

Antibacterial and antioxidant lemongrass essential oil Pickering emulsion stabilized by cellulose nanocrystals

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Received 23 May 2023; Revised 04 Jul. 2023; Accepted 08 Aug. 2023; Published 25 Aug. 2023.

DOI: <https://doi.org/10.54939/1859-1043.j.mst.89.2023.87-93>

ABSTRACT

An effective antibacterial system was developed by using cellulose nanocrystals (CNC) to stabilize lemongrass essential oil Pickering emulsion (PE-LEO) through ultrasonication technology. The factors affecting the formation and stability of PE-LEO were studied, such as ultrasonication times, CNC concentrations, lemongrass essential oil (LEO) concentrations. By size and zeta index, the most suitable sample was 8 ultrasonication times, 0.8% CNC, 15% LEO. The antibacterial and anti-fungal performance of PE-LEO was investigated by determining the minimal inhibitory concentrations (MIC). The results showed that for gram-positive bacteria (*E. faecalis*, *S. aureus*, MRSA), the MIC of PE-LEO was much higher than LEO, the opposite was true for gram-negative bacteria (*E. coli*) and fungi. Based on the concentrations of LEO, with IC₅₀ of PE-LEO is 0.30% vLEO/v, which is significantly lower than that of LEO (0.99%). The CNC-stabilized PE-LEO exhibited higher antioxidation activity at equivalent LEO concentrations. The fabricated CNC based Pickering emulsions provide a promising alternative for the delivery of antimicrobial essential oils in the food industries.

Keywords: Lemongrass essential oil; Cellulose nanocrystals; Pickering emulsion; Antimicrobial; Antioxidant.

1. INTRODUCTION

Essential oils (EOs) are attracted widely due to their effective antifungal, antibacterial, antiviral and antioxidant abilities [1]. The content and composition of bioactive compounds in EOs are very diverse and depend on the source of the EOs [2]. However, EOs have some drawbacks such as: low surface energy and high volatility and being highly sensitive to oxidation, light, and thermal decomposition [3]. Moreover, the hydrophobic nature of EOs also limits their application, especially for food and pharma [4].

Applying the Pickering emulsion (PE) technique for its active ingredients can circumvent stability issues. by using solid particles (metals, oxides, anisotropic particles, etc.) as stabilizers accumulated stabilizers, PE stabilizes the oil-in-water solution. PE technique stabilizes oil droplets by electrostatic and steric mechanisms [5]. This stabilization method decreases the droplet size and avoids the coalescence of the droplets, and are prepared using sufficient energy, such as homogenization, ultrasonication, and other [6]. The physical-chemical properties of PE (e.g. thermodynamically or kinetically stability, type of emulsion, droplet size) are influenced by several parameters, among others the size and surface properties of particles, the ratio of oil and water phase, the viscosity or density of dispersed phase [7]. The application of essential oil Pickering emulsions-ranging from drug carriers to active packaging-has been carried out in many studies [8-11].

Lemongrass (*Cymbopogon citratus*) essential oil (LEO), one of the common EOs, was found effective against *Staphylococcus aureus* (*S. aureus*), *Bacillus cereus* (*B. cereus*), *Bacillus subtilis* (*B. subtilis*), *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*) at low

concentrations (5%) [12]. Gram-positive organisms were found to be more sensitive to LEO as compared to gram-negative organisms. Therefore, LEO is suitable for application in food preservation, cosmetics, agriculture and farming [13]. Cellulose nanocrystals (CNC) are a class of rigid, hydrophilic, high in aspect ratios, and rod-shaped crystal cellulose whiskers of 1–20 nm in diameter, and tens to hundreds of nanometers in length [14]. With a large surface area (150–250 m²/g) and low coefficient of thermal expansion, CNC shows great potential in tissue engineering, fillers for injectable hydrogel, and drug carriers [15]. There is no available literature that provides a clear picture in univariate survey of lemongrass essential oil Pickering emulsion.

In this study, for the first time, CNC synthesized from rice was used to stabilize LEO Pickering emulsion (PE-LEO) with good emulsification properties without the use of any emulsifier. The stability, antibacterial and antifungal properties of PE-LEO were determined to show how well PE-LEO works.

2. MATERIAL AND METHODS

2.1. Materials

Cellulose nanocrystal (CNC) was attained from a treatment of rice straw from An Giang Province, Vietnam. Pure lemongrass (*Cymbopogon citratus*) essential oil (LEO) was recovered in Thanh Hoa province by fractional distillation and purchased from the commercial market. All the reagents, including sodium hydroxide (NaOH), hydrogen peroxide (H₂O₂), and sulfuric acid (H₂SO₄), were purchased from Xilong suppliers and used without further purification.

2.2. Preparation methods

2.2.1. CNC recovery

Cellulose was recovered from rice straw following a previously mentioned process to attain a purity of >90% [16]. Cellulose nanocrystal suspensions were prepared by hydrolysis using 62% H₂SO₄ solutions at a solid: liquid ratio of 1g : 12 mL with continuous mixing controlled at around 40 – 42 °C for 2 hours. Afterward, the reaction was stopped by diluting the solution by 10 folds and centrifuging. The final solution is neutralized by dialysis with water that is changed every 6 hours. The resulting CNC material was measured by dynamic light scattering (DLS) and SEM image to be around 20 – 30 nm in diameter and around 250 nm in length.

2.2.2. Emulsion preparation

The oil in water emulsion was prepared by combining LEO of different concentrations (5%, 10%, 15%, 20%, 25% v/v) and CNC suspension of different concentrations (0.2%, 0.4%, 0.6%, 0.8%, 1.0% w/v) by homogenization via sonication using a Hielscher UP400St sonicator (Germany). The sonication process was performed at a power of 200 W following a pulse sequence of 2 minutes of ultrasonication following with a 2 minute break, which is denoted as one time of ultrasonication (UT) where the total number of times was investigated (2, 4, 6, 8, 10 times), corresponding with an accumulation of 4, 8, 12, 16, 20 minutes.

2.3. Characterization methods

2.3.1. Droplet size and morphology

For analysis, the emulsions were diluted to a concentrations of 0.1% (v/v) LEO. The dynamic particle size and zeta potential (ZPV) of the emulsion were determined by the dynamic light scattering (DLS) method using a Malvern Zetasizer Nano ZS (UK) at a scattering angle of 173°. The droplet microstructures were observed using a BIOVAL L1000A optical microscope (Brazil) at x100 magnification.

2.3.2. In vitro antimicrobial and antifungal test

The antibacterial and antifungal activities of LEO and PE-LEO were conducted against *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*) (Gram negative bacteria);

Enterococcus faecalis (*E. faecalis*), *Staphylococcus aureus* (*S. aureus*), methicillin-resistant *Staphylococcus aureus* (Gram positive bacteria); *Candida albicans* (*C. albicans*) and *Aspergillus niger* (*A. niger*) (fungi) based on the agar well diffusion method. The culture medium were Mueller-Hinton agar (MHA) where for fungi, 2% glucose was introduced, melted and poured into a petri dish to obtain agar layers with a thickness of around 3-4 mm. The microorganism density was adjusted to reach around 1.5×10^8 CFU/mL for bacteria (McFarland 0.5) and around $1 - 5 \times 10^6$ CFU/mL (so that the absorbance at 530 nm is around 0.08 – 0.10) for fungi. After spreading the microbials onto the surface of the plate, 50 μ L of the test sample is introduced to 6 mm well that was previously carved out on the medium. After 24 hours of incubation at 37 °C, the diameter of the inhibition zone of the samples were measured.

For minimum inhibition concentrations (MIC) determination, different concentrations of test subjects were diluted with dimethyl sulfoxide (DMSO), mixed directly with specific volumes of sterilized MHA medium and let set at 45 - 50 °C. During determination, 1 – 2 μ L of microbial suspensions adjusted to 10^2 CFU/mL were added onto the layer of test subjects containing agar that is previously poured onto Petri disks. The availability of bacteria colonies observable on the disks was used to determine the MIC value.

DMSO was used as the negative control for the above assays.

2.3.3. *In vitro* antioxidant test

The antioxidant activity was performed based on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. Solutions of different concentrations of samples in 80% methanol and 0.1 mM DPPH in 80% methanol were first prepared. Afterward, 3.2 mL of DPPH solutions were added to 1.8 mL of samples and left in the dark for 30 min. The absorbance of the samples were measured using a UV-Vis 754 STECH INTERNATIONAL spectrometer (China) at 517 nm, which is characteristic of the peak of DPPH. The inhibition was calculated by:

$$I (\%) = \frac{A_{\text{control}} - (A_{\text{sample}} - A_{\text{color}})}{A_{\text{control}}}$$

Where A_{control} is the absorbance of the sample without test subjects, A_{sample} is the absorbance that includes the test subjects and DPPH, and A_{color} is determined by the absorbance of the sample without DPPH.

The concentrations at which the samples exhibit 50% scavenging activity (IC50) was used to compare the different samples.

2.3.4. Scanning electron microscopy (SEM)

Surface morphology of CNC was characterized using a scanning electron microscope (SEM) (Prisma E, US) at 10 kV of accelerated voltage.

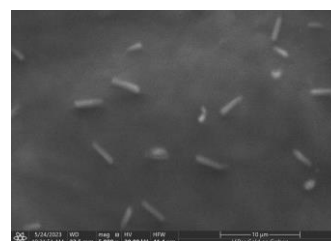


Figure 1. SEM image of CNC recovered.

3. RESULTS AND DISCUSSION

3.1. Surface morphology of CNC

The surface morphology of CNC was represented by SEM images. The CNC seemed to be rod-like particles with mean particle size that had typical dimensions of around 20 – 30 nm in diameter and around 250 nm in length, and exhibited excellent homogeneity and stability, as shown in fig.1.

3.1. Univariate survey on lemongrass Pickering emulsion (PE-LEO)

3.1.1. The influence of ultrasonication times

The PE-LEO samples contained 15% LEO; 0,8% CNC and different ultrasonication times (UT). As shown in Fig. 2a, the size of PE-LEO particles decreased with increasing UT from 2

times to 10 times. PE-LEO.2T with 2 UT exhibits a large size (5.820 mm), which was probably attributed to the fact that it was not having enough energy from the ultrasonication progress. With increasing of UT from 8UT to 10UT, the particle size of PE-LEO reached a plateau value of nearly 0.66 mm, which might indicate that PE-LEO had been supplied enough energy for the CNC particles to encase LEO.

The charge of the particle can be determined from the zeta potential value (ZPV). The higher the zeta potential, the smaller the potential for oil droplets to coalesce due to the repulsive force. Therefore, solutions with high zeta potential (negative or positive) have high stability while colloids with low zeta potential tend to coagulate or flocculate. [17]. The ZPV value of 30 mV is considered optimum for good stabilization of a nanodispersion [18]. PE-LEO with high ZPV (>28,3 mV) provides the system's stability and is less prone to form aggregates or increase in particle size. PE-LEO.8T reached plateau size value (672,8 nm) and the highest ZPV (38,42 mV). Therefore, 8 times, equivalent to a total of 16 minutes of ultrasonication was chosen for the preparation of the PE-LEO emulsion.

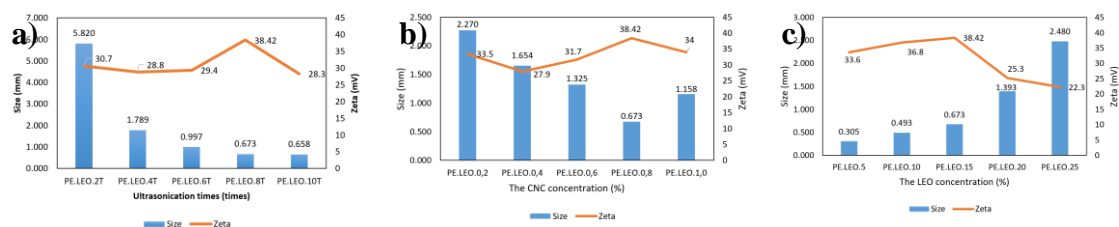


Figure 2. The influence of ultrasound times (a), CNC concentrations (b), LEO concentrations (c).

3.1.2. The influence of the CNC concentrations

In this experiment, PE-LEO samples included 15% LEO, 8UT and CNC concentrations varying from 0.2% to 1.0%. The influence of the CNC concentrations on the size and ZPV of PE-LEO was shown in Fig. 2b. From 0.2 to 0.8% CNC, the size of PE-LEO gradually decreased (0.227 mm to 0.673 mm) then increased slightly when CNC reached 1.0%. This was probably attributed to the fact that the CNC insufficiently covered the oil droplet surfaces during ultrasonication, when CNC concentrations increased, the amount of CNC coating LEO was increasing, separating the oil droplets into smaller particles, leading to a reduced size. According to ZPV, PE-LEO samples were almost stable (ZPV>27,9 mV) and PE-LEO.0.8 reached the stablest sample with ZPV=38,4 mV. So 0.8% CNC concentrations was selected to go on the next experiment.

3.1.3. The influence of the LEO concentrations

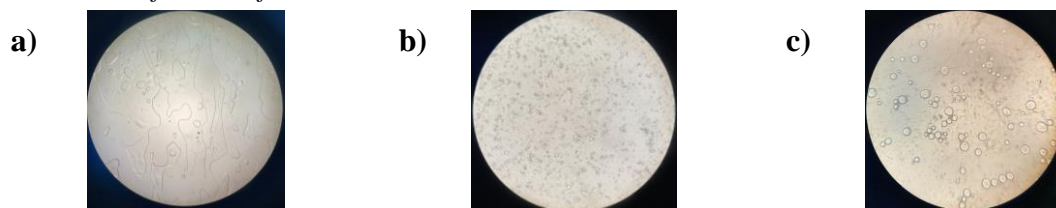


Figure 3. Optical microscopy images of LEO (a), PE-LEO.15% (b) and PE-LEO.25% (c).

The oil/water ratio is a remarkable factor affecting Pickering emulsion stability [19]. In this experiment, the concentrations of LEO changed from 5% to 25% with 0.8% CNC and 8UT. As shown in Fig. 2c, the oil/water ratio had an obvious influence on the drop diameter of the PE-LEO. The results showed that the CO-PEs with a high oil/water ratio possessed a larger droplet size as compared to those with a lower oil/water ratio at the equivalent CNC concentrations. The ZPV of PE-LEO was higher at 5%, 10% and 15% LEO, with highly stable (ZPV>30 mV). Since the CNC concentrations in the system was a certain number, it seemed that the addition of extra oil could increase the interfacial area which might not be sufficiently covered by the available

CNC. This could result in rapid coalescing and growing up of small drops in order to reduce the interfacial area [20, 21]. This can be shown by optical microscopy images of PE-LEO.15% and PE-LEO.25% (Fig. 4). The suitable condition for this experiment was 15% LEO.

3.2. Antimicrobial and anti-fungal features of PE-LEO

Based on the qualitative antibacterial and antifungal results, PE-LEO (15% LEO; 0.8% CNC; 8UT) was active against both gram-negative and gram-positive bacteria, and also has antifungal activity. The diameter of antibacterial and anti-fungal rings were shown in table 1, Fig. 4 and Fig. 5. The diameter of antibacterial and anti-fungal rings of PE-LEO were a little bit smaller than that of LEO. This can be explained by the low concentrations of essential oil in PE-LEO (only 15%), so the antibacterial and antifungal activity is smaller than that of the original LEO.

Table 1. Determination of antimicrobial and fungal resistance (diameter of antibacterial ring, mm).

	Sample	Diameter of antibacterial ring (mm)				
		<i>E. coli</i>	<i>P. aeruginosa</i>	<i>E. faecalis</i>	<i>S. aureus</i>	MRSA
1	PE-LEO	34	15	16	30	28
2	LEO	48	-	48	28	32
Negative sample	DMSO	-	-	-	-	-

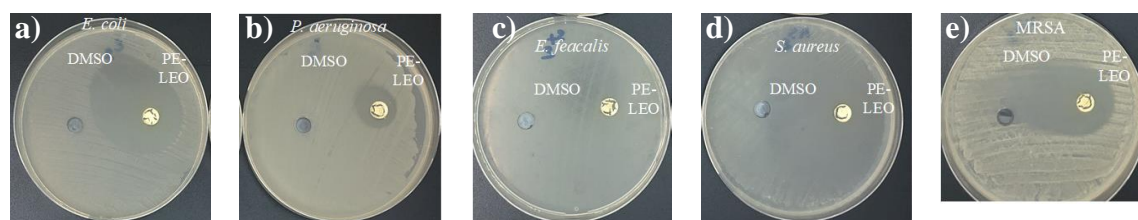


Figure 4. Inhibition zone diameter of PE-LEO against *E. coli* (a), *P. aeruginosa* (b), *E. faecalis* (c), *S. aureus* (d), MRSA (e).

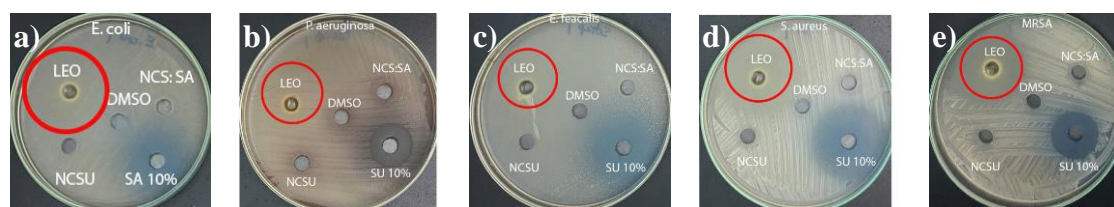


Figure 5. Inhibition zone diameter of LEO against *E. coli* (a), *P. aeruginosa* (b), *E. faecalis* (c), *S. aureus* (d), MRSA (e).

Minimal inhibitory concentrations (MICs) of LEO and PE-LEO against the seven tested microorganisms were investigated and the results were shown in table 2. PE-LEO was also converted into the same concentrations with LEO for comparison. For gram-positive bacteria (*E. faecalis*, *S. aureus*, MRSA), the MIC of PE-LEO was much higher than LEO, the opposite was true for gram-negative bacteria and fungi. For gram-negative bacteria and fungi, the negatively charged emulsion droplets would be repelled to approach the target microorganisms [22], which would inevitably affect the antimicrobial efficiency. Furthermore, PE-LEO had higher stability than unprocessed LEO due to CNCs as the stabilizer. Controlling the electrical characteristics of emulsion delivery systems may be an effective way of increasing their antimicrobial and anti-fungal activity. The CNC attached to the LEO surface further lowers the electricity of the PE - LEO generation, which helps to increase the contact between LEO and Gram-positive bacteria,

and increase the resistance to Gram-positive bacteria of the PE-LEO system.

Table 2. MIC results of PE-LEO and LEO.

	Sample	MIC results (%v/v)						
		<i>E. coli</i> (E)	<i>P. aeruginosa</i> (P)	<i>E. faecalis</i> (Ef)	<i>S. aureus</i> (MS)	MRSA (MR)	<i>C. albicans</i>	<i>A. niger</i>
1	PE-LEO	0,32	>5	1,25	1,25	2,5	0,08	0,04
2	PE-LEO (based on LEO concentrations of sample)	0,048	>0,75	0,1875	0,1875	0,375	0,012	0,006
3	LEO	0,125	>1	0,125	0,125	0,25	0,032	0,032

3.3. Analysis of antioxidant capacity- DPPH

The standard curve of antioxidant capacity I% against concentrations of PE-LEO was made at PE-LEO concentrations of 2.5% to 12.5% v/v, respectively, in triplicate. Similarly, the same experiment was performed with LEO at a concentrations of 1% – 5% v/v. Table 3 shows the antioxidant capacity of each sample based on the established calibration curve and the concentrations of the active compound concerning the total volume of the test vials. It is noteworthy that, even at a lower concentrations of LEO, the IC50 value of PE-LEO (2.02%) is close to LEO (0.99%). When calculating based on the concentrations of LEO, the IC50 of PE-LEO is 0.30% v_{LEO}/v , which is significantly lower than that of LEO, indicating that by encapsulating the oil particles in CNC, a synergistic behavior can be experienced, enhancing the antioxidating capacity of essential oil.

Table 3. Antioxidant capacity of PE-LEO and LEO for DPPH.

Sample	IC50 (%)
LEO	0.99%
PE-LEO	2.02% (%PE-LEO/v)
	0.30% (%LEO/v)

4. CONCLUSIONS

In this study, CNC was used for preparing PE-LEO as a stabilizer. The influence of ultrasonication time, the concentrations of CNC and the concentrations of LEO were analyzed. As the ultrasonication time increases, the average size decreases. Increasing the oil volume fraction at a fixed CNC particle concentrations causes an increase in the average emulsion droplet size. Antibacterial and DPPH antioxidation activity indicate that PE-LEO possesses similar bioactivity to LEO at the same oil concentrations. This demonstrates that CNC can help stabilize emulsions with a limited reduction in bioactivity. The results reported in the current study have the certain leading meaning for the design and use of PE-LEO stabilized by CNCs for the delivery of antimicrobial and antioxidant essential oils in the food and other industries.

Acknowledgment: We acknowledge the support of time and facilities from the Institute of Tropical Environmental and Ho Chi Minh University of Technology for this study.

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TÓM TẮT

Nhũ tương Pickering tinh dầu sả chanh kháng khuẩn và kháng oxy hóa ổn định bởi nano xenlulo tinh thể

Một hệ kháng khuẩn hiệu quả đã được phát triển bằng cách sử dụng các tinh thể nano cellulose (CNC) ổn định nhũ tương Pickering tinh dầu sả chanh (PE-LEO) thông qua công nghệ siêu âm. Các yếu tố ảnh hưởng đến sự hình thành và ổn định của PE-LEO đã được nghiên cứu như thời gian siêu âm, nồng độ CNC, nồng độ tinh dầu sả chanh (LEO). Theo kích thước và chỉ số zeta, mẫu phù hợp nhất là 8 lần siêu âm, 0,8% CNC, 15% LEO. Khả năng kháng khuẩn và kháng nấm của PE-LEO đã được nghiên cứu bằng cách xác định nồng độ ức chế tối thiểu (MIC). Kết quả cho thấy, đối với vi khuẩn gram dương (*E.faecalis*, *S.aureus*, MRSA), MIC của PE-LEO cao hơn nhiều so với LEO, đối với vi khuẩn gram âm (*E. coli*) và nấm thì ngược lại. Dựa trên nồng độ của LEO, IC50 của PE-LEO là 0,30% vLEO/v, thấp hơn đáng kể so với LEO (0,99%). PE - LEO ổn định bằng CNC thể hiện hoạt tính kháng oxy hóa cao hơn ở nồng độ LEO tương đương. Các nhũ tương Pickering ổn định bằng CNC hứa hẹn giải pháp thay thế cung cấp các loại tinh dầu kháng khuẩn trong ngành công nghiệp thực phẩm.

Từ khóa: Tinh dầu sả chanh; Nano xenlulo tinh thể; Nhũ tương Pickering; Kháng khuẩn; Kháng oxy hóa.