Preparation, characterization, and biological activity of Cinnamomum cassia essential oil nano-emulsion

Dongyi Liang a, Baijian Feng a, Na Li a, Linhan Su b, c, Zhong Wang b, c, Fansheng Kong a, Yongguang Bi a, b, c, *.

a College of Pharmacy, Guangdong Pharmaceutical University, China
b College of Chinese Medicine, Guangdong Pharmaceutical University, China
c Yunfu Traditional Chinese Medicine Hospital, China

A R T I C L E   I N F O

Keywords:
Essential oil
Cinnamomum cassia
Ultrasonic-assisted extraction
Nano-emulsion

A B S T R A C T

To solve the problems of low bioavailability and unstable properties of Cinnamomum cassia Essential oil (CCEO), encapsulation technology was introduced as an effective means to improve its shortcomings. In this study, Cinnamomum cassia Essential oil nano-emulsion (CCEO-NE) was successfully synthesized by the oil-in-water method and characterized by standard analytical methods, including dynamic light scattering (DLS), Scanning electron microscopy (SEM), and Transmission electron microscopy (TEM). The results show that the synthesized CCEO is spherical, smooth in surface, and uniform in shape, with an average particle size of 221.8 ± 1.95 nm, which is amorphous. In this experiment, by simulating the digestion of CCEO-NE in the gastrointestinal tract, it was found that CCEO-NE was undigested in the oral cavity, mainly in the stomach, followed by the small intestine. By understanding the digestion of CCEO-NE, we can improve the potential of CCEO bioavailability in food and drug applications. In addition, through the study of ABTS and DPPH free radicals by CCEO and CCEO-NE, it was found that the antioxidant activity of CCEO-NE was more potent than that of CCEO. When the concentration of CCEO-NE and CCEO is 400 μg/mL, the DPPH free radical scavenging rate is 92.03 ± 0.548% and 80.46 ± 5.811%, respectively. In comparison, ABTS free radical scavenging rate is 90.35 ± 0.480% and 98.44 ± 0.170% when the concentration of CCEO-NE and CCEO is 75 μg/mL, respectively. The antibacterial test shows that CCEO-NE can inhibit both Gram-positive and Gram-negative bacteria. Among them, CCEO-NE has a stronger antibacterial ability than CCEO, and the maximum inhibition zone diameter of CCEO can reach 15 mm, while that of CCEO-NE can reach 18 mm. Meanwhile, SEM and TEM showed that CCEO-NE treatment destroyed the ultrastructure of bacteria. Generally speaking, we know the situation of CCEO in the gastrointestinal tract. CCEO-NE has more potent antioxidant and antibacterial ability than CCEO. Our research results show that whey protein is an effective packaging strategy that can improve the effectiveness, stability, and even bioavailability of CCEO in various applications, including food and health care industries.

1. Introduction

Essential oil from aromatic plants is considered a vital medicine source containing unique bioactive compounds [1,2]. Essential oil is a volatile, natural, complex compound with a strong smell [2]. Essential oil is composed of some tiny molecules. These highly flammable substances can be absorbed by nasal mucosa and enter the body, which can send messages directly to the brain and regulate emotions and physiological functions of the body through the brain’s limbic system. In the middle Ages, they were usually obtained by steam or steam distillation, developed by Arabs. They are famous for their physical, chemical, fragrance, and antiseptic properties, such as sterilization, virus killing, and fungus killing [3]. They are used in many aspects, such as food preservation, antiseptic and antibacterial. Of course, there are different effects of different kinds of essential oils, and they can also relieve some diseases and symptoms of some diseases [5]. Cinnamomum cassia, an edible ingredient in food [6] belonging to Lauraceae, has been widely used. It is an aromatic tree species distributed in China and Southeast Asia, such as Vietnam and Myanmar [7]. Cinnamic acid, coumarin, cinnamaldehyde, diterpenes, and polyphenols...
are only a few bioactive chemicals discovered through chemical and pharmacological research. It can be used as raw material for pharmaceutical products and daily condiments. In addition, it has high economic value. It is a traditional herbal medicine with biological characteristics like anti-tumor, anti-diabetes, anti-inflammation, anti-bacteria, and anti-oxidation [8]. However, the essential oil was highly restricted because of its poor water solubility. To improve the solubility of critical oil, oil-in-water emulsion provides a good solution.

There are certain limitations because of the slow penetration of semi-solid preparations in the skin and transdermal administration, large pellets, rapid volatilization of highly volatile compounds, degradation due to environmental influence, light instability, etc. The fluid properties of nano-emulsion have the characteristics of rapid interaction with skin cells, small droplet size, and high permeability [9]. It is regarded as outstanding technology. Many researchers have preferred protein to inorganic substances in recent years in preparing nanoemulsions because the former is more nutritious and safe. As a protein macromolecule, Whey Protein Isolation can form a stable system [41,42]. Meanwhile, the solubility and bioavailability are improved. It can be seen from related literature that nanoemulsion technology is considered the best technology because it has fluid properties, remarkable interaction with skin cells, small droplet size, effective penetration, and even the protective ability to deliver irritating, volatile, and high molecular weight molecules [9].

Traditional extraction methods have the disadvantages of long extraction time, low extraction rate, and degradation of some components in the extraction process. Ultrasonic-assisted extraction can help to improve these problems. Besides, the operation of ultrasonic-assisted extraction is simple and reproducible [10]. Ultrasonic-assisted extraction can release the cell contents into the solvent to a greater extent through the cavitation effect so that the extraction rate can be significantly increased. Meanwhile, the degradation of essential oil can be avoided in ultrasonic extraction [11]. As a time-saving and labor-saving extraction method, ultrasonic-assisted extraction can protect the environment in the commercial production process [12].

This work aims to improve the stability and long-term antibacterial effect of CCEO-NE. The nano-emulsion was characterized by DLS, zeta potential measurement, and TEM. In addition, the experiment simulates the gastrointestinal tract (mouth, stomach, and small intestine), puts the nano-emulsion into the gastrointestinal solution, and judges the situation of nano-emulsion in the gastrointestinal tract by fluorescence microscope and the change of particle size potential.

2. Materials and methods

2.1. Materials

DPPH and ABTS were purchased from Shanghai Aladdin Biochemical Technology Co. Ltd. (China). Sodium tripolyphosphate (purchased from Tianjin Damao Chemical Reagent Factory). Bacterial strains, including Staphylococcus aureus (S. aureus) CMCC 26003, Bacillus subtilis (B. subtilis) CMCC (B) 63501, Salmonella typhimurium (S. Typhimurium) CMCC 50094, Pseudomonas aeruginosa (P. aeruginosa) CMCC 10104, Escherichia coli (E. coli) CMCC 44102, Staphylococcus epidermidis (S. epidermidis) ATCC1228 were purchased from Guangdong Institute of Microbiology (Guangzhou, China), mucin from the porcine stomach (M2378-100G); Bile salt (B22609-5G) from a pig; pepsin (P7000-25G) from porcine gastric mucosa; mucin (M2378-100G) from the porcine stomach; lipase (S10035-5G) from porcine pancreas; and Nile Red (N3013-100MG). Simulated Intestinal Fluid (PH1841); Simulated Gastric Fluid (PH1840); Artificial Saliva (PH1843). Whey glycoprotein (WGP) (G885091) is produced in Shanghai McLean Biological Co., Ltd (Le Sueur, MN, USA). All other chemicals used belong to the analytical grade in this study. All solutions and emulsions were prepared with double-distilled water.

2.2. Preparation of essential oil

According to the relevant literature, it has been modified [13]. Fresh cinnamon (100 g) is put into a grinder to be ground. Then, 800 mL of water and 2% sodium chloride were placed in a 60 °C trough ultrasonic for 30 min. After ultrasonic treatment, CCEO was separated from the water at 155°C and finally stored in the refrigerator (4°C) for later use.

2.3. Preparation of nano-emulsion

10% oil phase (CCEO) and 90% emulsifier solution (10% WGP in deionized water) (all the above units are w/w) is put into a beaker. To prepare a pre-emulsion, the water-in-oil emulsion is homogenized at high speed (18,000 rpm) for 5 min with a high shear stirrer (T18 digital Ultra-Turrax, IKA, Germany). Put the beaker into an ultrasonic pulverizer, set appropriate power, frequency, and time, and get a milky white oil-in-water emulsion with good fluidity after ultrasonic treatment.

2.4. Characterisation of nano-emulsion

The zeta potential and particle size of CCEO-NE by Delsa Nanometer Zeta Potential/Particle Size Analyzer (Delsa Nano C, USA). According to the literature [14], nano-emulsions are diluted 100 times for measurement to avoid multiple scattering effects.

The average zeta potential, particle size, particle size distribution, and polymer dispersion index (PDI) of nano-emulsion were measured at 25°C based on the zeta potential/particle size analyzer of DLS technology. All samples were tested in triplicate. The morphology of nanoemulsion was observed by TEM. The simulated gastrointestinal tract includes the oral cavity, stomach, and small intestine. The nano-emulsion was placed in the gastrointestinal solution. The nanoemulsion situation in the gastrointestinal tract was judged by a fluorescence microscope and the change of particle size potential [15].

2.5. Determination of rheological properties

According to relevant literature [16], the rheology of CCEO-NE was measured by a DHR-3 rheometer (TA USA). At 25°C, the viscosity was measured by the shear rate of 0.01–200 s⁻¹. The dynamic stress scanning method was estimated to be the linear viscoelastic region of nanoemulsion. The side pressure was 1 Pa. The viscosity was measured at an angular frequency of 0.1 to 10 rads⁻¹ [17].

2.6. Simulated gastrointestinal digestion

According to the above operation, CCEO and WGP were mixed to prepare CCEO-NE, preheated at 37°C for several minutes. Finally, the sample passed through the oral, gastric, and intestinal stages step by step. According to the related literature, this method is slightly different [18].

2.6.1. Initial system

At 37°C, the newly prepared nano-emulsion was preheated in a glass beaker, and the preparation method was as described above.

2.6.2. Mouth phase

An experiment was conducted with artificial saliva (APS) (pH6.8). APS was preheated in a water bath at 37°C, then α-amylase was added and stirred evenly, and then mixed with the preheated CCEO-NE according to the mass ratio of 1: 1. Finally, the mixture was placed in a shaking incubator (10 min, 120 rpm, and 37°C) to simulate buccal cavity conditions.

2.6.3. Stomach phase

Firstly, at 37 °C, simulated gastric fluid (SGF) (placed in a beaker) was heated in a water bath. After that, evenly mixed with 7.0 mg/mL.
beaker, and sodium hydroxide was added to adjust the pH to neutral of 1: 1 and put on a shaking table (120 rpm and 37 °C). Next, Simulated Intestinal Fluid (SIF) was heated in a water bath instead of DPPH solution, and the positive control was VcDPPH ethanol is added, mixed evenly, and placed in protection from ultraviolet light for 6 min, the absorbance was measured at 734 nm with an ultraviolet spectrophotometer. Vc is used as the positive control. Next, the treated bacteria were dried and sprayed with gold and positive control, respectively. Each group of experiments was carried out in the same way 3 times [21].

The sample’s minimum inhibitory concentration(MIC) was measured according to relevant literature [22]. Samples were diluted from 0.0888 μL/mL to 45 μL/mL with Mueller-Hinton broth (MHB). In a 96-well plate, 50 μL of bacteria solution (about 1 × 10⁸ CFU/mL) was mixed with 100 μL of samples with different concentrations and stored in an incubator at 37 °C for 16 h. Meanwhile, a positive control (levofloxacin) was set. MIC is the lowest concentration of visible bacteria that the naked eye or microscope [15].

2.9. Determination of antibacterial properties

2.9.1. Determination of minimum inhibitory concentration

The paper diffusion method was used to determine the inhibition diameter of CCEO and CCEO-NE to bacteria. The form of references has been slightly modified [20]. The target bacteria (bacterial suspension) were dispersed to 1 × 10⁶ CFU/mL with normal saline. Next, 100 μL of the regulated target bacteria were dispersed in 20 mL of culture medium using sterile cotton swabs. The filter paper with a diameter of 6 mm was soaked in CCEO-NE and CCEO for two hours, then clamped on the surface of the agar culture dish with tweezers. And keep these Petri dishes containing bacteria at 37 °C for a whole day. The diameter of the bacteriostatic circle indicates the antibacterial ability. Levofloxacin and sterile water represent positive control and negative control, respectively. Each group of experiments was carried out in the same way 3 times [21].

2.9.2. Bacterial ultrastructure

Combined with SEM and TEM, bacteria’s surface and internal morphology can be displayed more completely. With some modifications, SEM and TEM were used to image the surface structure of bacteria, as mentioned earlier [21]. In brief, 5 mg/mL samples were mixed with 1 × 10⁷ CFU/mL Escherichia coli and Staphylococcus aureus at 37 °C for 6 h. Next, the mixture was centrifuged at 8000 rpm for 5 min, and the supernatant was poured out. The sediment bacteria were washed in the lower layer with 0.1 mM PBS 2–3 times, added to 1.5 mL of 4% glutaraldehyde solution, and kept in the refrigerator for one night. The immobilized bacteria were taken out, washed, centrifuged at the same time and rate, and finally, 1.5 mL of 2.5% glutaraldehyde was added and put into the refrigerator for 1 h. Different percentages (20, 50, 80, and 100) of ethanol were used to dehydrate bacteria continuously, and then tert-butyl alcohol was used instead. Escherichia coli and Staphylococcus aureus without sample treatment were treated the same way as to control. Next, the treated bacteria were dried and sprayed with gold and finally observed by SEM and TEM.

2.9.3. Statistical analysis

Each set of experiments is performed no less than three times, and the results are expressed in mean ± standard deviations (SD). SPSS.25 software was used for regression analysis of the data. P < 0.05 was
3. Results and discussion

3.1. Characterization of nano-emulsion

The average particle size significantly affects drug-loaded particles’ absorption, biocompatibility, and bioactivity [15]. Fig. 1 (A) shows the average particle size distribution of nano-emulsion, similar to the normal distribution, indicating that nano-emulsion has a good consistency and uniform particle size distribution [24]. D50 of nano-emulsion is about 221.8 ± 1.95 nm. Although the stability of nano-emulsion cannot be determined by particle size alone, particle size has a significant influence on the stability of nano-emulsion [25]. Zeta potential can reflect the interaction of static electricity between particles. Because it is an essential reference for the physical and chemical stability of nano-emulsion during storage [26]. When the absolute value of zeta potential is higher, the system becomes more stable. However, when the total value of zeta potential exceeds 30 millivolts, it is relatively stable, and when zeta potential exceeds 60 millivolts, it indicates excellent stability. But the value of about 20 mV indicates that the short-term stability is limited, and the value below 5 mV usually leads to the rapid aggregation of nano-emulsion [27]. Fig. 1(B) shows that nano-emulsions have positively charged surfaces, which is 40.26 ± 0.58 mV, which indicates that nano-emulsions have high stability. Dispersion is usually measured by polydispersity index (PDI), which can reflect particle size dispersion in suspension. The lower the PDI value, the more uniform the particle size [28]. PDI<0.4 is usually an acceptable value. However, the PDI value of CCEO-NE is<0.3, which indicates that nano-emulsion is uniformly dispersed. The experimental results show that the PDI of nano-emulsion is 0.216 ± 0.0090, which means that nano-emulsion is evenly dispersed.

Through microscopic morphology, it was observed that CCEO-NE showed a bright monodisperse spherical shape in a dark environment. According to the literature report [17], the morphology of nano-emulsion is monodisperse spherical, which is similar to this study. Fig. 2 shows that the droplet size of CCEO-NE prepared by the ultrasonic-assisted method is about 100 nm. However, the droplet size measured by the laser particle size analyzer is 200–250 nm. On the one hand, it may be that the two measurement results are expressed in different ways. The laser particle size analyzer results show the average particle size under normal distribution, while TEM results show intuitive droplet size [17].

On the other hand, maybe the measurement status is different. The laser particle size analyzer directly measures the diluted nano-emulsion system, while the TEM measures the dry solid-state of nano-emulsion. The TEM measurement process was dried, which may lead to dehydration of nano-emulsion, thus changing the droplet size [17]. However, both characterization methods show that the particle size was less than 250 nm, which is enough to prove that CCEO-NE nano-emulsion has been formed.

Fig. 3 shows that the greater the shear rate of nano-emulsion, the lower its apparent viscosity. Before the shear rate was 25 s⁻¹, the apparent viscosity decreased significantly, but when the shear rate was greater than 25 s⁻¹, the apparent viscosity was infinitely close to 0. These characteristics indicate that nano-emulsions are all Newtonian fluids. With the increase of viscosity of nano-emulsion, the moving speed of oil droplets in the water phase decreases, which prevents further interaction between emulsifiers and oil droplets, thus affecting the stability of the nano-emulsion system [29]. With the increase in shear rate, the internal results of nano-emulsion gradually decompose, and the
3.2. Effect of time on the stability of emulsion in the gastrointestinal tract

3.2.1. Influence on particle size

The average particle size of CCEO-NE remained relatively constant after being exposed to the oral cavity (223.9 ± 1.25 nm), increased significantly after being exposed to the stomach, and decreased after being exposed to the small intestine (Table 1 and Fig. 4). The particle size distribution shows that a considerable part of the particles in the sample exposed to the oral phase has the same size (228.1 ± 2.65 nm). However, after being exposed to the stomach phase, the droplets aggregated widely because the particle size of the emulsion increased obviously. This result probably is due to many physical and chemical phenomena in the process of simulating the gastric phase, which includes the following factors: the change of pH and the strength between ions caused the repulsion between oil droplets to decrease, biopolymers such as mucin are adsorbed on the surface of droplets. And aggregation may be related to the change of pH value and ionic strength in the stomach. As shown in Fig. 6 (D) [31] that the granules in the stomach stage are significantly larger than those in the small intestine stage. The particles in the small intestine stage are smaller may be as previously mentioned. The mixture of undigested fat, digested fat products, and fatty acid calcium salt forms partially dense droplets are destroyed into single droplets [30].

3.2.2. Influence on particle charge

As shown in Fig. 5, the initial emulsion has a relatively high negative charge (-46.93 ± 0.874 mV). The main reason is that its isoelectric point (about 4.7) is lower than its pH value (6.8). After being exposed to the oral phase for 10 min (-36.03 ± 0.198 mV), the negative charge of particles in saliva decreased because of the reaction in simulated saliva (such as the electrostatic shielding effect of mineral ions and interaction with mucin) [31]. When the sample passes through the next stage, the stomach, the negative charge of the sample sharply decreases or even becomes a positive charge (21.77 ± 0.643 mV). The above results are due to many factors [31]: (i) The change of environmental pH value of the sample leads to the change of charge of protein; (ii) Electrostatic shielding effect (higher intensity of ions in gastric juice); (iii) Anionic biopolymers such as mucin are adsorbed on the surface of droplets. And (iv) Protease in gastric juice digests the protein layer. Some people also think that the stable oil droplets in protein will be positively charged under high acidity (pH 2.5), as the pH value is much lower than its isoelectric point. After being exposed to small intestinal fluid, protein-stable excipient emulsion becomes highly negative (~40 mV) due to the relatively high anionic substance (such as bile salt, phospholipid, and free fatty acid) in the intestinal fluid after digestion. In general, these results show again that emulsifiers affect the digestion of samples and the release of essential oils in the small intestine.

3.2.3. Influence on microstructure

The change information of emulsion microstructure in different areas was obtained through a confocal fluorescence microscope. Microscopic images show that after exposure to the oral cavity (Fig. 6(B)), the emulsion does not accumulate excessively, which is similar to colostrum (Fig. 6(A)). After being exposed to the stomach phase, it can be seen from microscopic images that there is obvious droplet flocculation in the stomach phase (Fig. 6(C)), which can also be ascribed to the decomposition of flocs in the process of sample preparation. Instability of emulsion in the stomach may occur due to various reasons: (i) The decrease of the stability of droplets to coalescence may be related to the hydrolysis of adsorbed protein. (ii) The decrease of the stability of droplets to aggregation may be related to the change of pH value and ionic strength (possibly to weaken the electrostatic repulsion). (iii) Polymers may boost protein dephosphorylation or its bridging flocculation. It can be found in (Fig. 6(D)) [31] that the granules in the stomach stage are significantly larger than those in the small intestine stage. The particles in the small intestine stage are smaller may be as previously mentioned. The mixture of undigested fat, digested fat products, and fatty acid calcium salt forms these particles. Generally speaking, lipid digestion in the small intestine and CCEO release will be affected by the aggregation of emulsion solution in the stomach.

3.3. Antioxidant activity

ABTS and DPPH are diamagnetic molecules that have the ability to change color. Because of the advantages of simple operation and accurate experimental results, DPPH and ABTS free radical scavenging tests are widespread for testing antioxidant capacity. As shown in Table 2, the 50% inhibitory concentration value of ABTS is significantly lower than that of DPPH. The IC50 of different antioxidant activities is different because of different free radical mechanisms. The mutual effect between antioxidants and stressors, the solubility of samples in different systems, and the stereoselectivity of free radicals will lead to different antioxidant activities of samples [32].

DPPH displays different colors when it is used. For example, it is dark purple in anhydrous ethanol solution. However, when DPPH is in the reaction process, the color will become lighter and yellow. The
scavenging rate of DPPH radicals depends on the kind of antioxidant and the concentration of the same antioxidant [33]. There is a significant difference in DPPH free radical scavenging rate between CCEO and CCEO-NE (Fig. 7(A)). Table 2 shows the IC_{50} of oxidation resistance of each sample. The DPPH clearance IC_{50} of CCEO is 208.7 ± 5.28 μg/mL, which is much higher than that of CCEO-NE (179.0 ± 2.62 μg/mL). In the saponification process, strong acid and alkali may decompose antioxidant substances, which leads to a content drop. Therefore, its antioxidant activity will also decrease. With the increase in concentration, the clearance rate of CCEO-NE and CCEO increased from 27.84 ± 2.482% to 92.03 ± 0.548% and 29.30 ± 1.403% to 80.46 ± 5.811%, respectively. CCEO-NE is a little stronger than CCEO in oxidation resistance, which may be because CCEO is protected and has a more potent antioxidant capacity. This study confirmed that the antioxidant properties of CCEO-NE and CCEO are quite different, which provides a theoretical basis for studying the difference between nano-emulsion and essential oil in the future.

ABTS free radical scavenging test is a test method that mainly studies the electron transfer of antioxidants [34]. Fig. 7(B) indicated that the higher the concentration of the sample, the stronger the ABTS radical scavenging ability. This shows that the concentration affects the dose-effect. When each concentration is 8.0 mg/mL, the scavenging capacity of CCEO-NE (98.44 ± 0.170%) is better than that of CCEO (90.35 ± 0.480%). But the effect of CCEO-NE is stronger than that of positive control (V_c), which is different from that of the DPPH method. In ABTS and DPPH, the same thing is that the ABTS clearance rate of CCEO-NE is higher than that of CCEO. However, the difference between them is that the scavenging ability of CCEO-NE to scavenge DPPH free radicals is weaker than that of positive control (V_c). The results of ABTS analysis (Table 2) showed that the IC_{50} values of essential oil and nano-emulsion.

### Table 2

Antioxidant capacity (IC_{50}) of CCEO-NE, CCEO, and Ascorbic acid.

|          | DPPH(μg/mL) | ABTS(μg/mL) |
|----------|-------------|-------------|
| CCEO     | 208.7 ± 5.28| 25.4 ± 1.55 |
| CCEO-NE  | 179.0 ± 2.62| 9.1 ± 0.71  |
| Ascorbic acid | 6.7 ± 0.08 | 5.8 ± 0.01  |

Fig. 6. The influence of nano-emulsion on the microstructure under simulated gastrointestinal conditions was measured by a confocal fluorescence microscope. The scale represents the length of 100 μm, and the red area represents oil.

Fig. 7. In vitro antioxidant activities of ascorbic acid, CCEO, CCEO-NE. (a) DPPH radical scavenging assay; (b) ABTS radical scavenging assay.
were 25.4 ± 1.55 μg/mL and 9.1 ± 0.71 μg/mL, respectively. According to the IC₅₀ value, the antioxidant capacity is CCEO-NE > CCEO. By combining DPPH and ABTS tests, it can be concluded that essential oil has a strong antioxidant capacity, which has also been reported in the literature. At the same time, the antioxidant capacity of CCEO-NE is more prominent, which can pave the way for the follow-up study of CCEO-NE.

### 3.4. Antibacterial activity

This experiment evaluated the antibacterial activities of CCEO, CCEO-NE, and levofloxacin against 6 strains of bacteria, including 3 strains of Gram-positive bacteria (S. aureus, B. subtilis, and S. epidermidis) and 3 strains of Gram-negative bacteria (E. coli, S. typhimurium, and P. aeruginosa). It can be seen from Table 3 and Fig. 8 that CCEO-NE has an influence on the growth of six bacteria. However, the inhibitory effect is relatively weak compared with essential oil nano-emulsion. Therefore, the prepared emulsion has a broader antibacterial effect. The antibacterial ability of CCEO-NE is more potent than that of CCEO. At the same time, it has better antibacterial performance [35]. Nano-emulsion destroys bacteria mainly by penetrating its cell wall and destroying the cell membrane. Therefore, they have great potential in antibacterial activities.

In Table 3 and Fig. 8, the results of the antibacterial activity of CCEO and CCEO-NE against six bacterial strains by agar well diffusion are expressed as the diameter of the inhibition zone. The results showed that CCEO and its CCEO-NE had sound inhibitory effects on S. aureus, B. subtilis, S. epidermidis, E. coli, S. typhimurium, and P. aeruginosa. The MIC and MBC values of CCEO-NE are between 0.118 and 0.355 μg/mL and 0.355-0.710 mg/mL, while the MIC and MBC of CCEO have the same concentration range (Table 4). The possible reason is that the concentration range of MIC and MBC is broad, but the difference between MIC and MBC is not particularly large. According to the analysis of the above results, the research substance is more sensitive to gram-positive bacteria (S. aureus, E. coli, and B. subtilis) than to gram-negative bacteria (P. aeruginosa, S. typhimurium, S. epidermidis). Many studies have proved that gram-positive bacteria are relatively easier to kill than gram-negative bacteria. Sugarcane molasses essential oil is stronger against Gram-positive bacteria (23.07 ± 0.0404 mm) than Gram-negative bacteria (17.73 ± 0.839–23.07 ± 3.101 mm) [21]. The antibacterial activity of citronella essential oil obtained by different methods against Gram-positive bacteria (10.33 ± 0.29–26.17 ± 0.29 mm) is higher than that against Gram-negative bacteria (7.67 ± 0.29–11.67 ± 0.29 mm) [36].

According to the bacteriostatic circle diameter, minimum inhibitory

### Table 3

| Microorganisms | Inhibition zone diameter (mm ± SD) |
|----------------|-------------------------------------|
|                | CCEO-NE  | CCEO  | Levofloxacin |
| E. coli        | 18.17 ± 0.764* | 14.00 ± 1.500 | 18.17 ± 1.258 |
| S. aureus      | 18.33 ± 1.528* | 15.00 ± 1.000 | 15.83 ± 1.155 |
| B. subtilis    | 16.23 ± 0.462* | 13.33 ± 1.258 | 25.83 ± 1.258*** |
| S. typhimurium | 13.00 ± 1.323* | 8.50 ± 1.323  | 16.33 ± 0.289* |
| P. aeruginosa  | 13.83 ± 0.577* | 9.33 ± 0.764  | 24.93 ± 0.764 |
| S. epidermidis | 14.67 ± 1.756* | 11.67 ± 0.289 | 24.47 ± 0.900** |

Note: *, CCEO-NE compared to CCEO, *P < 0.05, **P < 0.01, ***P < 0.001; \#, CCEO-NE compared to Levofloxacin, \#P < 0.05, \##P < 0.01, \###P < 0.001.

### Table 4

| Microorganisms | MIC(μg/mL) | MBC(μg/mL) |
|----------------|------------|------------|
|                | CCEO-NE    | CCEO       | Levofloxacin |
| E. coli        | 0.118      | 0.710      | 0.196        | 0.391 |
| S. aureus      | 0.355      | 0.710      | 0.196        | 0.391 |
| B. subtilis    | 0.355      | 0.710      | 0.196        | 0.391 |
| S. typhimurium | 0.118      | 0.355      | 0.196        | 0.391 |
| P. aeruginosa  | 0.118      | 0.710      | 0.196        | 0.391 |
| S. epidermidis | 0.355      | 0.710      | 0.196        | 0.391 |

**Fig. 8.** Inhibitory effects of CCEO, CCEO-NE, levofloxacin, and sterile water on six bacteria. A, B, C, D, E, F respectively indicate that it contains S. epidermidis, S. aureus, B. subtilis, E. coli, S. typhimurium, and P. aeruginosa. In contrast, a, b, c, d respectively indicate the inhibition of sterile water, levofloxacin, nano-emulsion, and CCEO.
concentration, and MBC value, S. aureus and E. coli are the representative test bacteria. To further understand the antibacterial ability of CCEO-NE and fully understand the surface morphology and internal changes of bacteria before and after transformations, the morphological characteristics of target bacteria were evaluated by SEM and TEM. Under normal conditions, E.coli has a complete smooth-faced, striped cell wall and regular short rod or cylinder shape (Fig. 11B (1–3)). However, after mixing with CCEO-NE for six hours, the shape of E.coli resembles a caterpillar, the cell wall and cell membrane become blurred, irregular wrinkles exist on the cell surface, and damaged cells or cell fragments are broken, adhered, and aggregated (Fig. 9 (C,D) and Fig. 11b (1–3)). Untreated Staphylococcus aureus cells were spherical, regular,
smooth, and complete (Fig. 10 A (1–3)). But after mixing with CCEO-NE for six hours, the shape of Staphylococcus aureus became irregular, the surface shrank, some cells were harmed, and the cell wall and cell membrane became blurred (Fig. 9 (A, B) and Fig. 10 a(1–3)). After treatment, Escherichia coli and Staphylococcus aureus showed cytoplasmic contents leakage, cell distortion, degradation of the cell wall, and leakage of cytoplasmic substances, indicating that the antibacterial mechanism could be the transformation of the cell wall, cell membrane, and mitochondrial membrane, causing the contents of the cell to leak out and bacterial cell death [37]. The antibacterial effect of CCEO-NE is ascribed to many elements, including the increase of cell membrane permeability and the remarkable depolarization of CCEO-NE [38]. The interaction between the negative charge of CCEO-NE and ions on the surface of bacterial cations is the fundamental reason why CCEO-NE can effectively mutually affect the cell membrane and changes the cell’s permeability, which makes the CCEO-NE combine with the cell membrane more effectively, thus resulting in harm to the membranes of the bacteria [39]. The antibacterial principle of essential oil usually was determined by the lipophilicity or hydrophilicity of microorganisms and the outer membrane arrangement and structure of microbial cells [40].

4. Conclusion

Encapsulation technology is an effective way to improve the bioavailability and stability of CCEO. In our research, CCEO-NE was successfully synthesized by the oil-in-water method, and its characterization is mainly analyzed by DLS, SEM, and TEM. The results showed that the synthesized CCEO-NE was spherical, smooth in surface, uniform in morphology, small in average particle size, and amorphous structure. In this experiment, by simulating the digestion of CCEO-NE in the gastrointestinal tract, it was found that CCEO-NE was undigested in the oral cavity, mainly in the stomach, followed by the small intestine. By understanding the digestion of CCEO-NE, we can improve the potential of CCEO bioavailability in food and drug applications. In addition, through the study of its biological activity, it was found that CCEO-NE has remarkable antioxidant activity. The antibacterial test shows that CCEO-NE can inhibit both Gram-positive and Gram-negative bacteria, and CCEO-NE has a stronger ability than CCEO. At the same time, SEM and TEM showed that the treatment of CCEO-NE destroyed the ultrastructure of bacteria. In general, we understand the situation of CCEO in the gastrointestinal tract, and the antioxidant and antibacterial abilities of CCEO-NE are more potent than those of CCEO. Our findings show that whey protein is an effective packaging strategy, which can improve the effectiveness, stabilities, and even bioavailability of CCEO in various applications, including food and health care industries. Nano-emulsion-loaded drug technology provides a new way and method to improve the solubility and bioavailability of insoluble drugs, realize sustained-release and targeted drug delivery, and even enhance the activity of some drugs. It is believed that with the essential theory research and technological innovation of nano-emulsion, its prescription is continuously optimized, and its manufacturing process is continually improved. This technology can be widely used in medicine, cosmetics, food, biotechnology, and other fields.

CRediT authorship contribution statement

Dongyi Liang: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization, Writing – original draft. Baijian Feng: Conceptualization, Methodology, Validation, Formal analysis, Visualization, Investigation. Na Li: Methodology, Investigation, Visualization, Writing – review & editing. Linhan Su: Methodology, Software. Zhong Wang: Conceptualization, Supervision, Project administration. Fansheng Kong: Supervision, Project administration. Yongguang Bi: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work is financially supported by Science and Technology Planning Project of Guangzhou Science and Technology Bureau.
