ORIGINAL ARTICLES

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Preparation, characterization and antimicrobial activity of inclusion complexes of *Myristica fragrans* Hott. essential oil in β-cyclodextrins

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Inclusion complexes of essential oils with cyclodextrins are an effective way to improve stability and solubility, and turn liquid materials into easy to handle powders. In this work, an essential oil of *Myristica fragrans* Hott. (MFEO), already used in the food and cosmetics industries, was formulated with beta-cyclodextrins (β-CD) using a co-precipitation method. The orthogonal array scheme was adapted for the optimization of preparation process. DSC and FT-IR spectroscopy analysis indicated the successful formation of MFEO/β-CD inclusion complexes, which improved the thermal stability of MFEO. Furthermore, comparing the antimicrobial activity of MFEO/β-CD inclusion complexes and free essential oil against *Staphyloccocus aureus*, *Staphyloccocus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, yeast *Saccharomyces cerevisiae* and *Bacillus subtilis*, it was found that the antimicrobial effect was enhanced after the formation of inclusion complexes. This study demonstrates the potential for the use of MFEO/β-CD inclusion complexes in the treatment of bacterial infection.

1. Introduction

Myristica fragrans Hott. is an evergreen tree indigenous to Moluccas of Indonesia, originally known as the Spice Islands (Van Gils and Cox 1994). It is also widely distributed in the tropics including Guangdong and Yunnan provinces in China, Malaysia, Kerala in India and South America. The seeds of *M. fragrans* are known as nutmeg (also known as *pala* in Indonesia) and it is a well-known spice for cooking sauce meat and fish as well as for formulation of medicated balm and perfume. Recent studies have shown that the essential oils of *Myristica fragrans* Hott (MFEO) shows a broad range of pharmacological effects, including anti-inflammatory (Olajide et al. 1999), antioxidant, antibacterial (Narasimhan and Dhake 2006), aphrodisiac (Tajuddin et al. 2003), memory enhancing and antidiarrheal properties (Grover et al. 2002).

In recent years, cyclodextrins have been successfully used to prepare inclusion complexes to improve the solubility and stability, mask unpleasant smell or taste, protect against oxidation and light-induced decomposition, regulate the release of drugs, and convert liquid substances to solids (Sebaaly et al. 2016; Wempe et al. 2008; Wen et al. 2016). Therefore, they are widely used in food (Hill et al. 2013), drug delivery systems (Vieira et al. 2015), pharmaceutical, and chemical industries, as well as agriculture and environmental engineering (Sharma and Baldi 2016). Cyclodextrins are a family of compounds constructed from a-(1,4)-linked-d-glucopyranoside units forming cyclic oligosaccharides, with a hydrophobic central cavity and hydrophilic outer surface (Szejtli 1998). This structure allows them to capture hydrophobic molecules in their interior cavity, while the polarity of their exterior ensures the water solubility of guest molecules (Liu et al. 2012). Most common native compounds are the α(alpha)-, β(beta)-, and γ(gamma)-cyclodextrins made up of six, seven and eight glucosyl units, respectively. Owing to its availability and cavity size suitable for guest molecules with molecular weights between 200 to 800 g/mol (Szente and Szejtli 2004; Waleczek et al. 2003), beta-cyclodextrins (β-CD) have become widely used pharmaceutical excipients for generating inclusion complexes (Galvão et al. 2015; Hill et al. 2013; Zhu et al. 2016). The interaction between β-CD (host) and active substances (guest) may involve complete inclusion or association with the hydrophilic or hydrophobic part of the molecules (Szente and Szejtli 2004). Inclusion complexes of essential oils with β-CD have been applied to prevent oxidation of volatile oil, evaporation, heat and light degradation. Once essential oils are within the inclusion complexes, they are less sensitive to external influence. Besides, inclusion complexes turn liquid volatile oils into water dispersible and well manageable powders (Fernandes et al. 2004).

Therefore, the purposes of the present study were to prepare MFEO/β-CD inclusion complexes using a co-precipitation method, characterize their physicochemical properties by Differential Scanning Calorimetry (DSC) and Fourier Transform Infrared Spectroscopy (FT-IR). Then, we have attempted to evaluate the potential use of MFEO/β-CD inclusion complexes for the treatment of infectious diseases produced by common pathogens.

2. Investigations, results and discussion

2.1. *Preparation procedure*

2.1.1. *Single factor experiment results*

As shown in Fig. 1a, it can be seen that the entrapment efficiency increased gradually with the decrease of MFEO/β-CD ratio. When the ratio of MFEO/β-CD varied from 1:4 to 1:8, the inclusion rate increased quickly. Thus, 1:8 of MFEO/β-CD ratio was selected for further study. Figure 1b shows the effect of inclusion time on the entrapment efficiency of MFEO/β-CD inclusion complexes. The inclusion rate reaches a maximum about 2 h after injection MFEO into β-CD saturated solution. The longer inclusion time is beneficial for improving the formation of inclusion complexes due to promotion collision chance between β-cyclodextrin and MFEO. However, the inclusion rate will not increase with the inclusion time prolonged when the inclusion complex is completely formed. Thus, 2 h was adopted in the next work. As can be seen from Fig. 1c, the inclusion rate increased with increasing temperature from 30 to 60 °C and then decreased. The reason for this trend is probably associated with volatilization of essential oil easily under high temperature. Therefore, an inclusion temperature of 60 °C was enough for the inclusion process.

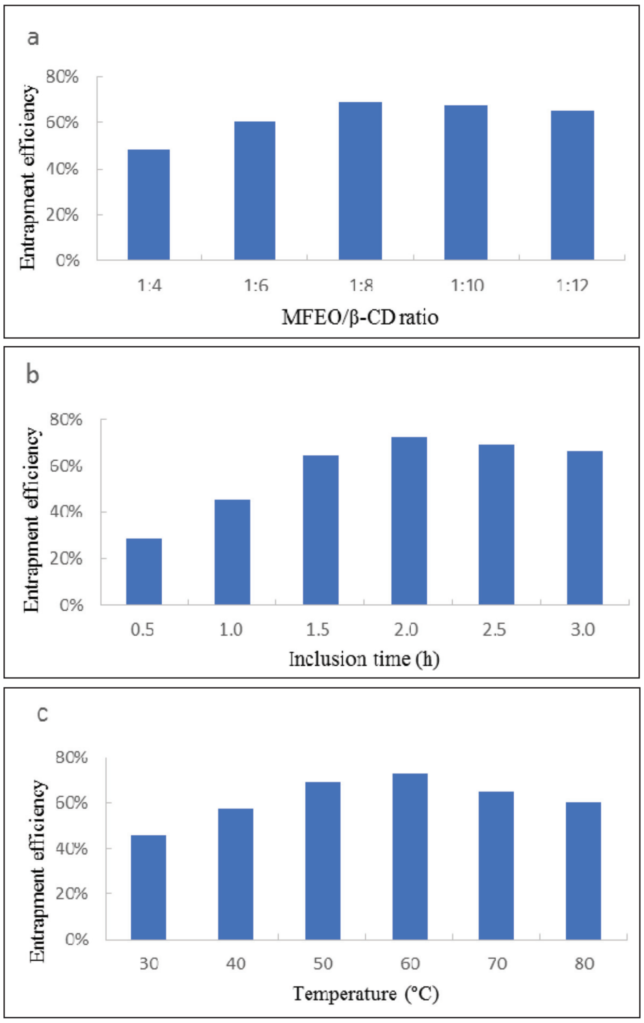

Fig. 1: Effect of different MFEO/β-CD ratio (a), inclusion time (B), and temperature (c) on the entrapment efficiency of β-CD/MFEO inclusion complexes

2.1.2. Orthogonal design $L_9(3^4)$ experiment

Orthogonal design involving three factors at three levels (Table 1) was used to optimize the preparation process of β-CD/MFEO inclusion complexes. Nine experimental trails were carried out with different condition arrays and the results were shown in Table 2. The numbers 1, 2 and 3 in the Table represent the level setting in the experiment. The optimum condition is identified by studying the main effects of each of the factors. *Km* is used to evaluate the effect of the factors on the response (entrapment efficiency). The *Km* (m=1-3) values in the three rows are the average of response with variable *m*. Range (R), also known as full range, is used to represent the variation in the statistics, equal to the difference between the maximum value and the minimum value. According to the largest donating rule, the largest *K* value which affects the entrapment efficiency of β-CD/MFEO inclusion complexes should be the selected value. Results show that inclusion time (B) obviously had much more effects on the preparation process of β-CD/MFEO inclusion complexes than β-CD/MFEO ratio (A) and inclusion temperature (C). The result of visual analysis on orthogonal test is A3B2C2, that is, β-CD/MFEO ratio of 1:10, inclusion time of 2 h, and the inclusion temperature of 60 °C, the entrapment efficiency under the optimal conditions was 85.76±1.04 %.

2.2. DSC thermograms

DSC is an effective tool to study the formation of inclusion complexes with cyclodextrin. The thermal curves of MFEO, β-CD, physical mixture and MFEO/β-CD inclusion complexes are shown in Fig. 2. The DSC curve of β-CD exhibited a wide endothermic peak in a range of 88-129 °C (Fig. 2a), which is associated with the release of water molecules from β-CD. Furthermore, decomposition and removal of carbonaceous occurred when temperature was raised above 310 °C (Galvão et al. 2015). As can be seen from Fig. 2c, MFEO shows one strong endothermic peak at 99.8 °C and a weak and broad endothermic event in the interval 140-220 °C, both possibly corresponding to the volatilization process or evaporation process. For the physical mixture (Fig. 2d), a large endothermic peak at 96 °C and decomposition peak above 320 °C were observed, offering the proof for the presence of the MFEO and β-CD. However, the thermogram of MFEO/β-CD inclusion complexes demonstrated that the peak of MFEO was completely disappeared and the peak originally in the β-CD also changed slightly (Fig. 2b). It is not merely a physical mixture, but molecular encapsulation of the MFEO within the cavity of β-CD.

Table 1: Factors and levels of the orthogonal design

level	A MFEO/β-CD ratio, ml/g	B Inclusion time, h	C Inclusion temperature, °C
1	1:6	1.5	50
2	1:8	2.0	60
3	1:10	2.5	70

Table 2 Orthogonal design and experimental results

	A	B	C	Entrapment efficiency (%)
1	1	1	1	55.56
2	1	2	2	72.84
3	1	3	3	63.46
4	2	1	2	60.14
5	2	2	3	66.67
6	2	3	1	54.44
7	3	1	3	51.42
8	3	2	1	85.67
9	3	3	2	64.41
K1	63.95	55.71	65.22	
K2	60.42	75.06	65.80	
K3	67.17	60.77	60.52	
R	6.75	19.35	5.28	

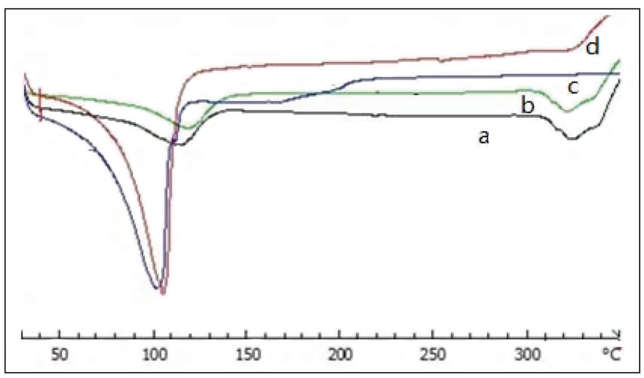

Fig. 2: DSC of β-CD (a), MFEO/β-CD inclusion complexes (b), MFEO (c) and physical mixture (d).

2.3. FT-IR

Infrared spectroscopy is often used to evaluate the interaction between cyclodextrin and guest molecules in solid state. By this means inclusion complexes can be determined by studying the changes of the peak shape, intensity and position. Figure 3 shows the FT-IR spectra of MFEO, β-CD, physical mixture and MFEO/β-CD inclusion complexes. The FT-IR spectrum of MFEO

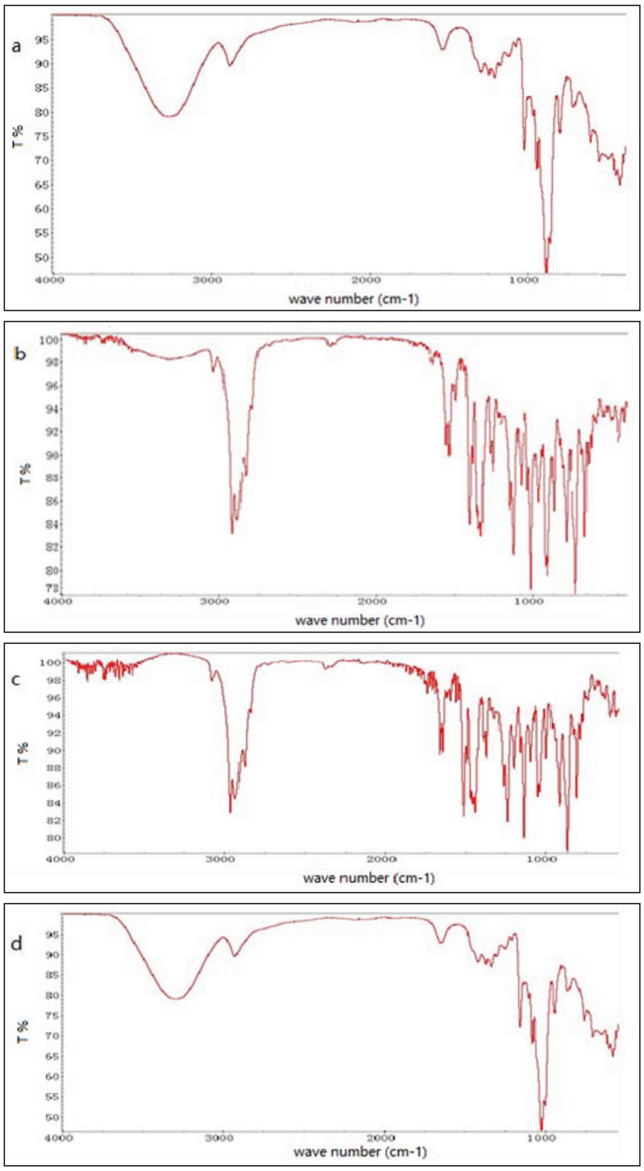

Fig. 3: IR spectra of β-CD (a), MFEO (b), MFEO/β-CD mixture (c) and MFEO/β-CD inclusion complex (d).

(Fig. 3a) consisted of the medium intensity absorption bands at 2870 cm^{-1} and 2957 cm^{-1} for symmetrical stretching vibration and asymmetrical stretching vibration of $-CH_3$, 2929 cm^{-1} for asymmetrical stretching vibration of $–CH_3$, and 1632 cm^{-1} for stretching vibration of C=C. The absorption bands at 1464 cm^{-1} and 1448 cm^{-1} correspond to C–H bending vibration, while the double peaks (1380 and 1369 cm^{-1}) of symmetrical deformation vibration of $–CH_3$ were due to the fact that two methyl groups attached to the same carbon atom. The intense peaks of 913, 862 and 804 cm^{-1} were characteristic absorption bands of MFEO attributed to out-of-plane deformation vibration of =CH, while the bands at 1261, 1237, 1194 and 1131 cm^{-1} were associated with the stretching vibration of C-O-C. The IR spectra of β-CD (Fig. 3b) exhibited absorption bands at 3365 cm^{-1} for O-H stretching vibration, 2926 cm^{-1} for C-H stretching vibration, 1636 cm^{-1} for H-O-H bending vibration, while the bands at 1156 cm^{-1} and 1039 cm^{-1} for asymmetrical stretching vibration and symmetrical stretching vibration of C-O-C. The IR spectra of physical mixture (Fig. 3c) displayed a combination of the individual spectrum of MFEO and β-CD, although slight changes in their intensity could be observed. In contrast, it can be observed that the characteristic absorption peaks of the MFEO/β-CD inclusion complexes (Fig. 3d) are similar to β-CD. However, the spectrum of MFEO/β-CD inclusion complexes did not show any feature similar to MFEO when examining the spectra between MFEO/β-CD inclusion complexes and MEFO. These results confirmed that the inclusion complexes have been successfully formed.

2.4. *Chemical composition of essential oils*

The major compositions in the essential oil of *M. fragrans* were identified as in decreasing order: β-pinene (28.22 %), α-pinene (9.25 %), cis-sabinen hydrate (8.41 %), 4-carene (8.03 %), sabinene (7.47 %), isoelemicin (6.77 %), γ-asarone (3.69 %), 2-carene (2.12 %), and α-terpineol (1.22 %). And the essential oil which obtained by β-CD/MFEO inclusion complexes contained β-pinene (28.47 %), α-pinene (9.45 %), cis-sabinen hydrate (8.39 %), 4-carene (8.15 %), sabinene (7.24 %), isoelemicin (6.64 %), γ-asarone (3.82 %), 2-carene (2.07 %), and α-terpineol (1.18 %). There are no significant differences in compositions and contents between free volatile oil and β-CD/MFEO inclusion complexes. According to previous reports (Mahdavi et al. 2017; Villa-Ruano et al. 2018), α-terpineol , β-pinene and sabinene are responsible for the antibacterial and antifungal effects.

Solubility of the essential oil in water was determined to investigate the difference between the inclusion complexes and the free volatile oil. It was found that the solubility of the components in free essential oils displayed 0.06 mg/L of β-pinene, 0.08 mg/L of α-pinene, 0.11 mg/L of cis-sabinen hydrate, 0.09 mg/L of 4-carene, 0.14 mg/L of sabinene, 0.13 mg/L of isoelemicin, 0.16 mg/L of γ-asarone, 0.04 mg/L of 2-carene, and 27.2 mg/L of α-terpineol. However, inclusion complexes have higher solubility of 0.25 mg/L of β-pinene, 0.34 mg/L of α-pinene, 0.46 mg/L of cis-sabinen hydrate, 0.37 mg/L of 4-carene, 0.48 mg/L of sabinene, 0.46 mg/L of isoelemicin, 0.53 mg/L of γ-asarone, 0.14 mg/L of 2-carene, and 104.3 mg/L of α-terpineol.

2.5. *Antimicrobial activity*

The antimicrobial activity of MFEO/β-CD inclusion complexes against *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumoniae*, yeast *S. cerevisiae* and *B. subtilis* was tested; the results are displayed in Table 3. The minimum inhibitory concentration (MIC) was used as an indicator of antimicrobial activity. The MFEO/β-CD inclusion complexes were shown to be able to inhibit bacteria in experiments at low concentration, and the MIC values were significantly lower than free essential oil, which is probably due to its increased water solubility that led to increased contact area between the pathogens and essential oil, thus improving antimicrobial activity.

The effect of MFEO/β-CD inclusion complexes on Gram-positive bacteria (*S. aureus*, *S. epidermidis*) was better than against Gram-negative bacteria (*E. coli* , *K. pneumoniae*), which is in agreement with previous results (Smith-Palmer et al. 2002; Sun et al. 2014). It can be explained that the antimicrobial properties of MFEO are related to the lipophilic character, leading to accumulation in membranes and the subsequent consequent such as energy expenditure (Sikkema et al. 1995). However, Gram-negative bacteria possess an external membrane, which provides a hydrophilic surface, that inhibit the association of the lipophilic compounds of the volatile with the cell membrane (Andrade et al. 2017; Helander et al. 1998).

Table 3: Minimum inhibitory concentration (MIC) of free essential oil and MFEO/β-CD inclusion complexes against different species

	MIC (mg/ml)	
	Free essential oil	MFEO/β-CD inclusion complexes
S. aureus	0.56±0.19	0.39±0.12
S. epidermidis	0.36±0.12	0.29±0.10
E. coli	2.75±0.31	1.88±0.15
K. pneumoniae	0.91±0.26	0.67±0.21
S. cerevisiae	9.62±1.08	6.37±0.75
B. subtilis	60.48±7.94	42.66±5.88

Note: the MICs for the MFEO/β-CD inclusion complex was based on the weight of oil in the complex

Furthermore, MFEO/β-CD inclusion complexes showed inhibitory effects against the yeast *S. cerevisiae*, and *B. subtilis.* Previous studies have shown that the antimicrobial activity of essential oil is related to the disruption and penetration the lipid structure of cell membrane, and leading to the destruction of cell membrane, cell lysis, and eventually cell death.

3. Experimental

3.1. Materials

The seeds of *M. fragrans* were obtained from An-Guo herbal market, Hebei province, PR China in March 2016. A voucher specimen (No. 2016-03) was deposited in the Department of Pharmacy, Hebei North University. β-CD was purchased from Aladdin. All other reagents were analytical grade.

3.2. Extraction of essential oil from M. fragrans

The seeds of *M. fragrans* were dried in a hot air circulating oven at 40 °C for 48 h, pulverized and submitted to hydrodistillation using a Clevenger's apparatus. The crushed samples (100 g) were boiled with water (700 ml) for 6 h in a 1000 ml round bottom flask equipped with a volatile oil extractor to collect essential oil. The extracted essential oil was separated from the aqueous, dried over anhydrous Na_2SO_4 and stored in amber glass at 4 °C until analysis and preparation of inclusion complexes.

3.3. Preparation procedure

3.3.1. Preparation of MFEO/β-CD inclusion complexes

The inclusion complexes of β-CD and MFEO were prepared by the co-precipitation method. Briefly, β-CD (50 g) and 250 ml distilled water were added into a beaker equipped with a magnetic stirrer. After stirring for 2 h at 50 °C, the β-CD saturated solution was obtained. 10 ml MFEO-ethanol solution (1:1, V/V) was then slowly added to the β-CD saturated solution with continuous agitation for 2 h at the same temperature and stirring speed. The beaker was covered by cling film to minimize the oxidation by air. After cooling the solution to room temperature, the final mixtures were refrigerated at 4 °C for 24 h. The precipitated MFEO/β-CD inclusion complexes were recovered by vacuum filtration, washed with ether to remove the residual MFEO and dried in a baking oven at 40 °C for 24 h. The dried powder was stored in a desiccator at room temperature.

3.3.2. Orthogonal array design

On the basis of single factor experiments, an orthogonal array ($L_9(3^4)$) was designed in the present experimental procedure to discover the optimal conditions for obtaining the maximum inclusion rate. The design involved β-CD/MFEO ratio (g/ml) (A), inclusion time (B) and inclusion temperature (C) three factors at three levels.

3.4. Characterization of inclusion complexes

3.4.1. Entrapment efficiency

Entrapment efficiency of MFEO in the inclusion complexes was evaluated by steam distillation method. MFEO (1 ml, 886.2 mg) and 300 ml distilled water were added into a round bottom flask equipped with volatile oil extractor, and then heated and collected the essential oil until the volume kept constant. MFEO was weighed accurately (798.8 mg) after removal of residual water with anhydrous Na_2SO_4. Percent blank recovery was calculated according to Eq. (1). Similarly, the amount of MFEO in inclusion complexes was also extracted in this way, and the entrapment efficiency was calculated according to Eq. (2).

$$\text{Blank recovery (\%)} = \frac{\text{mass of recovered MFEO}}{} \times 100\% \qquad (1)$$

$$\text{Entrapment efficiency (\%)} = \frac{\text{mass of recovered MFEO in inclusion complexes}}{\text{mass of added MFEO in inclusion complexes} \times \text{blank recovery}} \times 100\% \qquad (2)$$

3.4.2. Differential scanning calorimetry

Thermal analysis was carried out using a Mettler differential scanning calorimeter, with a heating rate of 10 °C/min from 30 to 300 °C. Before analysis, the instrument was calibrated using zinc and indium metals. Samples of MFEO and β-CD physical mixture were weighted approximately 2 mg. An empty aluminum pan was used as reference sample.

3.4.3. Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectra of MFEO, β-CD, physical mixture and MFEO/β-CD inclusion complexes were obtained from 4000 to 400 cm^{-1} on a Nicrolet IS5 FT-IR spectrometer (Thermo Scientific, USA) with an average of 64 scans at 4 cm^{-1} resolution.

3.5. Quantification of constituents of essential oils

Gas chromatography coupled with mass spectrometry (GC/MS) was performed on a Shimadzu model GCMS-QP2010 Plus apparatus. The column was a RTX-5MS capillary column (30 m × 0.25 mm i.d., film sickness 0.25 μm), and flow rate of helium carrier gas was set at 1.2 mL/min. Operation conditions were as follows: injector temperature 250 °C, injection volume 2 μL. The oven program temperature was maintained at 40 °C for 2 min after injection and then increased at 3 °C/min to 210 °C. The mass detector electron ionization was 70 eV. Identification of compounds was carried out using mass spectra library search (NIST 14) and compared with spectra existing in the literature (Wiley 8 and FFNSC 1.2).

3.6. Antibacterial activity tests

The following standard bacterial strains, obtained from Huankai Microbial Sci. & Tech. Co., Ltd. (Guangdong, China), were used as test organisms in the antibacterial assays: *Staphyloccocus aureus*, *Staphyloccocus epidermidis*, *Escherichia coli* , *Klebsiella pneumoniae*, yeast *Saccharomyces cerevisiae* and *Bacillus subtilis*. Antibacterial properties of the MFEO/β-CD inclusion complexes and free essential oils were determined by the disc diffusion in agar method according to the National Committee of Clinical Laboratory Standard protocol. The essential oils and MFEO/β-CD inclusion complexes were diluted in dimethyl sulfoxide (DMSO) at different concentrations (0.5, 1.5, 2.5, 5.0, 10.0, 15.0, 25.0, and 50.0 %) and with DMSO control. The bacterial assays were added to vials containing tryptic soy agar (TSA) to a concentration of approximately 10^{-8} CFU/ml. After solidification, filter paper disks (5 mm diameter) containing a volume of 5 μL MFEO/β-CD inclusion complexes solution or free essential oil solution at proper concentration were applied on the surface of agar plates previously seeded with standardized test microorganism. Then, the plates were incubated under suitable conditions, and the diameters of inhibition growth zones were measured using a caliper rule. The plate of bacteria, yeast and mold were incubated at 35 °C for 24 h, 28 °C for 48 h, and 28 °C for 72 h, respectively. The experiment was repeated three times.

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Conflicts of interest: None declared.

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