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Improving the antimicrobial activity of D-limonene using a novel organogel-based nanoemulsion

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ABSTRACT

The purpose of this research was to develop a novel antimicrobial delivery system by encapsulating D-limonene into an organogel-based nanoemulsion and investigating its antimicrobial activity. The D-limonene organogel-based nanoemulsion was prepared by high pressure homogenization method. The surfactant concentration had a major impact on the droplets' formation and distribution. At the optimal condition (10% w/w Tween 80, 100 Mpa, and 10 Cycles) the smallest droplet size ($d \approx 36$ nm) could be obtained, which has shown a narrow structure and good stability. Results from the antimicrobial activity have shown the encapsulation of D-limonene (4% w/w) into the organogel-based nanoemulsion contributed to the increase of its antimicrobial activity. In addition, the mechanism of D-limonene organogel-based nanoemulsion against the tested microorganisms was studied by the electronic microscope observation and the cell constituent release. This research would have an important implication for the design of more efficient antimicrobial systems for food preservation and production.

1. Introduction

Food-borne contamination has for a long time posed a major public health concerns worldwide (Settanni et al., 2012). Several pathogenic microorganisms such as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Listeria monocytogenes*, may lead to food spoilage and poisoning (Kordali et al., 2005). To deal with this health issue, food industry has used a wide range of synthetic antimicrobial agents to inhibit the growth of microorganisms (Al-Reza, Rahman, Lee, & Kang, 2010; Bajpai, Baek, & Kang, 2012). However, many of them have been found to cause respiratory allergies in humans and give a rise of carcinogens and toxic substances (Fleming-Jones & Smith, 2003; Gutierrez, Barry-Ryan, & Bourke, 2009). Therefore, natural antimicrobial compounds may be regarded as beneficial alternative that can be used against food-borne pathogens, since they may reach similar or ameliorate preservative effects.

D-limonene (4-isopropenyl-1-methylcyclohexene), a natural monoterpene with a lemon-like odor is a major constituent of several citruses derived essential oils such as orange, mandarin, lemon, lime, and grapefruit. Owing to its pleasant citrus fragrance, it is widely used in cosmetics, food, and consumer's products. D-limonene is listed in the code of federal regulation as generally regarded as safe (GRAS) for use as a flavoring agent and in food preservation (Sun, 2007). It is reported to have antimicrobial (Chikhoune, Hazzit, Kerbouche, Baaliouamer, & Aissat, 2013; Settanni et al., 2012; Van Vuuren & Viljoen, 2007), antioxidant (Roberto, Micucci, Sebastian, Graciela, & Anesini, 2010), chemopreventive (Crowell et al., 1992; Wattenberg & Coccia, 1991), anticarcinogen (Crowell & Gould, 1994), as well as antidiabetic proprieties (Murali & Saravanan, 2012).

However, D-limonene undergoes oxidative degradation under normal storage condition, leading to the loss of lemon-like flavor and the formation of off-flavors (Li & Chiang, 2012). Its oxidation initially results in the formation of D-limonene hydro-peroxides which undergo scission reactions to form alcohols, ketones, and epoxides (Nguyen, Campi, Roy Jackson, & Patti, 2009). In addition, its hydrophobic nature is another drawback to deal with, as it is difficult to achieve dispersion in water (Soottitantawat, Yoshii, Furuta, Ohkawara, & Linko, 2003). It requires the use of elevated concentrations to achieve equivalent antimicrobial efficiency in food systems. In order to ameliorate the limitations of oxidation and hydrophobic nature, many approaches have been explored to encapsulate D-limonene in different delivery systems.

Owing to its sub-cellular size, nanoemulsion has given an efficient approach to increase the stability and disruption of the encapsulated bioactive compounds, thereby improving their

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antimicrobial activities in food matrices (Weiss, Gaysinsky, Davidson, & McClements, 2009). The same bioactive compound encapsulated into nanoemulsion, exhibits higher antimicrobial activity than the conventional form due to its smaller droplet size. The formed droplets may fuse with the bacterial cell walls, leading to the destabilization of the pathogens' lipid envelope and initiating their disruption (Baker, Hamouda, Shih, & Myc, 2003).

In the literatures, different types of D-limonene lipid based formulations have been reported such as conventional O/W emulsions (Dickinson & Galazka, 1991; Jafari, Beheshti, & Assadpoor, 2012; Mohammadzadeh, Koocheki, Kadkhodaee, & Razavi, 2013), and nanoemulsions (Li & Chiang, 2012; Li, Zhang, Yuan, Liang, & Vriesekoop, 2013; Mahdi Jafari, He, & Bhandari, 2006). However, to the best of our knowledge, no D-limonene organogel-based nanoemulsion and its antimicrobial activity have been reported so far. The objective of the current study was to develop a D-limonene organogel-based nanoemulsion and investigate its antimicrobial activity against four food-borne pathogens, which includes *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Saccharomyces cerevisiae*, as well as its antimicrobial mechanism using the electronic microscope observation and the cell constituent release.

2. Materials and methods

2.1. Chemicals

D-limonene was obtained from Florida Worldwide Citrus Products Group Inc. (Bradenton, Florida, USA). Monostearin was purchased from Aladdin Reagents Co., LTD (Shanghai, China). Nonionic surfactant (Tween 80) was purchased from the sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Medium Chain Triglyceride (MCT oil) was purchased from Sigma Aldrich (Shanghai, China).

2.2. Preparation of D-limonene organogel and organogel-based nanoemulsion

D-limonene organogel was prepared as described by Yu and Huang (2012) with some modifications. Briefly, monostearin, MCT oil, and D-limonene were mixed at the weight ratio of 1.5:8.5:20 (w/w/w), followed by heating to ensure complete dissolution of monostearin. The mixture was set at room temperature, and organogel was formed within few minutes. D-limonene organogel-based nanoemulsion preparation was carried out by a high pressure homogenization procedure. D-limonene organogel was used as the oil phase (about 4% D-limonene in the nanoemulsion), and Milli-Q water containing different amounts of Tween 80 surfactant 2.5%, 5%, 10% (w/w) was used as the water phase. The organogel-based nanoemulsion formation was performed by mixing the oil phase together with the water phase in a high blending homogenizer (HENC, Shanghai, China) at 24,000 rpm for 5 min to initially form a coarse emulsion, followed by high pressure homogenization at 100 Mpa, and 10 Cycles to finally form the D-limonene oragnogel-based nanoemulsion.

2.3. Particle size measurements

The average particle sizes (*Z*-average) and particle sizes distributions of the organogel-based nanoemulsions were determined using Dynamic Light Scattering at 25 °C (Zetasizer Nano-ZS90, Malvern Instruments, Malvern Worcestershire, UK). The measurements were carried out at a scattering angle of 90°. Each individual measurement was recorded in an average of 10 scans. The samples were diluted approximately 1000 types with Milli-Q water. The particle size of the emulsions was described by the mean particle size (*Z*-average) diameter, and the size distribution was described by the polydispersity index (PdI). Each measurement was repeated in triplicate.

2.4. Turbidity measurements

The turbidity was assessed in triplicates using UV–visible spectrophotometer (Ultra-spec 2450 pr, Shimadzu Ltd, Kyoto, Japan) at 600 nm. Distilled water was used as a reference to the blank cells.

2.5. Antimicrobial activity

2.5.1. Microbial strains

Four food-borne pathogens were used to assess the antimicrobial proprieties, which includes the gram-negative bacteria *E. coli* ATCC 8739, the gram-positive bacteria *B. subtilis* ATCC 6633, *S. aureus* ATCC 6538, and the yeast *S. cerevisiae* ATCC 9763. These strains were obtained from China General Microbiological Culture Collection Center (Beijing, China). They were maintained at 4 °C on slants of Nutrient Agar for bacteria, and Yeast Peptone Dextrose (YPD) for the yeast (Abxing, Beijing, China).

Active cultures were prepared by transferring a ring loop of cells from the agar slant to a test tube containing 5 mL of Nutrient broth for bacteria and YPD for the yeast. The bacterial and yeast cultures were then incubated overnight at 37 °C (6–10 h) and 30 °C (12–16 h) for the bacteria and yeast respectively. The purity of the cultures was examined by streaking each culture on plates of Nutrient Agar for the bacteria and YPD for the yeast (Gilles, Zhao, An, & Agboola, 2010). The turbidity of the cell cultures was measured at 600 nm using UV spectrophotometer, and adjusted to the required concentration (1×10^8 CFU/mL) (Firuzi, Asadollahi, Gholami, & Javidnia, 2010; Liang, Yuan, Vriesekoop, & Lv, 2012).

2.5.2. Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of free D-limonene and D-limonene organogel-based nanoemulsion were determined by broth dilution method as described by Weerakkody, Caffin, Turner & Dyke (2010) with minor modifications. Briefly, after the addition of an appropriate amount of free D-limonene or D-limonene organogel-based nanoemulsion to the first tube containing 6 mL, serial two-fold dilutions in the tubes containing the same nutrient broth for bacteria and YPD for yeast was carried out. A 400 μL of tested microorganism suspensions (1×10^8 CFU/mL) was added to each tube. Meanwhile, a positive control tube (50 μg/mL of Kanamycin sulfate) and negative control in broth and microorganisms were prepared separately. MIC was defined as the lowest concentration in the serial dilution of the free D-limonene or D-limonene organogel-based nanoemulsion, which resulted in the lack of visible microorganism growth in tubes after 24 h for bacteria and 48 h for the yeast (Al-Reza et al., 2010; Lv, Liang, Yuan, & Li, 2011).

2.6. Mechanism of D-limonene organogel-based nanoemulsion against the cell membranes

2.6.1. Scanning electron microscopy analysis

In order to examine the mechanism of D-limonene organogel-based nanoemulsion against cells' membrane of the four target microorganisms, SEM studies were performed as previously reported with minor modifications (Moosavy et al., 2008). Logarithmic growth phase of the four tested microorganisms (each approximately 1×10^8 CFU/mL), were treated with each MICs of D-limonene organogel-based nanoemulsion, and free D-limonene at