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# Ultrasonic nanoemulsification of food grade *trans*-cinnamaldehyde: 1,8-Cineol and investigation of the mechanism of antibacterial activity

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ABSTRACT

Using ultrasonic technology, *trans*-cinnamaldehyde as a natural antibacterial compound was used to prepare nano size emulsions to increase its bioavailability and therefore bactericidal action. Nanoemulsions containing *trans*-cinnamaldehyde as an active agent and 1,8 cineol as co additive oil (Ostwald ripening inhibitor) were formulated using probe sonicator. Three different determining factors, namely time of sonication, surfactant to oil ratio and type of emulsifier (Tween 80 and Tween 20) were investigated to enhance the stability profile. In addition, the effect of changes in the particle size and emulsifier on the antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were examined using agar dilution method. Then, the effect of optimized formulation on the membrane fluidity and cell constituent release, were investigated by analysis of membrane lipids using GC–MS and IR spectrometry, respectively. The data showed that a 15 min sonication of the formulation containing Tween 80 as emulsifier with surfactant to oil ratio of 2:1 (w/w) resulted in a significant stability for 6 months with considerably small particle size of 27.76 ± 0.37 nm. Furthermore, the nanoemulsion showed great antibacterial activity and could reduce the minimum inhibitory concentration (MIC) from 8 to 1 mg/mL against *E. coli* and *S. aureus*, and from 16 to 2 mg/mL against *P. aeruginosa*. Interestingly, *E. coli*'s membrane fluidity increased dramatically after treatment with the optimum nanoemulsion (T804). This study revealed that nanoemulsion of *trans*-cinnamaldehyde and 1,8 cineol has substantial antibacterial activity against selected microorganisms.



## 1. Introduction

Natural antimicrobial compounds such as essential oils (EOs) can inhibit the food-born microorganisms and help to supply food quality and safety [1]. It has been suggested that EOs exert their antibacterial activity through destruction of the bacterial cell membrane [2]. Encapsulation of EOs in suitable delivery system such as nanoemulsions represents an efficient way to increase the bioavailability and physical stability of active lipophilic compounds [3]. Nanoemulsion are colloidal delivery system with mean particle size less than 100 nm [4], which protect the lipophilic bioactive compounds from chemical degradation [5]. There are several studies that have evaluated the antimicrobial activity of nanoemulsions [5–10], showing that nanoemulsification of EOs is an efficient delivery technique to increase the antimicrobial activity. Nanoemulsions can be produced from both high-energy and low-energy methods [11]. High energy methods are utilizing devices with high energy such as high pressure homogenizer, ultrasonicator and high shear homogenizer [12]. Application of ultrasonication waves is very common due to its economical and easy to use advantages, which can effectively disperse oil phase into water phase by providing the required energy [13]. There are several studies that using ultrasonic technique for nanoemulsion preparation in recent years [4,14,15]. *trans*-Cinnamaldehyde is a natural compound that is commonly used in food products as flavour and texture enhancer. It is also known as natural antimicrobial, antifungal, antiseptic and antioxidant agent [16,17]. Therefore, preparation of a *trans*-cinnamaldehyde nanoemulsion could potentially enhance its biological activities. However, the main instability process of nanoemulsions, i.e. Ostwald ripening (OR) has to be controlled for *trans*-cinnamaldehyde nanoemulsions [18]. In OR process small molecules of the dispersed phase diffuses to the larger droplets due to the differences of internal pressure in the droplets of different sizes [19]. There are several methods for controlling the OR [18,20,21], including the usage of carrier oils

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such as corn oil, soybean oil and sunflower oil [1,22]. These oils dissolve the EOs and prevent the transmission of polar components to the aqueous phase and therefore, prevent the OR. However, the use of oil phase usually results in reduced biological activity, perhaps due to the limited diffusion of active compounds to the bacterial cell membrane [22,23]. Therefore, as a new approach to control OR, we have considered using a mixture of nonpolar and miscible compounds to decrease the overall polarity of the dispersed droplets. So, nanoemulsions from *trans*-cinnamaldehyde as active compound and 1,8-cineol as co additive with antibacterial features [24] with small particle size and transparent appearance were prepared and the effect of sonication time, emulsifier type and concentration of components on the nanoemulsion stability were investigated. Also, the antibacterial activity of the prepared nanoemulsions and the mechanism of antibacterial action were investigated against selected pathogenic bacteria. Moreover, the influence of nanoemulsion on the bacterial membrane permeability was monitored using lipid membrane analysis. Finally, the effect of nanoemulsion on the bacterial membrane destruction was evaluated using IR analysis.

## 2. Materials and methods

### 2.1. Chemicals

*trans*-Cinnamaldehyde (⩾98%), 1,8-cineol (⩾98%), Tween 80 and Tween 20 were obtained from Merck Millipore (Darmstadt, Germany).

### 2.2. Fabrication of nanoemulsions using probe sonication

Nanoemulsions were prepared using Tween 80 or Tween 20 as emulsifier and *trans*-cinnamaldehyde as oil phase and 1,8-cineol as ripening inhibitor. Based on the preliminary stability studies, the ratio of *trans*-cinnamaldehyde to 1,8-cineol was fixed on 1:4. Different surfactant to oil ratio (SOR 2:1, 1:1, 1:2, 1:3) and different time of emulsification (5, 10 and 15 min) were chosen for fabrication of stable nanoemulsions with the smallest particle size based on the previous experimental data and instrumental limits. The emulsions were formed using a 20.5 kHz probe sonicator (MPI, Switzerland) with a maximum power output of 400 W. A piezoelectric crystal sonotrode transferred input energy into the mixture with a maximum probe diameter of 8 mm. The volume of mixture was 3 mL and the sonotrode was immersed at the center of the reaction vessel, using an ice bath to maintain a constant temperature during sonication. Different formulation preparations are summarized in Table 1.

### 2.3. Nanoemulsion stability

The accelerated stability test of nanoemulsions were performed by centrifugation at 3500 rpm for 30 min [25] and any phase separation or particle size enhancement was recorded. The rotor radius of centrifuge apparatus (Hettich, EBA 20, Tuttlingen, Germany) was 8.6 cm. Moreover, the particle size and size distribution for samples stored at room temperature were monitored for a six month period.

### 2.4. Nanoemulsion characterization

Morphology and structure of optimum nanoemulsion were evaluated using transmission electron microscopy (TEM). TEM micrographs were obtained using Zeiss-EM10C (Oberkochen, Germany) at an accelerating voltage of 80 keV. Also, droplet size measurements were carried out using a Dynamic Light Scattering (DLS) instrument (Nanophox Sympatec GmbH, Claushtal, Germany).

### 2.5. Antibacterial activity

#### 2.5.1. Bacterial strains

Antibacterial activity of the nanoemulsions and *trans*-cinnamaldehyde oil were analyzed against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* PTCC 1430, and *Staphylococcus aureus* ATCC 25923.

#### 2.5.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC)

Antibacterial activity of nanoemulsified and pure *trans*-cinnamaldehyde with incorporated 1,8-cineol (CIN:CIN) was affirmed through the microbroth dilution method and using MIC. The MICs value were determined according to the amount of CIN:CIN oil in nanoemulsions. Also, the influence of particle size and emulsifier type on the antibacterial activity of nanoemulsions was evaluated. For this purpose, aliquots of samples were serially diluted in a 96 well plate containing Mueller Hinton Broth (MHB) medium to produce a concentration range of 0.0078–32 mg/mL. The final concentration of microorganisms was attuned to $5 \times 10^6$ CFU/mL. 0.5% v/v Tween 80 solution was added to the medium of CIN:CIN oil for MIC analysis. Also, Tween 80 and Tween 20 in the same concentrations as in formulations were utilized as a control for MIC determination. Plates were incubated at 37 °C for 24 h and the lowest concentration that showed no visible microbial growth were determined. The MBC of the samples were determined using incubation of 100 μL of the broth from wells that contained no growth and were plated onto Mueller Hinton Agar (MHA) at 37 °C for 24 h. The MBC was the lowest concentration that could kill 99.9% of the treated microorganisms.

#### 2.5.3. Killing kinetics assay

The killing kinetics of T804 nanoemulsion and CIN:CIN oil were studied against a model bacteria (*E. coli*). For this purpose, 5 mL of overnight culture in Nutrient Broth (NB) medium added to 150 mL cultures and incubated at 37 °C under agitation. This step was continued until an $OD_{600}$ of the suspension reached 0.5–0.6 $cm^{-1}$, which related to the exponential phase of the bacterial growth. In the next step, the MICs concentration of the T804 nanoemulsion and equivalent amount of CIN:CIN oil were added to the cell suspension and incubated under shaking condition. For the CIN:CIN oil 0.5% w/w Tween 80 was poured on cell suspension. For viable count, after 0, 5, 10, 15, 30, 45, 60 min from incubation time, 100 μL of samples were serially diluted on nutrient agar (NA) and incubated at 37 °C for 24 h.

**Table 1**
The characteristic of nanoemulsions with different formulations.

| Surfactant to oil ratio (w/w) | Oil concentration | Surfactant concentration | Nanoemulsion with Tween 80 no. | Nanoemulsion with Tween 20 no. |
|---|---|---|---|---|
| 1:3 | 15% | 5% | T801 | T201 |
| 1:2 | 10% | 5% | T802 | T202 |
| 1:1 | 5% | 5% | T803 | T203 |
| 2:1 | 5% | 10% | T804 | T204 |

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