Isolation, Antibacterial, Nematicidal and Anxiolytic Activities of Essential Oil from *Cinnamomum longepaniculatum* (Gamble) N. Chao ex H. W. Li Leaves

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Abstract: The utilization of natural product resources is significant for economic growth and health care. Herein, *Cinnamomum longepaniculatum* essential oil (CLEO) was isolated by microwave-assisted hydrodistillation (MAH). The composition of CLEO was determined by gas chromatography/mass spectrometry (GC/MS), and the antibacterial, nematicidal and anxiolytic activities of CLEO were evaluated. GC/MS results revealed that 33 compounds were identified, accounting for 99.87% of the total identified compounds. The major components of CLEO were monoterpenes, including β-myrcene (22.55%), eucalyptol (11.59%), α-pinene (11.56%), terpinen-4-ol (8.63%). Further research found that CLEO can inhibit the growth of *Staphylococcus aureus* (MIC 7.13 mg/mL, MBC 14.25 mg/mL), *Escherichia coli* (MIC 14.25 mg/mL, MBC 57 mg/mL), and *Pseudomonas aeruginosa* (MIC 14.25 mg/mL, MBC 28.50 mg/mL), the zone of inhibition were 17.49 ± 0.51 mm, 13.35 ± 0.27 mm and 15.15 ± 0.31 mm, respectively. CLEO could increase cell membrane permeability and make β-galactosidase, protein and other substances leak from the cell. Importantly, CLEO was first used to kill pine wood nematodes (*PWN, Bursaphelenchus xylophilus*). It was found that CLEO was toxic to *B. xylophilus* (LC₅₀ = 30.81 mg/mL), and PWN treated with CLEO rapidly died and extended. Furthermore, CLEO was used in anxiolytic for the first time. CLEO inhalation can effectively relieve anxiety-like behaviors in open-field test (OFT) and elevated plus-maze test (EPT), and restore neurotransmitters (5-HT, NE, GABA) in brain tissue to normal levels. This study provides a strategy for the industrial production of CLEO and discovers its potential role in food transportation and preservation, forest pest control, and relief of emotional disorders such...
as anxiety and stress, which are beneficial to health care and economic development and provides a basis for broader application of CLEO.

**Keywords:** Antibacterial, Anxiolytic, *Cinnamomum longepaniculatum*, Essential oil, Isolation, Nematicidal.

**Introduction**

*Cinnamomum longepaniculatum* (Gamble) N. Chao ex H. W. Li. is an endemic tree species in China, mainly distributed in Yibin, Sichuan Province. *C. longepaniculatum* is an evergreen tree, up to 20 m tall. Leaves alternate, petiole greenish red, leaf blade gray-green and opaque abaxially, dark green and shiny adaxially, ovate or elliptic, 6-12×3.5-6.5 cm, thinly leathery, glabrous on both surfaces. The total area of *C. longepaniculatum* in Yibin is 33000 hm², accounting for about 85% and 65% of Chinese and global *C. longepaniculatum* resources, respectively. It has become one of the main economic crops in Yibin. *C. longepaniculatum* has attracted researchers because its leaves are rich in essential oils. Moreover, the large amount of eucalyptol in *C. longepaniculatum* essential oil (CLEO) is an important raw material for the production of perfume, medicine, industrial consumer goods, and various chemical products. There are many methods for the isolation of CLEO, such as ultrasound-assisted extraction, enzyme-assisted extraction, microwave-assisted extraction, and solvent-free microwave-assisted extraction. Microwave-assisted hydrodistillation (MAH) has been widely used to isolate essential oils, which retains the eco-friendliness of the traditional method and uses the characteristics of microwave radiation to destroy cells rapidly and efficiently isolate essential oil. Considering efficiency, convenience, and environmental protection, MAH is a good choice for the isolation of CLEO.

Foodborne bacteria in fruits, vegetables, and meat pose a risk for humans to cause severe foodborne illnesses, which includes *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. However, the chemical preservative is marked as unhealthy due to possession of toxic, mutagenic, and carcinogenic properties. In combination, *Monochamus alternatus* and pine wood nematodes (PWN, *Bursaphelenchus xylophilus*) have been linked to widespread damage to pine forests in China. PWN spreads in pine trees through *M. alternatus*, causing pine diseases, leading to pine wilting, drying and death, and ultimately decay. This disease spreads rapidly and is hard to cure. At present, ecological benefits have been damaged by pesticides. Anxiety is the most common mental health disorder. The existing anxiolytic (such as chlordiazepoxide or diazepam) have side effects such as memory disturbance, dependence, and tolerance. Consequently, safe essential oils may provide significant benefits in the nursing of patients with anxiety.

So far, isolation of CLEO by a similar method using microwave and its chemical constituents have been reported, but the isolation mechanism is still unknown. Studies have shown that CLEO has antibacterial, anti-inflammatory and anticancer activities. But the antibacterial mechanism of CLEO remains largely unexplored. In addition, the chemical components of CLEO, such as eucalyptol, α-terpineol, and terpinen-4-ol, have been confirmed to have insecticidal or mood-regulating activities, which increases the application scope of CLEO. To the best of our knowledge, the nematicidal and anxiolytic activities of CLEO have not been reported. Therefore, the current main work is to isolate CLEO by MAH, analyze chemical components by GC/MS and explore the isolation mechanism, and evaluate the potential activity of CLEO on foodborne bacteria (*S. aureus*, *E. coli*, and *P. aeruginosa*), PWN (*B. xylophilus*) and anxiety mice, to make it valuable.

**Materials and methods**

**Materials, reagents and organisms**

*Cinnamomum longepaniculatum* leaves (10 kg) were collected from Yibin, Sichuan Province, China (Latitude: 27°50′ N, Longitude: 105°20′ E and Altitude: 399 m), and authenticated by Professor Kailin Mo (Sichuan Academy of Forestry Sciences, China). A voucher specimen...
has been deposited in the Herbarium of Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University (voucher No. CL-140804-021). Afterward, the leaves were dried in the shade (moisture content was 19.39%). Penicillin G, Ampicillin and \( n \)-alkane (C7-C40) were purchased by Shanghai Yuanye (Shanghai, China). Tryptic Soy Broth (TSB) and Tryptose Soya Agar (TSA) were procured by Beijing Aoboxing (Beijing, China). O-nitrophenyl-\( \beta \)-D-galactoside (ONPG), bovine serum albumin (BSA) were obtained from Shanghai Aladdin (Shanghai, China). 5-hydroxytryptophan (5-HT), norepinephrine (NE) and \( \gamma \)-aminobutyric acid (GABA) ELISA kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Diazepam was procured by Guizhou Guangzheng Pharmaceutical (Guizhou, China).

Staphylococcus aureus (CGMCC 1.2465), Escherichia coli (CGMCC 1.8723), and Pseudomonas aeruginosa (CGMCC 1.6723) were obtained from China General Microbiological Culture Collection Center (CGMCC, Beijing, China). Bacteria were used to evaluate the antibacterial activity of CLEO. Bursaphelenchus xylophilus (FSBx) were obtained from Liaoning Provincial Key Laboratory of dangerous forest pest management and control (Liaoning, China). B. xylophilus was reared on a lawn of Botrytis cinerea at 25°C in the dark. All live nematode experiments were completed in Liaoning Provincial Key Laboratory of dangerous forest pest management and control. Male Kunming mice (18-22 g) were housed in a sterile room maintained at a controlled temperature (22 ± 1°C) and humidity (50 ± 5%) with a 12 h light/dark cycle. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Harbin Medical University (approval No. HMUIRB-2008-06).

Microwave-assisted hydrodistillation (MAH)
CLEO was isolated from C. longepaniculatum leaves using MAH method. A microwave oven (P70F23P-G5(S0), Galanz, China, 23 L, 2.45 GHz) was perforated above (6 cm diameter) for MAH operation. Fifty grams of C. longepaniculatum leaf powder (10 mesh) was placed in a 1 L flask containing distilled water (500 mL). The flask was placed in a microwave oven (600 W for 10 min, 3 times) and the essential oils were collected using a condenser on top of the microwave oven. After that, the CLEO was collected and centrifuged at 3500 rpm for 10 min. After dehydration of anhydrous sodium sulfate, the mass was accurately weighed, recorded, and stored at 4°C after sealing. The yield was calculated on w/w basis. All experiments were performed in triplicate.

Optimization of isolation conditions

Single-factor test
We investigated the effects of the solid-liquid ratio (1:5 to 1:25, g: mL), extraction time (6 to 14 min) and extraction power (100 to 800 W) on the yield of essential oil. The experiment was performed three times.

Response surface methodology (RSM)
To obtain the optimal isolation conditions of CLEO, we used Design Expert 11 software to design the experiment. The Box-Behnken design is shown in Table S1. The yield of essential oil (\( Y \)) was used as the response value, and the solid-liquid ratio (\( A \)), extraction time (\( B \)) and extraction power (\( C \)) were used as the factors. To verify the accuracy of the Box-Behnken model, the CLEO was isolated by the optimal process. Each experiment was performed in triplicate.

Hydrodistillation
Hydrodistillation was used as a traditional method to obtain CLEO by a Clevenger apparatus. C. longepaniculatum leaf powder (10 mesh, 50 g) was placed in a flask and heated for 240 min by a heating jacket at 1 kWh, and the other procedures are the same as MAH method. The experiment was performed three times.

Gas chromatography/Mass spectrometry (GC/MS) analysis
GC/MS analysis of CLEO was performed by Agilent 7890A GC system with Agilent 7000B MS. The chromatographic conditions were as follows: HP-5MS column with 5%
phenyl methyl silox film (Agilent 19091S-433, 30 m × 0.25 mm × 0.25 μm), the injector and detector temperatures were 280°C and 180°C, respectively. Helium was the carrier gas (flow rate 1 mL/min) and an injection volume of 0.1 μL was employed (split ratio 20:1). Oven temperature program conditions were as follows: initial temperature of 50°C for 2 min, ramped at 3°C/min to 180°C, where it remained for 2 min, then ramped at 8°C/min to 240°C for 5 min, total run time 60 min. Mass spectra was taken at 70 eV, the scanned mass range was set at 50 to 620 m/z.

Each component was confirmed by National Institute Standard and Technology (NIST) database identification, literature searches, and manual analysis. The retention indices (RI) of a homologous series of \( n \)-alkanes (C7-C40) were determined under the same operating conditions. The concentration of each component content was calculated by the peak area normalization method 37.

**Antibacterial activity**

**Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)**

The MIC and MBC of CLEO against bacteria were determined by a broth dilution assay 38,39. Briefly, CLEO was prepared as 2-fold serial dilutions from 228 mg/mL to 1.78 mg/mL concentrations (with 1% DMSO) and loaded into a 96 well plate (100 μL/well). The resulting bacterial suspension was adjusted to a concentration of \( \sim 10^6 \) CFU/mL with TSB and placed into a 96 well plate (100 μL/well). Penicillin G-1, Ampicillin, and Penicillin G-2 were used as positive controls for *S. aureus*, *E. coli* and *P. aeruginosa*, respectively. Wells with TSB alone served as negative control. The bacteria were cultured at 37°C for 24 h. Then, the turbidity was determined using a microplate reader (Infinite 200 PRO NanoQuant, Switzerland) at 600 nm. No visible growth (OD value) was identified as the MIC of CLEO after 24 h. After that, MBC was carried out by inoculating bacterial inoculum on TSA plates by spread inoculation, incubated at 37°C for 24 h 40. Each assay was performed in triplicate.

**Aagar diffusion method**

The zone of inhibition of CLEO against three bacteria was determined by improving the agar diffusion method 41. The zone of inhibition refers to the bacterial clearance zone around the central well, and its diameter represents the bacteriostatic capacity. Bacterial inoculum (100 μL, \( 10^5 \) to \( 10^6 \) CFU/mL) were seeded on TSA. Then, sterile filter paper and Oxford cup were successively placed on the plate, and CLEO was added (91.20 mg) and cultured at 37°C for 24 h. In addition, Penicillin G-1 (1 μg), Ampicillin (5 μg), and Penicillin G-2 (40 μg) were used as positive controls for *S. aureus*, *E. coli* and *P. aeruginosa*, respectively. TSB was used as a negative control. The results were expressed by the zone of inhibition and measured in millimeters (mm). All antibacterial tests were replicated three times and average values were taken for record.

**Antibacterial kinetic**

The antibacterial kinetic of CLEO was assessed by measuring the growth curve 42. *S. aureus*, *E. coli* and *P. aeruginosa* were mixed with 2 MIC, MIC and MIC/2 of CLEO, respectively, and cultured at 37°C. The supernatant was collected at 2, 4, 6, 8, 10, 12 and 24 h, respectively. Then, the OD value was determined at 600 nm. Penicillin G-1 (0.0004 mg/mL), Ampicillin (0.04 mg/mL), and Penicillin G-2 (0.40 mg/mL) were used as positive controls for *S. aureus*, *E. coli* and *P. aeruginosa*, respectively. TSB was used as a negative control. The effect of CLEO on the bacterial growth curve was reflected by the bacterial concentration versus incubation time. Tests were performed in three replicates for each sample.

**Cell membrane integrity**

Inner membrane (IM) permeabilization assays: ONPG cleavage experiment was carried out to evaluate the effect of CLEO on bacterial inner membrane permeability. *S. aureus*, *E. coli*, *P. aeruginosa* were diluted to \( 1 \times 10^7 \) CFU/mL. Then CLEO (2 MIC, MIC and MIC/2) and ONPG (25 mM) were added. Penicillin G-1 (0.0004 mg/mL), Ampicillin (0.04 mg/mL), and Penicillin G-2 (0.40 mg/mL) were used as
positive controls for *S. aureus*, *E. coli* and *P. aeruginosa*, respectively. TSB was used as a negative control. Strains were incubated at 37°C and the absorbance (420 nm) was detected at 24 h. Each treatment was replicated three times.

Leakage of proteins: We used Bradford’s method to determine the protein content released in the supernatant. In brief, bacteria cultures in the logarithmic growth phase were treated with different concentrations of CLEO (2 MIC, MIC and MIC/2) at 37°C for 24 h. Subsequently, the supernatant was obtained after centrifugation at 5000 rpm for 5 min at 4°C and detected the protein content. Penicillin G-1 (0.0004 mg/mL), Ampicillin (0.04 mg/mL), and Penicillin G-2 (0.40 mg/mL) were used as positive controls for *S. aureus*, *E. coli* and *P. aeruginosa*, respectively. TSB and BSA were used as the negative control and the standard, respectively. Each experiment was repeated three times.

Nematicidal activity of CLEO

Since CLEO is not miscible with water and floats to form a capping layer, this leads to death of PWN due to lack of oxygen. To exclude the death of PWN in such cases, we prepared CLEO as *C. longepaniculatum* essential oil emulsion (CLEOE) dissolved in water. The emulsifier was prepared by mixing Span-80 and Tween-60 (HLB = 14). 1 g emulsifier was dissolved in 4.50 g distilled water, and 4.50 g CLEO was slowly added. Finally, milky white CLEOE was obtained by stirring at room temperature for 30 min and ultrasonic treatment for 10 min.

Nematocidal experiments were performed in 96-well culture plates as described previously. Briefly, PWN in a 96-well plate was adjusted to 50 nematodes/well, and 200 μL CLEOE (40% CLEO, 390 mg/mL), blank solvent (negative control, emulsifier at the same concentration as CLEOE) and sterile distilled water were added to the wells, respectively. The mortality of *B. xylophilus* was calculated after 24 h of dark treatment at room temperature. Mortality (%) = [(mortality percentage in treatment-mortality percentage in the negative control)/(100-mortality percentage in the negative control)]×100. According to the insecticidal effect, CLEOE was diluted step by step until the mortality of *B. xylophilus* was 0. The LC50 of CLEOE on *B. xylophilus* was calculated and verified. Each treatment was replicated three times and repeated twice.

Animotropic activity

**Anxiety model and treatment**

Chronic emotional stress anxiety model was established by uncertainty empty bottle stimulation for 21 days. Forty mice were trained to drink water from 09.00 h to 09.10 h and 21.00 h to 21.10 h by allowing them access to water bottles only during periods for seven days. After that, mice were irregularly given empty water bottles during one of the two watering periods for 14 days to induce emotional stress. The control group was given food and water ad libitum normally. Stress procedure were carried out according to Table 1.

Anxiety mice were grouped (n=8) and treated for 14 days. Grouping: model group, low-dose group (inhaled 0.1% CLEO), middle-dose group (inhaled 1% CLEO), high-dose group (inhaled 10% CLEO) and the positive control group (DZP, 1 mg/kg, i.p.). In addition, eight mice were randomly selected as the control group. The control group and the model group were treated with jojoba oil. All mice were treated for 30 min each day in a closed space.

**Behavioral tests**

Open-field test (OFT): The apparatus of OFT

| Day     | 1-7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---------|-----|---|---|----|----|----|----|----|----|----|----|----|----|----|----|
| Time    | 09.00 h to 09.10 h | N | EB | N | EB | N | N | EB | N | EB | N | N | EB | N | N |
|         | 21.00 h to 21.10 h | N | N | EB | N | EB | N | EB | N | EB | N | EB | N | EB | N |

EB, empty water bottle; N, normal drinking water at trained time

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was divided into peripheral and central zone (20 cm × 20 cm, length×width). Each animal was placed in the corner of the apparatus, and the motor activity was monitored for 5 min. The total distance traveled and the entry number to the central zone were measured with the video tracking system software (Shanghai Xinruan Information Technology Co., Ltd, China). After each trial, the apparatus was cleaned thoroughly with 70% ethanol. Each experiment was repeated three times.

Elevated plus-maze test (EPT): EPT takes advantage of the tendency of mice to darkness, enclosed space and fear of height or open spaces, which can be used as a classic model to evaluate rodents’ anxiety. The device of EPT consisted of two open arms (30 cm × 5 cm × 0.25 cm, length×width×height) and two closed arms (30 cm × 5 cm × 15 cm, length×width×height) with a central platform (5 cm × 5 cm, length×width) above the floor. The test consisted in placing a mouse in the center of the device (facing a closed arm) and allowing this mouse to explore the maze for 5 min. The percentage of duration in the open arms (OT%, duration in the open arms/5 min×100%) and entry number to the open arms (OE%, entry number to the open arms/entry total number to the open arms and close arms×100%) were parameters for anxiolytic behavior. The apparatus was cleaned thoroughly with 70% ethanol. Each experiment was repeated three times.

Detection of monoamine neurotransmitters in mouse brain
Neurotransmitters (5-HT, NE and GABA) levels were measured by Elisa kit. 10% brain tissue homogenate was prepared by liquid nitrogen grinding and centrifugation (2500 rpm for 10 min). All operations were carried out at between 0°C and 4°C. Each sample was repeated three times.

Statistical analysis
The data were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) test was used for statistical analysis. Statistical analyses were conducted using OriginPro 2021 software.

Results and discussion
Optimization of isolation conditions of CLEO by MAH
As shown in Fig. 1 a, b and c, the results showed that the yield of CLEO reached the maximum at 1 g: 10 mL (solid-liquid ratio), 10 min (extraction time) and 600 W (extraction power), respectively. According to the results of the single-factor test, 17 groups of experiments were generated by Design Expert 11 software. The experimental design and results are shown in Table S2. The quadratic regression Eq. (2):

\[Y = 4.26 + 0.07A + 0.375B + 0.4875C + 0.0275AB - 0.0225AC - 0.0975BC - 0.2918A^2 - 0.4268B^2 - 0.7568C^2\]

where \(Y\) is the yield of CLEO, %. \(A\) is solid-liquid ratio, g: mL. \(B\) is extraction time, min. \(C\) is extraction power, W.

According to ANOVA for the quadratic model (Table S3), \(B\), \(C\), \(A^2\), \(B^2\), \(C^2\) were significant model terms (\(p<0.05\)). Non-significant lack of fit was good. The predicted \(R^2\) was in reasonable agreement with the adjusted \(R^2\), which shows a linear relationship between the predicted value and the actual value (Fig. 1e). The coefficient of variation (C.V.) was less than 10%. Adeq precision was greater than 4. In summary, this model had high accuracy. The normal probability plot followed the straight line (Fig. 1d). Moreover, the residuals and the ascending predicted response values were random scatter, which test the assumption of constant variance (Fig. 1f). As shown in Fig. 1 g-l, the contour map between \(C\) and \(A\) was elliptical, and the 3D response surface was steep, with strong interaction. Similarly, there was a strong interaction between \(C\) and \(B\). The optimum isolation conditions were solid-liquid ratio 1 g: 11 mL, extraction time 11 min, extraction power 660 W. Under these conditions, the theoretical yield of CLEO was 4.41%. After verification (three repeats), the actual value of CLEO was 4.39 ± 0.09%, which was consistent with the predicted value, indicating that the MAH isolation process of CLEO was reasonable.

In the process, higher power can make the
Figure 1. Selection of CLEO isolation conditions using MAH. a-c, Single-factor experiment results; d-f, Diagnostics plots; g-l, Contour line map and 3D response surface diagram of the interaction between various factors.

Material heated from the inside absorb microwave radiation, generate pressure on the cell wall and break quickly, which is more conducive to obtaining CLEO. The yield reached the maximum when the extraction time and power reached 11 min and 660 W, respectively. It is
worth noting that too high power may lead to the cell wall’s rapid rupture, and some essential oil chemical components are decomposed to affect the yield \(^{51-53}\). In literature, CLEO was obtained by MAH concatenated double-column liquid-liquid extraction. The essential oils of the three separation columns were 29.04 ± 0.87, 2.03 ± 0.07 and 0.90 ± 0.03 g/kg, respectively, which effectively separated oxy-compounds. The safety and anesthesia of ether should be considered in the separation process, which increases the difficulty of separating essential oil \(^8\).

**High efficiency and low energy consumption of CLEO by MAH**

The efficiency and energy consumption were compared with those of the traditional method to evaluate the efficiency and environmental benefits of MAH (Table 2).

Here, the efficiency represents the isolation speed of CLEO, and the shorter the isolation time, the higher the efficiency when the yield is the same. The data revealed that the MAH had high efficiency and low energy consumption compared with the hydrodistillation. The yield by MAH for 11 min (4.39 ± 0.09%) was higher than that by hydrodistillation for 240 min (4.39 ± 0.18%).

To clarify the isolation process of CLEO, the equation for the kinetic study is based on the method described by Guo et al.\(^{54}\). According to the first-order kinetic model, initial conditions and boundary conditions \((Y_i = 0\) to \(Y_e\) at \(t = 0\) to \(t\)), the equation of the kinetic model is:

\[
Y_t = Y_e (1 - \exp^{-kt})
\]

where \(Y_t\) is the CLEO’s yield at any time, %; \(Y_e\) is the CLEO’s yield at equilibrium; \(t\) is the extraction time, min; \(k\) is the rate constant.

The results showed that the experimental data of hydrodistillation \((R^2 = 0.995)\) and MAH \((R^2 = 0.994)\) fitted well with the predicted values of the kinetic model, indicating that this model could better describe the kinetic process of extracting essential oil from C. longepaniculatum leaves (Table 3). Fig. 2a and Fig. 2b were the kinetic curves of extracting CLEO by hydrodistillation and MAH, respectively. The results illustrated that the yield of essential oil reached equilibrium at 11 min by MAH, while the yield reached equilibrium at about 120 min by hydrodistillation. MAH greatly shortened the extraction time of CLEO. In addition, the rate constant of the MAH kinetic curve was much larger than that of hydrodistillation (26.07 times). The high efficiency of MAH is further illustrated (Table 3).

Energy consumption \((E)\) was the multiplication of applied power \((P)\) and extraction time \((t)\) given by Eq. (4).

\[
E = P \times t
\]

where \(P\) (W) is the applied power, \(t\) is the extraction time (s), and energy \(E\) is expressed as (J).

**Table 2. Isolation yield and energy consumption**

| Isolation method          | Yield (%) | Energy consumption (J) |
|---------------------------|-----------|------------------------|
| Hydrodistillation (240 min, 450 W) | 4.36 ± 0.18 | 6.48 × 10^6          |
| MAH (11 min, 660 W)       | 4.39 ± 0.09 | 4.36 × 10^5          |
| MAH: microwave-assisted hydrodistillation |           |                       |

**Table 3. The rate constant and the yield of CLEO at equilibrium of hydrodistillation and MAH**

| Isolation method          | \(Y_e\) | \(k\) | \(R^2\) |
|---------------------------|---------|-------|--------|
| Hydrodistillation          | 4.54    | 0.02  | 0.995  |
| MAH                       | 4.57    | 0.49  | 0.994  |
| MAH: microwave-assisted hydrodistillation |         |       |        |

\(Y_e\) is the CLEO’s yield at equilibrium, \(k\) is the rate constant.
In terms of energy consumption, the energy consumption of MAH (660 W, 11 min, 4.36×10^5 J) was only 1/14.88 of that of hydrodistillation (450 W, 240 min, 6.48×10^6 J). Several reports indicate that MAH enables rapid heat generation inside the sample, destroying the cell structure, which may be the reason for its isolation of essential oils in a short time. In addition, MAH is more energy-saving, high-efficiency and eco-friendly than conventional hydrodistillation, with higher yield and volumetric mass transfer coefficient, a greater proportion of oxygenated compounds, lower electricity consumption, and lower CO₂ release and wastewater. These findings are consistent with our conclusions.

**Chemical component of CLEO**

The result (Table 4) showed chemical constituents of CLEO. Herein, total of 33 compounds were identified, accounting for 99.87% of the total compounds. The major components of CLEO were terpenes, including β-myrcene (22.55%), eucalyptol (11.59%), α-pinene (11.56%), terpinen-4-ol (8.63%). Similar results were detected in the literature, including eucalyptol, β-myrcene, α-terpineol, terpinen-4-ol. Monoterpene hydrocarbons accounted for 50.61%, whereas oxygenated monoterpenes, sesquiterpene hydrocarbons and oxygenated sesquiterpenes represented 33.26%, 12.28% and 2.14% of the total oil, respectively (Table 4).

**Antibacterial action of CLEO on S. aureus, E. coli and P. aeruginosa**

To find a safe alternative to chemical antibacterial agents, the antibacterial effect and mechanism of CLEO were studied. As shown in Table 5 and Fig. 3, CLEO showed a strong inhibitory effect on **S. aureus** (MIC = 7.13 mg/mL, MBC = 14.25 mg/mL, the inhibition zone diameter = 17.49 ± 0.51 mm). Interestingly, the MIC of CLEO on **E. coli** and **P. aeruginosa** was the same (14.25 mg/mL), but the MBC of **E. coli** (57 mg/mL) was higher than that of **P. aeruginosa** (28.50 mg/mL), indicating that the antibacterial effect of CLEO on **P. aeruginosa** was stronger than that of **E. coli**, which was consistent with the results of inhibition zone diameter (Table 5).

The action of CLEO was investigated against **S. aureus**, **E. coli** and **P. aeruginosa** using antibacterial kinetic and cell membrane integrity assays. At MIC/2, the growth of **S. aureus** and **E. coli** was inhibited within 10 h, after which the inhibition was reduced, but the growth of **S. aureus** was more affected than **E. coli**. However, the inhibition of **P. aeruginosa** growth by MIC/2 lasted for 12 h. Furthermore, the growth kinetics of the three bacteria seems to be stable at MIC, whereas at 2MIC they undergone complete bacterial inhibition. The results showed that CLEO had a significant effect on the growth kinetics of three foodborne bacteria (**S. aureus**, **E. coli** and **P. aeruginosa**) at MIC concentrations. This effect was detected even in the exponential phase, indicating the high bactericidal power of

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**Figure 2.** The kinetic curves of CLEO were obtained through hydrodistillation (a) and MAH (b)
Table 4. Chemical composition of CLEO by GC/MS

| No. | Components                        | RT<sup>a</sup> (min) | RI<sup>b</sup> | RI<sup>c</sup> | Concentration (%) |
|-----|-----------------------------------|-----------------------|---------------|---------------|-------------------|
| 1   | α-Thujene                         | 7.47                  | 925           | 925           | 2.83              |
| 2   | α-Pinene                          | 7.73                  | 932           | 936           | 11.56             |
| 3   | Camphene                          | 8.21                  | 945           | 951           | 0.41              |
| 4   | β-Myrcene                         | 9.33                  | 975           | 975           | 22.55             |
| 5   | β-Pinene                          | 9.39                  | 977           | 980           | 3.27              |
| 6   | α-Phellandrene                    | 9.95                  | 992           | 996           | 4.32              |
| 7   | 3-Carene                          | 10.43                 | 1004          | 1005          | 0.21              |
| 8   | α-Terpinene                       | 10.96                 | 1016          | 1017          | 0.25              |
| 9   | Eucalyptol                        | 11.74                 | 1034          | 1038          | 11.59             |
| 10  | β-Ocimene                         | 12.37                 | 1049          | 1050          | 0.09              |
| 11  | γ-Terpinene                       | 12.80                 | 1059          | 1060          | 4.07              |
| 12  | (+)-Isomenthol                    | 13.15                 | 1067          | -             | 1.84              |
| 13  | Terpinolene                       | 14.05                 | 1087          | 1088          | 1.05              |
| 14  | Linalool                          | 14.52                 | 1098          | 1098          | 0.88              |
| 15  | cis-β-Terpinol                    | 15.57                 | 1121          | 1121          | 0.53              |
| 16  | (E)-p-Menth-2-en-1-ol             | 16.43                 | 1140          | 1142          | 0.27              |
| 17  | L-α-Terpineol                     | 17.71                 | 1169          | 1172          | 4.33              |
| 18  | Terpinen-4-ol                     | 18.18                 | 1179          | 1177          | 8.63              |
| 19  | α-Terpinol                        | 19.04                 | 1198          | 1200          | 5.19              |
| 20  | α-Terpinal acetate                | 25.14                 | 1336          | 1341          | 1.59              |
| 21  | Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1α,2β,4β)]- | 27.49                | 1391          | 1391          | 0.32              |
| 22  | Caryophyllene                     | 28.62                 | 1418          | 1417          | 4.72              |
| 23  | γ-Elemene                         | 29.21                 | 1432          | 1431          | 0.14              |
| 24  | α-Guaiene                         | 29.38                 | 1436          | 1433          | 0.13              |
| 25  | Humulene                          | 29.99                 | 1452          | 1452          | 2.42              |
| 26  | Alloaromadendrene                 | 30.27                 | 1458          | 1459          | 0.27              |
| 27  | Germacrene D                     | 31.12                 | 1479          | 1480          | 0.46              |
| 28  | Bicyclogermacrene                | 31.78                 | 1495          | 1496          | 3.69              |
| 29  | δ-Cadinene                       | 32.84                 | 1522          | 1519          | 0.13              |
| 30  | Spathulenol                      | 34.99                 | 1578          | 1571          | 1.77              |
| 31  | Guaiol                            | 35.82                 | 159            | 1595          | 0.36              |

Monoterpene hydrocarbons (No. 1-8, 10, 11, 13) 50.61
Oxygenated monoterpenes (No. 9, 12, 14-19) 33.26
Sesquiterpene hydrocarbons (No. 21-29) 12.28
Oxygenated sesquiterpenes (No. 30, 31) 2.14
Total identified compounds 99.87

<sup>a</sup>Retention time; <sup>b</sup>Retention index on HP-5MS column determined by using C7-C40 alkanes; <sup>c</sup>Retention index from literature

CLEO. This conclusion is consistent with the findings of other studies. To evaluate the effect of CLEO on the cell wall and cell membrane of three bacteria (S. aureus, E. coli and P. aeruginosa), ONPG was used to monitor IM permeabilization. Meanwhile, leakage of proteins was measured. When the permeability of the cell wall and...
Table 5. The inhibitory effect of CLEO on *S. aureus*, *E. coli* and *P. aeruginosa*

| Strains               | MIC (mg/mL) | MBC (mg/mL) | Inhibition zone diameter (mm) |
|-----------------------|-------------|-------------|------------------------------|
| *S. aureus*           | 7.13        | 14.25       | 17.49 ± 0.51                 |
| Penicillin G-1        | 0.0002      | 0.008       | 15.37 ± 0.44                 |
| *E. coli*             | 14.25       | 57.00       | 13.35 ± 0.27                 |
| Ampicillin            | 0.02        | 0.06        | 19.09 ± 0.60                 |
| *P. aeruginosa*       | 14.25       | 28.50       | 15.15 ± 0.31                 |
| Penicillin G-2        | 0.20        | 0.40        | 13.45 ± 0.52                 |

Penicillin G-1, Ampicillin, and Penicillin G-2 were used as positive controls for *S. aureus*, *E. coli* and *P. aeruginosa*, respectively. For the inhibition zones, CLEO was added 91.2 mg, the positive controls were Penicillin G-1 (*S. aureus*, 1 μg), Ampicillin (*E. coli*, 5 μg) and Penicillin G-2 (*P. aeruginosa*, 40 μg), respectively. TSB was used as a negative control.

membrane increase, ONPG is hydrolyzed into yellow O-nitrophenol (ONP) by the leakage of β-galactosidase from the cytoplasm. In addition, cytoplasmic contents including proteins also leak. The result (Fig. 4) illustrated that the cell membrane integrity of three bacteria was affected by CLEO. For *S. aureus*, ONP absorbance increased with the increase of essential oil concentration (*p*<0.05), and MIC and 2 MIC showed significant and highly significant differences compared with the control (Fig. 4a, *p*<0.05, *p*<0.01). Similarly, the higher the concentration of CLEO, the greater the degree of protein leakage (Fig. 4b). In addition, CLEO also showed the same inhibitory effect on *E. coli* and *P. aeruginosa*. This indicated that CLEO might destroy the integrity of the bacterial cell membrane, and destroy its structure, resulting in the leakage of bacterial content. Notably, CLEO showed the most tremendous damage to the cell membrane of *S. aureus*.

The biological activity of essential oils is assessed by their main components 57. In this study, CLEO found interesting bacterial inhibition, with MIC and the zone of inhibition results allowing conclusions on inhibition and MBC results showing bactericidal action concentrations. The inhibition kinetics further validated the inhibition and bactericidal concentrations. In addition, the cell wall and membrane of bacteria were disrupted, which may be related to the antimicrobial effect of some chemical components in CLEO 58-60. On the other hand, CLEO, as a natural antimicrobial agent, can be developed as an antibacterial retardant for the transportation and preservation of food products 51,62.

**Nematicidal activity of CLEO**

Synthetic chemicals have negative ecological effects, and CLEO may prove to be an environmentally friendly alternative to inhibit nematodes. As can be seen in Fig. 5a, milky-white CLEOE contains 40% CLEO (390 mg/mL). To detect the insecticidal activity, nematodes were treated in CLEOE, blank solvent and distilled water for 24 h. The study found that CLEOE had insecticidal effect, and the mortality of nematodes was 100%. No nematodes died in blank solvent and distilled water. Further, diluted with distilled water, the concentrations of CLEO were 78 mg/mL, 39 mg/mL, 15.60 mg/mL and 3.90 mg/mL, respectively, and nematodes were treated for 24 h. The mortality was 53%, 56%, 48% and 0, respectively. According to the experimental data, the LC50 of CLEO was 30.81 mg/mL (Fig. 5b). Interestingly, after seven replicates, the mortality of nematodes treated with 78 mg/mL CLEOE was lower than that of nematodes treated with 39 mg/mL CLEOE, which may be due to the stress resistance of nematodes. In addition, the state of death nematodes was mostly stiff. Here, the nematodes killed usually exhibited an extended shape (Fig. 5c and 6d).

To the best of our knowledge, the nematicidal activity of CLEO has not been reported. Pine
Figure 3. The inhibition zones (a, b, c) and antibacterial kinetic curves (d, e, f) of CLEO against three bacteria. For the inhibition zones, CLEO was added 91.20 mg, the positive controls were Penicillin G-1 (a, S. aureus, 1 μg), Ampicillin (b, E. coli, 5 μg) and Penicillin G-2 (c, P. aeruginosa, 40 μg), respectively. TSB was used as a negative control. For antibacterial kinetic, the positive controls were Penicillin G-1 (d, S. aureus, 0.0004 mg/mL), Ampicillin (e, E. coli, 0.04 mg/mL) and Penicillin G-2 (f, P. aeruginosa, 0.40 mg/mL), respectively
Figure 4. Effect of CLEO treatment on *S. aureus*, *E. coli* and *P. aeruginosa* cell membrane integrity. 

a, Inner membrane permeabilization assays, b, Leakage of proteins. The positive controls were Penicillin G-1 (*S. aureus*, 0.0004 mg/mL), Ampicillin (*E. coli*, 0.04 mg/mL) and Penicillin G-2 (*P. aeruginosa*, 0.40 mg/mL), respectively. *p<0.05 and **p<0.01 compared with the control group.

Figure 5. Nematicidal activity of CLEO: (a) Nematicidal effect of CLEOE; (b) Nematicidal effect of CLEO at different concentrations; (c) Nematodes treated with distilled water; (d) Nematodes treated with CLEOE.

Wilt disease caused by PWN, is not only in China, but even threatens forests, tourism and the environment worldwide. CLEO can act as a potential natural insecticide by eliminating...
PWN or vectors. In addition, Jae Soon Kang et al. believed that the mechanism of essential oil against PWNs was related to the inhibition of *B. xylophilus* acetylcholinesterases (BxACEs), which is our next attempt in our research direction.

**Anxiolytic activity of CLEO**

After inhaling CLEO, behavior (OFT and EPT) and neurotransmitters (5-HT, NE and GABA) in the brain were affected (Fig. 6A). DZP reduces anxiety in two important rodent defense behavior assays: OFT and EPT. In OFT, DZP and CLEO treatment reduced anxiety-like behavior, including a significant decrease in the total distance traveled (*p*<0.01, Fig. 6Ba) and an increase entry number to the central zone, but especially 1% (*p*<0.01, Fig. 6Bb). In EPT, lower OT% and OE% were considered to reflect severe anxiety-like behavior. DZP and CLEO treatment showed higher OT% and OE%, but especially 1% (*p*<710.01, Fig. 6bc and 7bd), which alleviated the anxiety-like behavior of anxious mice. The study showed that inhaling CLEO alleviated anxiety-like behaviors in mice, consistent with previous findings.

Neurotransmitters in the brain, including 5-HT, NE and GABA, are associated with anxiety, and dysregulation of them can lead to a variety of psychiatric disorders, such as social phobia and anxiety. Studies have found that the increased concentrations of 5-HT and NE may be related to the occurrence of emotions such as anxiety and depression. As can be seen in Fig. 6Ca and 6Cb, DZP and CLEO treatment decreased the levels of 5-HT and NE in the brain of mice as compared with the model group. As can be seen in Fig. 6Ca and 6Cb, DZP and CLEO treatment decreased the levels of 5-HT and NE in the brain of mice as compared with the model group.

![Figure 6. Mice alleviate anxiety by inhaling CLEO (A) and its effects on behavior (B: OFT (a, b) and EPT (c, d)) and neurotransmitters (C: a, b and c) in the brain (n=8). *p*<0.05 and **p*<0.01 compared with the model group.](image-url)
CLEO treatment decreased the levels of 5-HT and NE in the brain of mice as compared with the model group. Especially 0.1%, the levels of 5-HT ($p<0.01$) and NE ($p<0.05$) in brain of mice were equivalent to DZP. Moreover, 1% showed a significant difference in 5-HT level and NE level compared with the model group ($p<0.05$). GABA is an important inhibitory neurotransmitter in the central nervous system. The decrease of GABA level in the brain can lead to depression, tension, anxiety, insomnia and other neurological problems. On the contrary, it can effectively treat anxiety. Inhalation of CLEO, especially 0.1% and 1%, resulted in a significant increase in GABA concentrations in the brain tissue of anxious mice and returned to normal levels ($p<0.05$, Fig. 6Cc). DZP treatment as a positive control significantly increased GABA levels in anxious mice ($p<0.01$).

Essential oils can cross the blood-brain barrier and enter the brain, relieving anxiety, depression, and mood disorders. The anxiolytic effects of CLEO may be closely related to its chemical composition. Studies have shown that eucalyptol is effectively reduces anxiety before the selective nerve root block (SNRB). In addition, inhaled eucalyptol can be used to relieve anxiety before, during, and after various operations. On the other hand, the application of CLEO can be expanded in daily life to relieve people’s tension and anxiety.

**Conclusions**

In this study, the CLEO in *C. longepaniculatum* leaves was isolated by MAH method and the main components of the essential oil were identified as β-myrcene (22.55%), eucalyptol (11.59%), α-pinene (11.56%). Moreover, the antibacterial, nematicidal and anxiolytic activities of CLEO were further confirmed, providing a basis for the development and application of *C. longepaniculatum* resources in cosmetics, medicine, food, forest pest control and public health.

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**Declaration of interest**

The authors declare there are no conflicts of interest.

**Data availability statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Credit author statement**

Yuanyuan Zhang: Investigation, Formal analysis, Data curation, Writing-original draft; Lin Zhang: Methodology, Investigation, Writing-review & editing; Yongbin Meng: Data curation, Software; Yuanang Zu: Project administration, Funding acquisition; Xiuhua Zhao: Supervision, Methodology, Conceptualization, Formal analysis, Writing-review & editing; Feng Han: Supervision, funding acquisition, Resources.

**Supplementary file**

Table S1-S3: RSM experimental data: Box-Behnken design, Box-Behnken experimental design and results and ANOVA for quadratic model.

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