



## Research Article

# Chlorogenic acid nanoemulsion for *Staphylococcus aureus* causing skin infection: Synthesis, characterization and evaluation of antibacterial efficacy

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## ABSTRACT

*Staphylococcus aureus* (*S. aureus*) causes many skin infections such as impetigo, infected abrasions, cellulitis, folliculitis, subcutaneous abscesses, infected ulcers and sores. In this study, it was aimed to develop a nanoformulation of chlorogenic acid that was efficient against *S. aureus*. In this context, ultrasonic emulsification method was used for production of the chlorogenic acid (CA) nanoemulsion formulation and was characterized in detail. In addition, the kinetic and thermodynamic stability of the CA nanoemulsion formulation was examined. Finally, the broth microdilution method was used to determine the antibacterial activity of the formulation. It was determined that the average droplet size of the CA nanoemulsion formulation was  $120.4 \pm 6.39$  nm, the polydispersity index (PdI) was  $0.180 \pm 0.018$ , and the zeta potential value was  $-11.5 \pm 1.15$  mV. As a result of the thermodynamic stress tests of the CA nanoemulsion formulation, it was observed that there was no precipitation or phase separation. Moreover, *in vitro* release study showed a CA release of 75.49% after 48 hours. The antibacterial results revealed that the CA nanoemulsion formulation was efficient (95% inhibition) against *S. aureus* (ATCC 25923). As a result, it is thought that CA nanoemulsion is an effective, nano sized and controlled release system based formulation candidate that may be used in the topical treatment of *S. aureus* causing skin infection.

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## INTRODUCTION

Bacterial infections of the skin and soft tissue are among the most common infections worldwide [1]. A well-known bacterium, *S. aureus*, a well-known bacterium, is the leading cause of infections in impetigo and infected abrasions. It is known that *S. aureus* causes superficial skin infections and soft tissue infections such as impetigo and infected abrasions. In addition, it causes more complex skin infections such as cellulitis, folliculitis/furunculosis, subcutaneous abscesses, infected ulcers, and lesions [2,3]. *S. aureus* usually starts with an inflammatory process in the epidermis, dermis, or subcutaneous tissues [4] with no pathogeny or symptoms of infection [5], and can spread to other parts of the body, cause severe infections such as endocarditis, osteomyelitis, necrotizing pneumonia, and sepsis [6,7]. It is known that topical antibiotics do not provide an adequate influence on the treatment of deep tissue infections due to their poor penetration ability [8]. In addition, most antibiotics may lose their effectiveness over time because of antimicrobial resistance [9]. The reason *S. aureus* infection is becoming increasingly difficult to treat is the breakout and rapid spread of methicillin-resistant *S. aureus* (MRSA) [10]. This bacterium is resistant to all known  $\beta$ -lactam antibiotics. Worryingly, Nannini et al. reported that there was a resistance to linezolid and daptomycin and reduction susceptibility to vancomycin in clinical strains of MRSA [11]. This issue is a major challenge in clinical practice globally [12-14]. The emergence of resistance to conventional antibiotic treatments has become a forefront the need for novel and safer antibacterial drugs and alternative controlled release systems [15-17]. The complex action mechanism of the plant polyphenols on bacteria compared to conventional antibiotics make them a strategic antibacterial candidate [18,19]. CA is a polyphenolic natural compound that has antibacterial, antifungal, antiviral, antiphlogistic, and antioxidant features [20,21]. It is commonly found in tea leaves, coffee beans, apples, and grapes [22]. It has been reported that antimicrobial activities on various Gram-positive and Gram-negative bacterial pathogens are through cell membrane permeability [20,23].

Intravenous injection (IV) and oral administration of antimicrobial agents for infections in the deep layers of the skin is more successful than the topical drug delivery system. However, these administration methods may become ineffective because of vascular insufficiency, diabetes and similar reasons [8]. For these reasons, the development of new nanocarriers, topical drug delivery systems that can carry the antimicrobial agent to the deep layers of the skin and exert its antimicrobial activity are of great importance [24-26].

Nanoemulsions (NEs) are nano-sized droplets used as carriers against enveloped viruses, fungi and bacteria [27-30]. Meral et al., (2019) synthesized thyme oil NEs and investigated their antibacterial effects on *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhimurium*.

Zeta potential and PdI of thyme oil NEs (TONa and TONb) with diameters of 219 nm and 163 nm, respectively, were defined as 19.77, 0.24 and -24.80, 0.054, and they reported that these NEs have effective antibacterial effects [31]. Ceylan et al., (2020) synthesized curcumin and rosemary oil NEs (CUR and RON) with average diameters of 184.3 nm and 158.3 nm by sonication technique [32]. They evaluated the effectiveness of NEs against *P. aeruginosa*, *E. coli* and *S. typhimurium*. It has been reported that CUR shows varying levels of antibacterial activity against three bacterial species, while RON shows antibacterial activity only against *S. typhimurium*. In another study, a nanoemulsion was developed with glyceryl monocaprylate (GMCY), which is known to have bactericidal activity for the treatment of infected wounds. Three common wound bacteria (*S. aureus*, *P. aeruginosa* and *E. coli*) were used for this [33]. As a result of the study, it was shown that GMCY nanoemulsion exhibited antibacterial properties against *S. aureus* and *E. coli*. NEs are thermodynamically stable systems with a translucent appearance [34,35]. It can be prepared using high-energy methods such as ultrasonication or homogenization [36,37]. NEs are formulations with small droplet size (<200 nm) that provide a large surface area in contact with the skin [38-40]. These systems are widely used for the controlled release of nutraceuticals and active pharmaceutical ingredients into the skin layers of the skin [41,42].

In our study, CA-NE formulation was produced by using the ultrasonic emulsification method, and then characterized. The parameters such as average droplet size, zeta potential PdI were analyzed by Dynamic laser light scattering (DLS). Drug content assay and *in vitro* release profile were determined by UV-Vis spectrophotometer. The kinetic and thermodynamic stability of the CA-NE formulation was examined by accelerated stability tests (centrifuge and thermal stress tests). Finally, the antibacterial effect of CA-NE formulation against *S. aureus* was determined by Minimum Inhibitory Concentration (MIC) test.

## MATERIALS AND METHODS

### Materials

Pluronic® F-68, ethanol, DL-alpha tocopherol acetate, Muller-Hinton broth and Muller Hinton agar from Sigma-Aldrich; Chlorogenic Acid from Alfa Aesar; Isodecyl neopentanoate (DUB VCI 10) from Stearinerie Dubois; Undecyl alcohol (Sensiva® PA 30) was purchased from Schülke and Caprylic/capric triglyceride (Labrafac Lipophile WL 1349) was purchased from Gattefosse.

### Methods

#### Preparation of CA-NE

CA-NE was synthesized using ultrasonic homogenization method in NE type, in which the aqueous phase is a continuous phase (O/W) [43]. The water phase of NE was

prepared with distilled water to be 15% Pluronic® F-68. In the oil phase, 1% Isodecyl neopentanoate, 2% undecyl alcohol, 11.675% Caprylic capric triglyceride, and 0.2% DL-alpha tocopherol acetate, 2.5% Transcutol, 5% Labrafil and 17.8 mg CA were used. The water phase and oil phase were prepared in separate beakers and then the water phase was mixed into the oil phase. It was then mixed for 5 minutes at 8100 rpm using a homogenizer (Witeg, Germany). After premixing, the emulsion was ultrasonically emulsified using a 20 kHz and 750 W Sonicator (Ultrasonics, USA). Ultrasonication was performed at 50% amplitude for 20 minutes.

#### Dynamic Light Scattering (DLS) Analysis of CA-NE

The critical parameters such as average droplet size, PDI and zeta potential values of the NE were analyzed at 25 °C by using Zeta Sizer Nano ZS (Malvern Instruments, UK). Samples were prepared in distilled water (1:100 v/v), and then analyzed within 24 hours in triplicate [44].

#### Electrical Conductivity and pH Measurements of CA-NE

The probes of a pH meter (Ohaus® STARTER 3100M) were used for the determination of the electrical conductivity and the pH values of formulation. The analyzes were determined at 25 °C in triplicate.

#### Accelerated Stability Studies of CA-NE

The accelerated thermodynamic stability of NE was performed within 24 hours by centrifugation and thermal stress tests. 15 g of CA-NE formulation was centrifuged at 25±1°C at 4,500 rpm for 20 minutes. Macroscopically, any phase separation or turbidity was evaluated [45]. Thermal stability test was accomplished to determine the organoleptic analysis of CA-NE formulations, such as color, odour, consistency, presence of precipitate and phase separation. 10 g of CA-NE formulation was exposed to a temperature increase of 5 °C from 40 °C to 80 °C in a water bath. Data on the appearance (such as no phase separation, agglomeration or creaming) of the formulation were obtained as a result of centrifugal and thermal stress tests.

#### Analysis of Active Substance Content

For the determination of active substance (CA) content, 0.5 mL of CA-NE was taken into a flask, its volume was completed to 10 mL with methanol. After it was extracted in an ultrasonic bath for 30 minutes, it was analyzed by UV-Vis spectrometer at 324 nm. Finally, the concentration of CA was calculated with the calibration curve ( $R^2 = 0.999$ ).

#### In Vitro Release Study of CA-NE

Since the CA-NE formulation was developed to use for topical application, the *in vitro* release study was conducted at skin pH (5.0-6.0) and temperature (32°C) [46]. 1 mL of CA-NE was put in a dialysis capsule, and the samples were taken from 5 mL of release medium at specified time intervals in a shaking water bath at 120 rpm, and replaced with

an equal amount of fresh release medium. The absorbance values of each sample were analyzed by the UV-Vis spectrometer at 324 nm. The release (%) of CA was calculated using Equation (1).

$$\text{Release \%} = \frac{\text{Released CA}}{\text{Total CA}} \times 100 \quad (1)$$

#### Antibacterial Activity

The antibacterial activity of the free CA and CA-NE formulation on *S. aureus* ATCC 25923 was determined by the broth microdilution method. Bacterial culture was activated on Mueller-Hinton Agar (MHA) at 37°C for 24 hours. After incubation, serial dilutions were prepared by adding compounds to 96-well plates with 100 µL of Mueller Hinton Broth (MHB). Serial dilutions were prepared to start from 0.178 mg/mL for the free CA, and 1 mg/mL for the CA-NE formulation. Bacteria were incubated at 35-37 °C for 18-24 hours. The plate was analyzed with an ELISA reader. The percentage (%) of bacterial growth inhibition was determined as Equation (2); where  $A_c$  is the absorption value of the negative control and  $A_t$  is the absorption value of samples.

$$\text{Inhibition (\%)} = \frac{A_c - A_t}{A_c} \times 100 \quad (2)$$

## RESULTS AND DISCUSSION

#### Characterization of CA-NE

It has been stated in the literature that the ultrasonic homogenization method is quick and effective technique in order to prepare stable NE with the desired droplet size and low PDI [47,48]. In our study, NE formulation was synthesized by ultrasonic homogenization method, and then characterization and stability studies were conducted. DLS, which is one of the most preferred method, was used to determine the hydrodynamic size, PDI and zeta potential values of the synthesized NE [49-53]. The results were given in Figure 1 and Figure 2. The blank NE had average droplet size of 110.8 ± 0.14 nm. On the other hand, CA-NE had average droplet size of 120.4 ± 6.39 nm. It is known that the nanoemulsions are nanosystems with smaller droplet size between 20 and 200 nm compared to emulsions [54,55]. NEs with the small droplet sizes provides advantages in order to overcome the challenges in nano-size study such as gravity separation, flocculation, and coalescence [31,54]. In many studies in the literature, it has been stated that the droplet sizes ranging from 100 to 200 nm can provide advantages for NEs in topical applications because of their superior penetration and controlled release properties [37,55,56]. Based on this information and our average droplet size result, CA-NE formulation is considered suitable for topical applications.

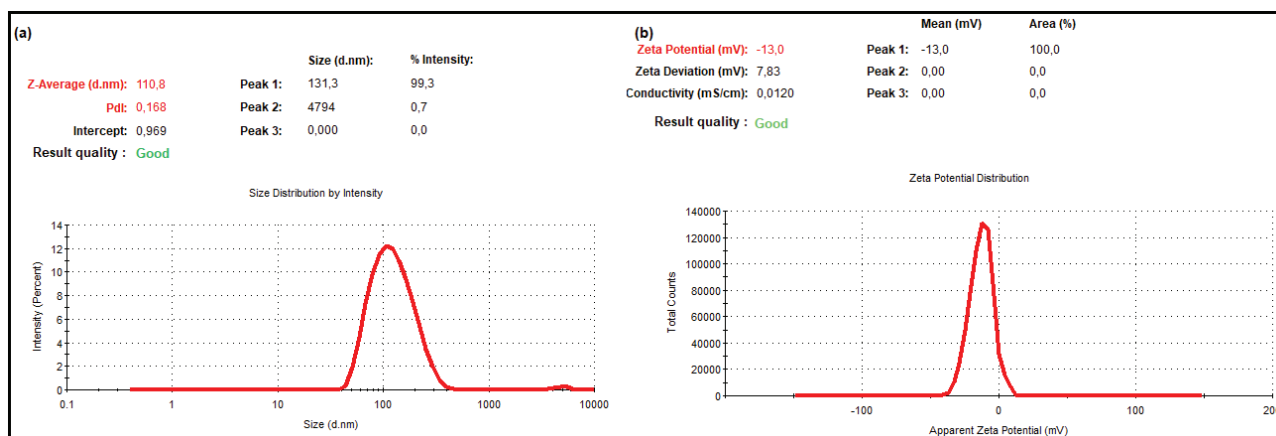


Figure 1. DLS analysis of blank NE; (a) average droplet size, and (b) Zeta potential graphs.

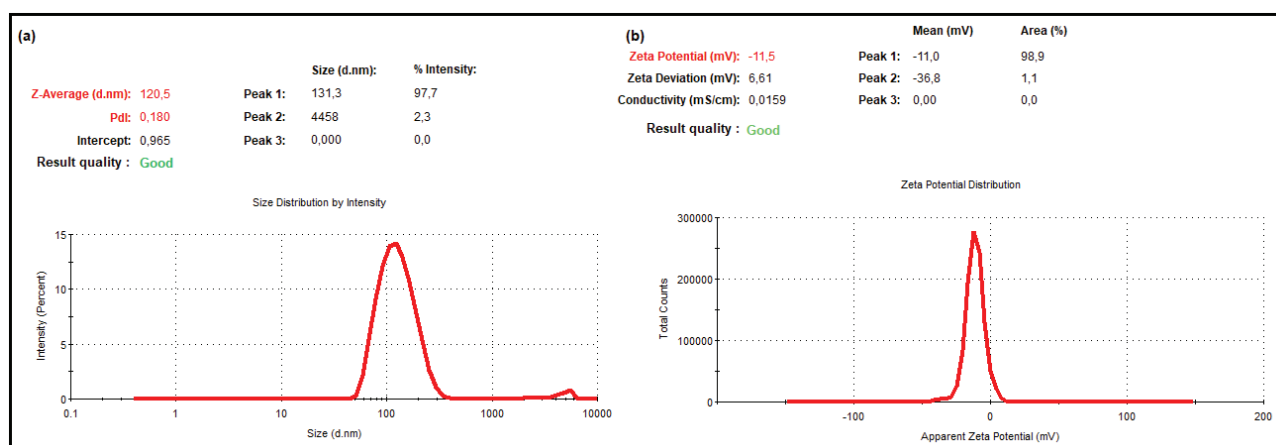


Figure 2. DLS analysis of CA-NE; (a) average droplet size, and (b) Zeta potential graphs.

The Pdl value is a measure of the homogeneity and stability of the droplet size in the NE. A smaller value of less than 0.1 for Pdl means that monodisperse size distribution for droplets, while large value greater than 0.2 for Pdl means heterogeneity within the size distribution of droplets [31]. It was found that the blank NE had Pdl value of  $0.168 \pm 0.006$ , and CA-NE had Pdl value of  $0.180 \pm 0.018$ . From the obtained results, it was determined that the formed droplets had a narrow size distribution.

The zeta potential is an indirect measurement method of the electric charge of particles in the colloidal system, and it is used to determine approximately the surface charge of NE droplets [57]. The oils used in the oil phase of the formulation consist of fatty acids. The negative charge of the droplets' zeta potential values is due to the carboxylic acid groups of fatty acids [58]. It was found that the blank NE had zeta potential of  $-13 \pm 4.03$  mV, and CA-NE had zeta potential of  $-11.5 \pm 1.15$  mV. These results were similar to the reported in previous studies in the literature [56,59-62].

### pH and Conductivity

The pH value is crucial to evaluate the stability of nanoemulsions. pH changes that may occur in the formulation may indicate the possibility of chemical reactions that may cause problems in the quality and stability of the final product [63]. The pH value of human skin is approximately between 4.6 and 5.8 [46]. It was found that the pH values obtained from our study were suitable for topical application (Table 1).

Similar to pH change, electrical conductivity changes can also provide information about the quality and stability of NEs [45,64]. Electrical conductivity measurement for NE

Table 1: pH and conductivity results of NE formulation. (Data are presented as mean  $\pm$  SD)

Formulation	pH	Conductivity ( $\mu$ S/cm)
Blank-NE	$5.70 \pm 0.06$	$87 \pm 0.30$
CA-NE	$5.31 \pm 0.33$	$96.2 \pm 1.58$



is a simple, inexpensive, and essential method. Electrical conductivity measurement means that the aqueous phase is the continuous phase (O/W), and high electrical conductivity values are observed in such nanoemulsions [64]. In the present study, high conductivity values were obtained for the blank NE and CA-NE formulations. It indicated that the external phase of the synthesized NE formulation was water (Table 1).

#### Accelerated Stability Tests

Accelerated stability tests provide useful information in predicting the stability of colloidal systems [65]. Centrifugal and thermal stress test were performed to evaluate the thermodynamic stability of the formulation. Thermodynamic stability problems in colloidal systems are observed as coalescence, creaming, and phase separation. The results showed that none of the stability problems were observed in the developed formulation (Table 2).

**Table 2: Accelerated stability test results of NE formulation**

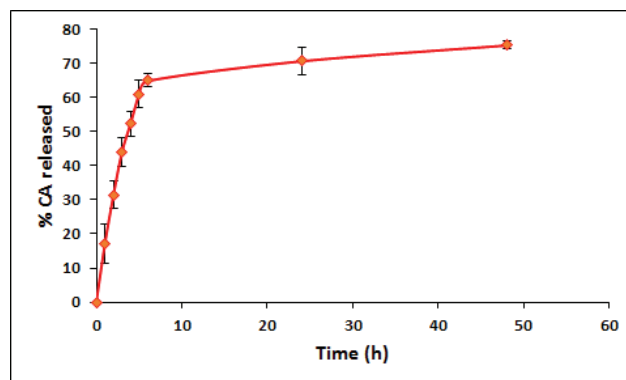
Formulation	Organoleptic characteristics
Blank-NE	milky and bluish aspect
CA-NE	milky and bluish aspect

#### Analysis of Active Substance Content

In this study, CA-NE formulation was synthesized as oil-in-water type nanoemulsion, and its continuous phase was the aqueous phase. Extraction was performed by using methanol to determine the active ingredient content (CA) of the formulation. Then, the sample was analyzed with a UV-Vis spectrometer [66] at 324 nm. The active substance content of CA-NE formulation was calculated via the calibration curve of CA ( $R^2 = 0.999$ ), and it was found to be  $99.7 \pm 0.96\%$ .

#### In Vitro Release Profile of CA-NE Formulation

NEs are unique systems for the delivery of active ingredients to the target area without degradation, and their controlled release ability. In our study, the *in vitro* release study was conducted by simulating skin pH and temperature to determine the controlled release profile of the CA-NE formulation when applied to the skin. In Figure 3, it was shown that *in vitro* release plot of CA-NE was plotted depending on time and cumulative release of CA (%). As seen in the graph, the release profile shows that  $65.04 \pm 2.00\%$  of CA is released in the first 6 hours. This may be due to its initial burst release [67]. It was determined that  $70.85 \pm 4.00\%$  and  $75.49 \pm 1.09\%$  of CA was released in the first 24 hours and 48 hours from the nanoemulsion formulation, respectively. In conclusion, our results showed that the CA-NE formulation released CA in a controlled and exhibited a slow release profile after 24 hours.



**Figure 3.** *In vitro* release profile of CA (%).

#### Bacterial Activity Assay

The antimicrobial activity of CA, CA-NEs was evaluated by using the broth microdilution method. The initial concentration of CA and CA-NE was determined according to the *in vitro* release profile. Bacterial growth inhibition values of free CA and CA-NEs on *S. aureus* were found to be 85% and 95%, respectively (Table 3).

**Table 3: Determination of % inhibition values of CA and CA-NE**

Treatment	% inhibition
CA	85%
CA-NE	95%

Up to now, various studies have been carried out to develop a nanoemulsion formulation with desired physicochemical properties and biological activity such as antibacterial efficiency [57, 74-78]. In many studies in the literature, it has been reported that nanoemulsion formulations have a suitable surface property that provides an easier contact area with microorganisms owing to their nano-sized droplets. Thus, it was observed that the antimicrobial activities of bioactive contents formulated as NEs increased [28,68,69].

The bacteria cell membrane contains charged lipids with high binding efficiency and electrostatic attraction potential [70,71]. On the other hand, nanoemulsions have a structure containing charged lipids in the outer shell layers and bioactive compounds in the core. NEs are colloidal solutions consisting of nanosized droplets and have physicochemical properties that remain stable for a long time [57]. NEs interact with lipids of bacteria to cause bacterial death [72]. Majeed et al., (2016) developed a clove oil nanoemulsions and evaluated its antibacterial effect on gram positive bacteria (*L. monocytogenes* ve *S. aureus*) and gram negative bacteria (*E. coli*) [73]. The results reported that negatively charged clove oil nanoemulsion were effective against *L.*

*monocytogenes* and *S. aureus*, but the effect was low against *E. coli*. Dilen et al., (2008) reported that positively charged polymeric nanoparticles interact with the lipopolysaccharide of gram-negative bacterial cell walls [74]. The results of the studies clearly show that the nanomaterial load plays an important role in the antibacterial activity on gram positive and gram negative bacteria. In our study, the negatively charged CA-NE formulation may have interacted well with the LPS of the gram-positive bacterial cell wall. Therefore, it may have shown antibacterial activity on *S. aureus* strains.

Electrostatic attraction increases their possibility of combining with charges on the bacterial surface. When NEs bind with bacteria, they empty their internal contents resulting in cell lysis [69,75]. Therefore, the NE formulation of bioactive content shows a more effective antibacterial activity than the free bioactive content. In this study, when the inhibition results were examined, it was determined that the CA NE formulation provided a more effective bacterial inhibition against *S. aureus* compared to free CA. Our study overlaps with the articles reporting that the bioactive content formulated as a NE has a more effective bacterial inhibition than the free bioactive content [16,76-81].

## CONCLUSION

NEs have been widely used in the fabrication of bioactive content loaded nanocarriers for topical applications. These formulations are not only guaranteeing a controlled release profile but also high penetration with small droplet size and large surface area for an efficient topical application.

Based on overall our findings, it was concluded that CA-NE formulation had an effective inhibition property on *S. aureus* with the nano-size formulated and controlled release of CA.

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## AUTHORSHIP CONTRIBUTIONS

Authors equally contributed to this work.

## DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

## CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## ETHICS

There are no ethical issues with the publication of this manuscript.

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