

## Improvement of citral antimicrobial activity by incorporation into nanostructured lipid carriers: a potential application in food stuffs as a natural preservative

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### Abstract

At the present time, utilization of essential oils in food preservation to prevent bacterial and fungal growth and improve shelf life and safety of the food products has notably gained increased interest. The aim of the present study was to improve the antimicrobial efficacy of citral as a natural preservative using nanostructured lipid carriers (NLCs). Formulations of NLCs were characterized using particle size analysis and scanning electron microscopy methods. Possible citral-carrier interaction and citral encapsulation efficiency percent (EE%) were assessed by Fourier transform infrared (FTIR) spectroscopy and gas chromatography techniques, respectively. Antimicrobial activity, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) of citral-loaded NLCs were evaluated and compared with the conventional citral emulsion against various gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*), gram-negative bacteria (*Escherichia coli*), and fungi (*Candida albicans*). Citral-loaded NLCs were spherically shaped nanosized (74.8 nm) particles with narrow size distribution, high EE% (99.84%), and appropriate physical stability during 90 days of storage period. FTIR spectra indicated the interaction between citral and formulation ingredients, which justified the obtained high EE% value. The MIC and MBC values of citral-loaded NLCs were lower than those of citral emulsion for all microorganisms. NLCs formulation showed remarkable capability of encapsulating essential oil and increasing antimicrobial properties to offer effective preservation in food industry.

**Keywords:** Citral; Nanostructure lipid carriers (NLCs); Antimicrobial activity; Food preservative

### INTRODUCTION

Besides many improvements taking place in food production methods, food safety is still an important issue. It has been estimated that each year about 30% of people in developed countries are affected by foodborne diseases such as diarrheal diseases (1). Many methods have been presented for decreasing or preventing foodborne pathogens produced by modified atmosphere packaging and food preservation systems such as refrigeration, heating, and addition of antimicrobial compounds (2). Synthetic chemicals have been widely used to control the microbial growth

and reduce the incidence of food poisoning and spoilage. However, synthetic antimicrobial chemicals are sometimes associated with adverse effects including hypersensitivity, allergic reactions, and immune suppression. Hence, there is a growing interest to offer alternative natural antimicrobial agents such as extracts and essential oils of plants, which relatively indicate less hazardous effect on human health (3). Essential oils are volatile compounds formed by aromatic plants as secondary metabolites and have been widely used in agricultural, sanitary, cosmetic, pharmaceutical, and food industries.

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Essential oils reveal significant potential to be used in foodstuff preservation process due to their antimicrobial property. Nevertheless, they can easily undergo oxidation reactions leading to reduced biological activity (4). Citral (3,7-dimethyl-2,6-octadienal) is the mixture of two geometric isomers, i.e. geranial and neral), and exhibits inhibitory effect on oil and vapor form against both gram-positive and gram-negative bacteria (5). However, similar to other essential oils, citral is highly vulnerable to oxidation, and its application as a preservative is restricted to fatty foodstuffs due to its hydrophobic property.

Lipid-based nanocarriers can protect essential oils against thermal- or photo-degradation, increase the stability of the product and consequently extend the final product shelf life, and do not affect product appearance, texture, and taste (6). Furthermore, utilization of such delivery systems can increase the concentration of antimicrobials in food areas in which microorganisms are preferably located. Furthermore, they potentially increase the passive cellular absorption mechanisms which affect stability of lipid membrane and as a result increase the antibacterial activity. Incorporation of active ingredients as the oil part of nanostructured lipid carriers (NLCs) would be an interesting and novel idea in the field of nanoparticle preparation (7). The present study aimed at loading citral, as a whole structural component substituted for oil phase, into NLCs with the maximum encapsulation efficiency for improvement of its antibacterial efficacy.

## MATERIALS AND METHODS

### Materials

Citral, Tween<sup>®</sup> 80, Mueller Hinton agar, Mueller Hinton Broth, and nutrient dextrose agar were supplied from Merck Chemicals Co. (Germany). Poloxamer<sup>®</sup> 407 and Miglyol<sup>®</sup> 812 were obtained from Sigma-Aldrich Company (USA) and Sasol Company (Germany), respectively. Glyceryl palmitostearate (Precirol<sup>®</sup> ATO-5) was kindly donated by Gattefossé Company (France).

### Preparation of NLCs

Blank NLCs were prepared by hot melt homogenization method as described previously (8). Briefly, 200 mg Miglyol<sup>®</sup> was dissolved in 800 mg melted Precirol<sup>®</sup> at about 70 °C.

Afterward, Poloxamer<sup>®</sup> was dissolved in water and added dropwise into the oil phase under stirring at 20000 rpm (DIAX 900 homogenizer, Heidolph, Germany) and 70 °C. Finally, after 15 min, formulation was allowed to cool down at room temperature. Citral-loaded NLCs were prepared by using 200 mg citral instead of Miglyol which resulted in higher loading capacity of citral in solid lipid core matrix that can solubilize lipophilic molecules. Citral emulsion was also prepared by gradually addition of water into citral, Miglyol<sup>®</sup>, and Tween<sup>®</sup> 80 mixture under stirring. Each formulation was prepared and characterized in triplicate.

### Characterization of citral-loaded NLCs

The mean particle size, size distribution, and zeta potential were analyzed using photon correlation spectroscopy (Malvern Zetasizer Nano, UK) and reported as intensity-weighted average (z average) and polydispersity index (PDI). The morphology of prepared nanoparticles was analyzed using scanning electron microscope (SEM) (MIRA3 TESCAN, UK) after gold coating (DST1, Nanostructured Coating Co., Tehran, Iran). Fourier transform infrared spectroscopy (FTIR, 8400S, Shimadzu, Japan) spectra of citral, Precirol<sup>®</sup>, Poloxamer<sup>®</sup> 407, and lyophilized optimized formulation were obtained from 400 to 4000 cm<sup>-1</sup> (9). The EE% and LC% values were determined by centrifugation method using Amicon<sup>®</sup> Ultra-15 tube (Millipore, Germany). The EE% and LE% values of citral-loaded NLC formulations were mathematically calculated according to the following equations:

$$EE (\%) = \frac{W_{(Initial\ citral)} - W_{(Free\ citral)}}{W_{(Initial\ citral)}} \times 100 \quad (1)$$

$$LE (\%) = \frac{W_{(Entrapped\ citral)}}{W_{(Total\ lipid)}} \times 100 \quad (2)$$

where,  $W_{(\text{Initial citral})}$  is the amount of citral initially used and  $W_{(\text{Free citral})}$  is the amount of free citral detected in the lower chamber of Amicon<sup>®</sup> tube after centrifugation of the NLCs formulation. Accordingly,  $W_{(\text{Entrapped citral})}$  is the amount of loaded citral and  $W_{(\text{Total lipid})}$  is the amount of used lipid in the preparation process (10). The rinsed formulations were used for further experiments.

The clear solution in the bottom chamber of Amicon<sup>®</sup> tube was used for the determination of unloaded citral by gas chromatography (Fisons 8160, Milan, Italy, equipped with a flame ionization detector). Samples of each formulation were stored at room temperature and the physical stability was evaluated in term of the mean particle size after 30, 60, and 90 days.

### **Microbial strains**

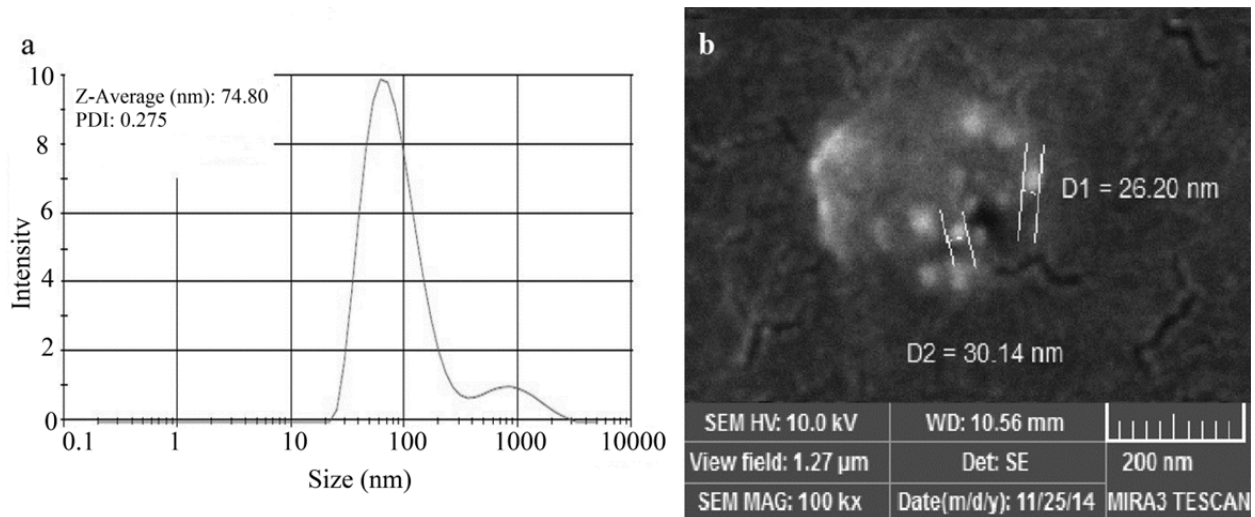
The antimicrobial activity of the citral essential oil formulations was evaluated against four food-related microorganisms including a gram negative bacteria (*Escherichia coli* ATCC 25922), two gram positive (*Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 11778) as well as a fungi (*Candida albicans* ATCC 10231). A single colony from the stock was transferred into Mueller Hinton Broth (MHB, Merck, Germany) and incubated over night at 37 °C. After incubation time, the cells were harvested by centrifugation at 3000 rpm for 15 min, washed twice, and re-suspended in saline solution to provide an optical density equal to 0.5 McFarland standard turbidity (equivalent to  $1.5 \times 10^8$  colony forming units (CFU)/mL of microorganisms). The MIC values were assessed using the broth microdilution method in sterile 96-well microtiter plates (Greiner, Germany) (11). Bacterial strains were cultured overnight at 37 °C in MHB medium. Two-fold serial dilutions of the citral-loaded NLCs were prepared in concentration of 3.81 to 2000 µg/mL and 1× MHB medium for MIC assessment of *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*. Prepared diluted solutions (180 µL) were transferred in to 96-well microtitre plates and then 20 µL of standardized microorganism suspensions (0.5 McFarland) was added and

incubated at 37 °C for 24 h. After incubation time, turbidity of tubes was evaluated to determine bacterial growth and last dilution with no turbidity at wavelength of 620 nm (lack of growth) was considered as MIC. Subsequently, to determine the MBC, samples (5 µL) from tubes with no growth was cultured in Mueller Hinton agar medium plate and incubated for 24 h at 37 °C. To determine the MIC value for *Candida albicans*, sixteen dilution series from 1 to 2048 µg/mL of citral were prepared and 50 µL of fungi suspension was added and incubated at 37 °C for 24 h. The MIC value was determined as the lowest concentration of essential oil inhibiting visible growth of fungi on the agar plate when there was visible growth on the control plates. All procedures were performed under sterile conditions and reproducibility was examined by triplicate examination. In each test, microorganism strain in MHB (with blank NLCs or emulsion formulation) and MHB alone were used as a positive and negative growth controls, respectively.

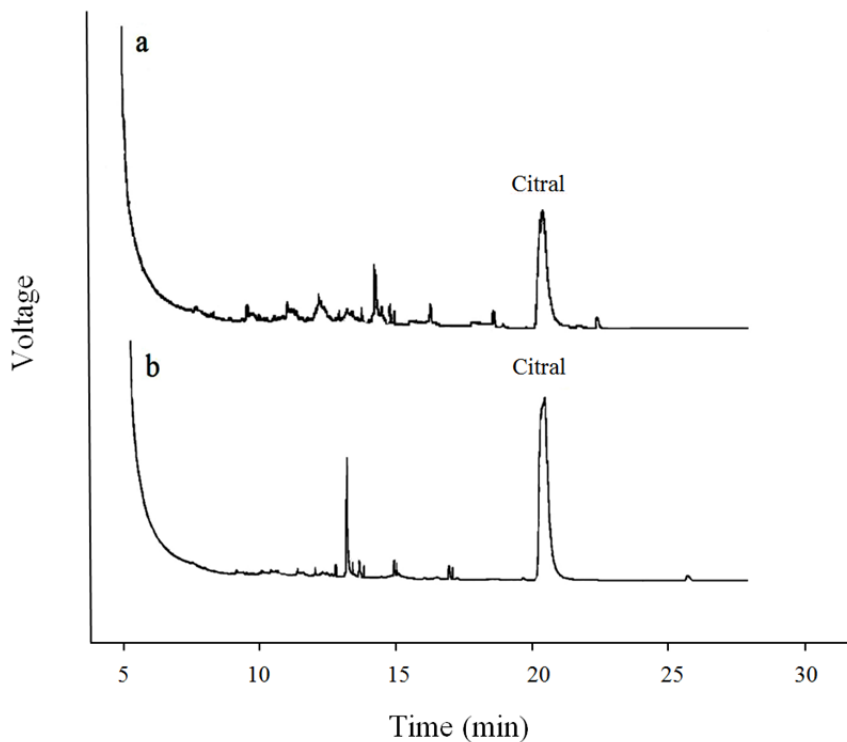
## **RESULTS**

### **Preparation, characterization, and stability of citral formulations**

Particle size and size distribution are the key parameters that may have significant effects on the ultimate performance of nanoparticles such as bioavailability, stability, and microbial activity (12). Particle size and PDI values of the prepared emulsions were 1.02 and 0.726 µm, respectively. The particle size and size distribution profile of the optimized formulation were  $78.8 \pm 5.3$  nm (z average intensity). Furthermore, the PDI value of the optimized formulation was  $0.266 \pm 0.08$  (Fig. 1a). SEM images revealed that nanoparticles were spherical in shape matching with size analysis data and confirming the narrow size distribution of NLCs (Fig. 1b). SEM image showed particle size smaller than the one exhibited by PCS. This is due to the lipophilic characteristics of the nanoparticles which tend to associate in close proximity. EE% of the optimized NLCs formulation was found to be  $99.84\% \pm 0.05$  with LC% of  $12.5\% \pm 0.5$ .



**Fig. 1.** (a) Particle size distribution profile and (b) scanning electron microscopy (SEM) image of optimum nanostructured lipid carrier (NLC) formulation.



**Fig. 2.** Representative gas chromatography (GC) chromatograms of (a) citral standard solution and (b) free citral after separation from citral-loaded nanostructured lipid carrier (NLCs) formulation.

The physical stability experiment indicated lack of citral leakage and particle size growth during 90 days of storage period (Table 1). Representative GC chromatograms of citral standard solution and unloaded citral after separation from citral-loaded NLCs are presented in Fig. 2. The peak value of citral

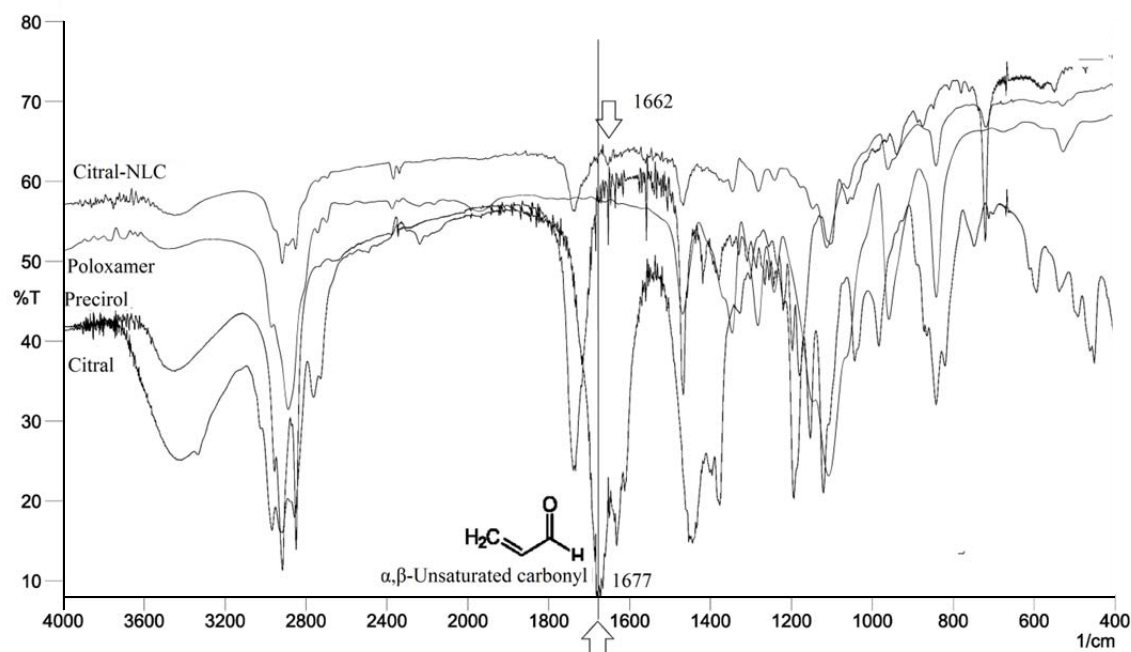
appeared at 21.9 min. FTIR spectra exhibited movement of  $\alpha,\beta$ -unsaturated carbonyl group peak of citral from  $1677\text{ cm}^{-1}$  to  $1652\text{ cm}^{-1}$  (Fig. 3).

This shift indicates interaction of citral with lipids via hydrogen bond in the process of NLCs preparation.

### Determination of the MIC and MBC values of citral formulations

MIC and MBC values of citral-loaded NLCs and citral emulsion formulations against different microorganisms are shown in Table 2. Mean MIC values of citral-loaded NLCs against all microorganisms were significantly lower than those of citral emulsion. On the other hand, citral-loaded NLCs and emulsion

formulations showed lower MIC values, which resulted in higher antibacterial effect on *Bacillus cereus* in comparison with other microorganisms. Correspondingly, both of the NLCs and emulsion formulations exhibited the highest MIC values in the case of *Escherichia coli*, suggesting that citral presented higher antibacterial efficiency against gram-positive bacteria compared with gram-positive ones.



**Fig. 3.** Fourier transform infrared (FTIR) spectra of (a) Poloxamer<sup>®</sup>, (b) Precirol<sup>®</sup>, (c) citral, and (d) citral-loaded nanostructured lipid carriers (NLC).

**Table 1.** Physical stability of optimized nanostructured lipid carriers (NLCs) just after preparation and during storage period (data are presented as mean  $\pm$  standard deviation, n = 3).

Time	Initial	Days of storage		
		30	60	90
Size (nm) <sup>a</sup>	78.80 $\pm$ 5.10	80.31 $\pm$ 4.70	89.15 $\pm$ 4.10	95.30 $\pm$ 6.10
PDI <sup>b</sup>	0.27 $\pm$ 0.09	0.27 $\pm$ 0.05	0.29 $\pm$ 0.07	0.24 $\pm$ 0.05
EE (%) <sup>c</sup>	99.80 $\pm$ 1.00	99.50 $\pm$ 2.00	97.60 $\pm$ 2.00	97.10 $\pm$ 1.00

(a) Z-average diameter

(b) Poly dispersity index

(c) Encapsulation efficiency

**Table 2.** Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of citral-loaded nanostructured lipid carriers (NLCs) and emulsion formulation.

Microorganisms	MIC ( $\mu\text{g/mL}$ )		MBC ( $\mu\text{g/mL}$ )	
	Emulsion	NLCs	Emulsion	NLCs
<i>Staphylococcus aureus</i>	500	125	2000	500
<i>Bacillus cereus</i>	500	31.25	1000	125
<i>Escherichia coli</i>	1000	250	> 2000	> 2000
<i>Candida albicans</i>	125	-	250	-

## DISCUSSION

A good number of studies such as one conducted by Cristani, *et al.* evaluated several parameters to explain the antibacterial mechanism of essential oils. In this regard, it was proposed that terpenes presented antimicrobial effect due to their capability of interrupting lipid section of bilayer membrane of the bacteria. Furthermore, after crossing the membrane, terpenes disturbed some main regions inside the cells, which led to the antibacterial effect (13). Nanocarriers could also protect essential oils against possible thermal- or photo-degradation, oxidation or evaporation, which assures increased stability, flavor and function, and consequently extend the final product shelf life (14). The proposed underlying mechanisms of antimicrobial effect of terpenes such as citral are the interaction with cytoplasmic membrane resulting in loss of membrane integrity, inhibition of respiratory enzymes, and subsequently dissipation of the proton-motive force (15). Lipid-based nanocarriers can also facilitate antimicrobial activity of the essential oils by providing diverse diffusion properties through biological membranes due to nanoscale and lipophilic nature (16). Addition of encapsulated citral in lipid-based nanoparticles with lower amounts of citral rather than using citral emulsion is not only economical, but also could prevent any changes in the taste and color of foodstuffs. One of the possible mechanisms justifying the superiority of nanoparticles presenting elevated microbial activity of active ingredients is the enhancement of NLCs transport through cell membrane of the microorganisms and increment of the antibacterial and antifungal activity (17). One of the strategies to enhance the stability is decreasing the particle size, which results in reduction of the sedimentation rate. Consequently, the particles remain suspended for longer periods in the suspension. Surface-area-to-volume ratio is dependent on diameter of nanoparticles. Smaller nanoparticles result in larger surface area, and therefore more loading sites will be available. The obtained results indicated that particle size and size distribution of the

produced nanoparticles were less than 100 nm and relatively stable during the storage period. This can be considered as a critical advantage in formulation of nanoparticles, warranting their stability in the distribution media such as drinking stuffs. The low PDI values indicated the narrow size distribution of the prepared formulation. Due to Ostwald ripening phenomenon, a narrow size distribution is critical to inhibit the particle growth (18). Monodispersity decreases the saturation solubility difference and gradients of drug concentration within the medium and therefore help to prevent occurrence of Ostwald ripening phenomenon (19). High EE% and LC% were predictable considering high lipophilicity of citral. Furthermore, nonporous structure of solid lipid matrix in NLC formulation can be regarded as another reason for higher encapsulation capacity of NLCs formulations (20). The observed interaction in FTIR experiment also provided the justification for the observed high loading capacity of citral in NLCs formulation.

## CONCLUSIONS

Encapsulation of citral in NLCs achieved in the current study could be a promising strategy to improve the efficiency of these essential oils as an effective antimicrobial. Encapsulation of the oil in NLCs could offer prolonged preservative effect in cosmetic and food industry as they are protected from physicochemical instability. NLCs could be used as a highly efficient carrier system to improve citral antibacterial and antifungal activity. The obtained results showed lower MIC and MBC values of citral-loaded NLCs compared with citral emulsion.

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