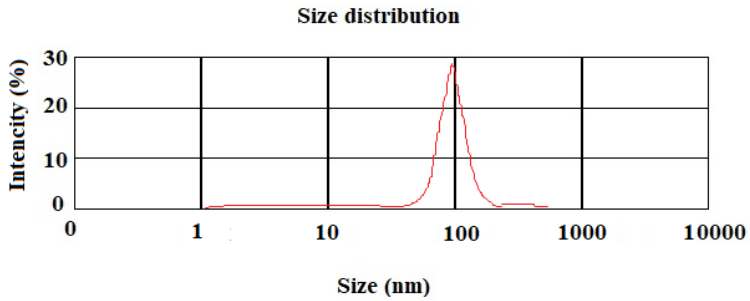

Fig.1. The PSD of optimum formulation of NLCs.

from a scanning electron microscope (MIRA3, TESCAN, Czech Republic). Metal stud and a double-sided adhesive tape have employed. They were then covered with gold in an argon atmosphere (DST1, Nanostructured Coatings Co. (NSC), Tehran, Iran) in a vacuum.

Culture conditions and inoculum preparation

First, the supplier protocol has applied to activate standard *S. epidermidis*. A suitable agar medium has then selected to keep the bacterial cultures at 4 °C. A single colony has transferred into Mueller-Hinton broth (MHB, Merck, Germany) and incubated at 37 °C overnight. An optical density (OD) of a 0.5 McFarland standard (equivalent to 1.5×10^8 CFU/mL of microorganisms) has achieved as follows: First, the cells have collected by centrifugation at 3000 rpm for 15 min. They have incubated and at last, were washed and suspended once more in saline to form an OD equivalent to a 0.5 McFarland turbidity standard (equivalent to 10^7 CFU/mL of bacteria, 10^8 CFU/mL of fungi).

Calculation of MICs

MICs have estimated to analyze the antimicrobial activity of *MA* oil-loaded NLCs against *S. epidermidis*. MIC values have obtained using sterile 96-well microtiter plates (Greiner, Germany) and the broth microdilution method. The bacterial strain has cultured in the MHB medium at 37 °C for 24 h. To estimate MIC, two-fold serial dilutions from *MA* oil-loaded NLCs were prepared in 0.1-10 µg/mL concentrations using the medium. The standardized microorganism suspension (20 µl) was added to the diluted solutions (100 µL) once transmitted into the 96-well microtiter plates. The resulting mixture has then incubated overnight at 37°C. Afterward; the turbidity of the tubes was determined to analyze bacterial growth. However, MIC has known to be the last dilution without turbidity (without growth). Various groups have provided containing media, media + blank NLCs, media + *MA* oil-loaded NLCs, to which the bacteria have then added. The turbidity of wells has defined via spectrophotometry (Ultrospec-2000, Pharmacia Biotech, UK) at a wavelength of 620 nm. All tests have conducted three times, and the analyses have carried out under sterile conditions.

Stability test

In order to study the stability of the prepared NLCs, the effect of acidity investigated. To investigate the influence of the acidity on the impairment of NLCs, MA oil-loaded NLCs have divided into two parts. One part has kept at the pH of 7.4, and the other part has adjusted to the pH of 5.5 using citric acid (10% w/w). Then, the samples have inspected macroscopically and compared.

Statistical analysis

Statistical analysis was performed using GraphPad software version 8 (using one-way ANOVA test).

RESULTS AND DISCUSSION

The obtained values for the polydispersity index (PDI) and the product's particle size were 0.22 and 104.5 nm, respectively. Fig. 1 shows the results of the analysis of the PSD of the provided NLCs. The obtained NLCs with narrow size distribution exhibit a low PDI value. According to Fig. 2, the zeta potential value of the provided NLCs was -16.12 mv. Also, Table 1 shows the amounts for PSD and ZP for the prepared NLCs in several times (1, 6, 12 and 24 days) after production.

According to the SEM images, NPs had a spherical shape, indicating the narrow size

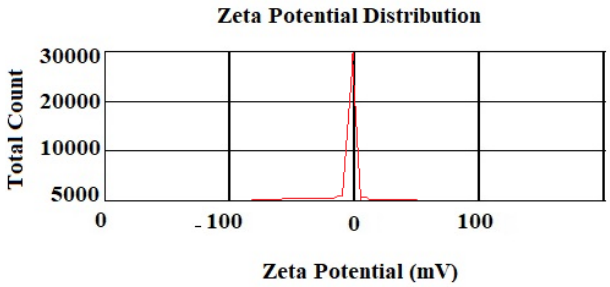

Fig.2. Zeta potential values of the prepared nanomaterial.

Table 1. The amounts for PSD and ZP for the prepared NLCs in several times (1, 6, 12 and 24 days) after production.

Times (days)	PSD (nm)	ZP (mV)
1	104.50	-16.12
6	110.23	-15.13
12	109.00	-17.02
24	125.69	-15.89
Statistical analysis (one-way ANOVA)	P=0.82	P=0.23

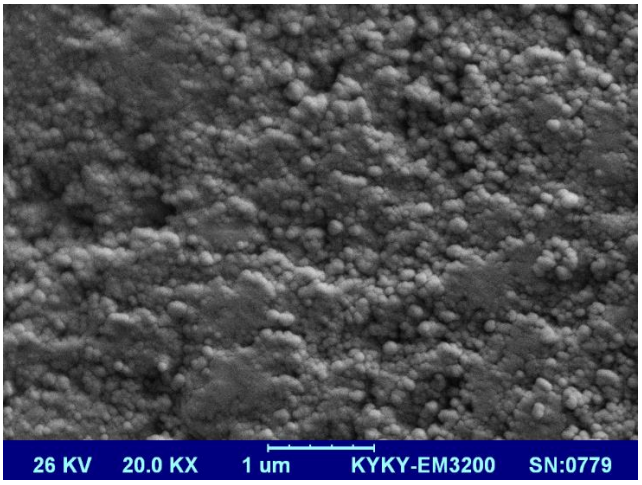

Fig.3. SEM image of optimum formulation of NLCs.

distribution and NLC size determination data (Fig. 3).

This paper has utilized spectrophotometry to estimate the absorbance of microtiter plates before and after bacteria incubation. For this purpose, different concentrations of emulsion and *MA* oil-loaded NLCs have employed. According to NLC formulation, a concentration of 0.63 µg/mL of *MA* oil prevents *S. epidermidis* growth. The MIC value of *MA* oil was 1.5 µg/mL,

In order to study the stability of the prepared NLCs, the effect of acidity investigated. The results showed that no aggregation are visible for NLCS with a pH of 7.4 (Fig. 5a). However, high aggregation was detected for the system with a pH of 5.5 (Fig. 5b).

Different important parameters, such as preparation, characterization, stability, and PSD, are key to determining the final behavior of NPs, including bioavailability, dissolution, stability, and content uniformity in colloidal systems. The particle size analyzer is an effective and non-destructive method used to characterize particles, such as assessing the size distribution of suspended small nanometer-sized particles in a liquid [20-22]. PDI, a PSD width index, has used to demonstrate the size distribution. The values for narrow and broad PSD were 0.1- 0.25 and above

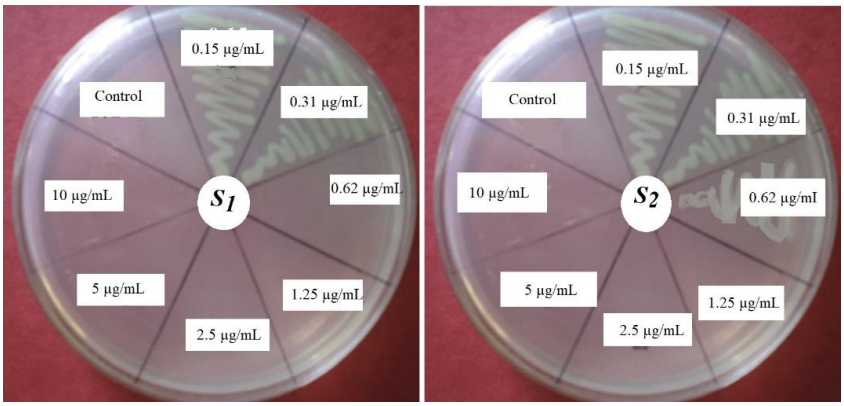

Fig.4. Image of microbial plates (S1; MA oil-loaded NLCs, S2; MA oil).

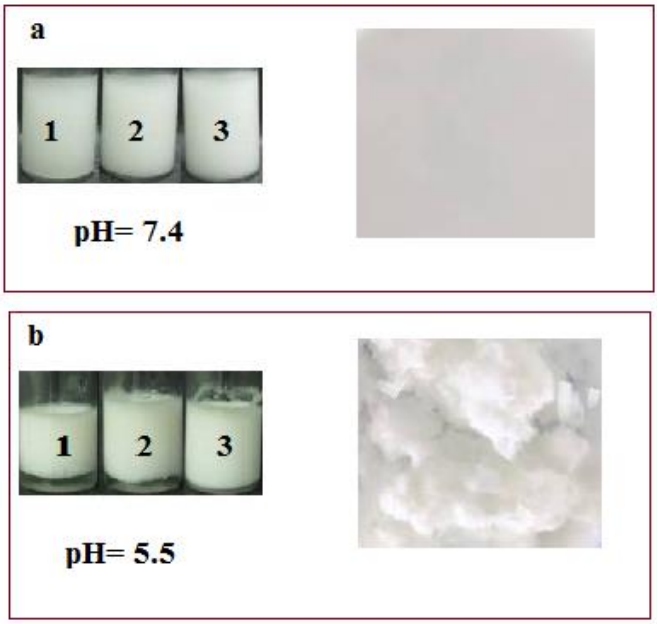

Fig.5. The effect of acidity for MA oil-loaded NLCs; with a pH of 7.4 (Fig.5a), with a pH of 5.5 (Fig.5b).

0.5, respectively [23].

Table 1 shows the amounts for PSD and ZP for the prepared NLCs in several times (1, 6, 12 and 24 days) after production. There are no statistical differences between the groups (P≥0.05), that means the stability of the prepared NLCs during 24 days. The smaller size of NPs is the result of the provided nanoscale suspension with lower turbidity. This may be beneficial for the formulation of beverages. Particle size is an alternative strategy to improve stability. Small particles make lower sedimentation and, thus, can stay more in the suspended form of nanoformulations. The surface-area-to-volume ratio could be measured based on the diameter of NPs. Accordingly, smaller-size NPs have larger surface ranges and, therefore, more loading positions. Additionally, the saturation solubility difference has reduced by narrow PSD, such as the decrease in the drug-concentration gradients in the medium. Hence, the particle size aggregation would be prevented according to Ostwald ripening phenomenon [24].

To reach a polar medium containing an aqueous medium, all materials may suddenly require a surface electric charge. Some mechanisms in which the components may require a surface charge include differential ion dissolution from a crystal lattice, electron affinity difference

between two phases, ionization of surface groups, surface anisotropy, differential ion adsorption of the electrolyte solution, and isomorphic substitution. Ionization of surface groups is crucial for metal oxide surfaces and materials with amino or carboxyl groups, such as ionic polymers, proteins, and polyelectrolytes used extensively in pharmaceutical preparations. In general, acceptable stability would be obtained when the zeta potential value has reached ±60 mV.

On the other hand, particle aggregation would result when zeta potentials have reached lower than ±5 mV. The values between these ranges demonstrate considerable stability or beneficial short-term stability [25, 26]. The particle size of NLCs has assumed to be affected by formulation parameters, such as homogenizer speed, temperature, type of surfactant, liquid-to-solid lipid ratio, and lipid to surfactant ratio.

The MIC values of emulsions of *MA* oil-loaded NLCs could be determined upon increasing quantities of bacteria by incubation. In the event of the production of an NLC, a concentration of 0.63 µg/mL of *MA* oil inhibited the growth of *S. epidermidis*. However, the MIC value for emulsion was 1.5 µg/mL, while the blank NLC had no antimicrobial activity. In other words, when *MA* oil has used against *S. epidermidis*, the mean MIC value was significantly higher than when *MA* oil-loaded NLCs have used. That is, encapsulating *MA* oil by NLCs reduces the required levels of *MA* oil to prevent microorganism growth, while using *MA* oil in the form of emulsion does not display such a beneficial ability. To advance antimicrobial properties, cell membrane transport mechanisms in microorganisms could be influenced by loading *MA oil* in NLC formulation [27]. *MA* oil owes such properties to altered permeability in the plasma membrane and interaction with various agents inside the microorganism. Despite various studies on the antibacterial activity of *MA oil*, the detailed mechanism of its performance has not been elucidated. Enzymes or substrates, toxic action on membranes, and complexation of metal ions that may account for *MA oil* materials may be the parameters that interfere with the possible mechanisms [27-29].

The acidity is so vital for the physical stability of a colloidal system, as it powerfully affects the zeta potential. Therefore, alterations in the pH can be a reason for instability (agglomeration) of a colloidal system owing to a reduction in the zeta potential [30].

CONCLUSION

Nanostructured lipid carriers loaded with *MA* oil (*MA* oil-loaded NLCs*)* were about 104.5 nm in diameter with a narrow size distribution (PDI = 0.22). According to the antimicrobial properties of *MA* oil-loaded NLCs, the MIC value in the emulsion form of *MA* oil was higher than NLCs. *MA* oil-loaded NLCs have recommended as an alternative for antimicrobial agents. However, further evaluations are required to define the antibacterial activity of such nanostructures against the drug-resistant microorganisms.

AUTHORS' CONTRIBUTIONS

FL, HH, MM, and SSh contributed to the research procedure, drafting, and scientific revision of the manuscript. SS and AE contributed to the scientific revision of the revised manuscript. SM is the corresponding author of the manuscript and contributed to the research procedure, drafting, and scientific revision of the manuscript. All authors read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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