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Revealing the composition and mechanism of the antibacterial activity of essential oil from the fruits of *Litsea mollis* Hemsl.

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Abstract

Essential oils (EOs) have received increasing attention due to their safety and effective antibacterial activity. The chemical components and antibacterial activity of *Litsea mollis* Hemsl. essential oil no. 2 (LMEO2) was investigated in the present work. GC-MS analysis was performed, and 24 bioactive compounds were detected with citral being the most predominant (46.1482%). LMEO2 had high antibacterial activity against the foodborne pathogens *Escherichia coli* O157 and *Salmonella* Enteritidis. The minimum inhibitory concentration (MIC) was 0.01% for *E. coli* O157, and 0.05% for *Salmonella* Enteritidis, and the minimum bactericidal concentration (MBC) was 0.05% for *E. coli* O157, and 0.1% for *Salmonella* Enteritidis. Furthermore, the alkaline phosphatase (AKP) leak assays revealed that LMEO2 destroyed the integrity of cell wall. This was consistent with the nucleic acid leakage assays which revealed that LMEO2 enhanced the membrane permeability. Meanwhile, the scanning electron microscopy (SEM) and transmission electron microscopy (TEM) assays also demonstrated that LMEO2 could damage the cell wall integrity, thus leading to cellular content leakage. The main bioactive component of LMEO2 was citral. LMEO2 could be a promising plant-derived antibacterial agent.

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Introduction

Foodborne diseases caused by foodborne pathogens threaten human health, and incur heavy economic burden in the food industry as well as healthcare system (Ye *et al*., 2013; Lee and Je, 2013). The use of chemical preservatives to combat food poisoning has attracted huge attention over the last 30 years (Tian *et al*., 2014), but synthetic chemical preservatives can potentially cause harmful side effects, and lead to serious health problems (Naufalin, 2019). To mitigate this, natural preservatives have emerged as an attractive alternative (Goñi *et al*., 2009). Natural preservatives in foods are also urgently required due to emerging antibiotic resistance. To this end, essential oils (EOs) that are extracted from plants, and characterised by their fragrance have emerged as suitable candidates (Lila *et al*., 2015).

EOs have many physiological and biochemical properties including antibacterial, antioxidant, and antifungal activity (Hu *et al*., 2017). EOs are generally recognised as safe (GRAS), and have been touted as new candidates to replace traditional preservatives (Doughari *et al*., 2012). *Litsea mollis* Hemsl. belongs to the genus Lauraceae, and is distributed in the mountain areas of the Yangtze River in China. *Litsea mollis* essential oil (LMEO) is effective in the treatment of malaria, gastroenteritis, rheumatic pain, and hip injury (Sun *et al*., 2010). In a previous study (Yang *et al*., 2014), it has been demonstrated that LMEO consisted of methyl-anthranilate, citronellol, and eugenol. However, detailed information regarding its composition and

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mechanistic information on its antibacterial activity are lacking.

In the present work, the chemical components of *L. mollis* EO no. 2 (LMEO2) were analysed through GC-MS. The antibacterial activity of LMEO2 against foodborne pathogens including *Escherichia coli* O157 and *Salmonella* Enteritidis were then characterised, and the mechanism of the antibacterial activity were revealed. Citral was the major bioactive compound of LMEO2. To the best of our knowledge, the present work served as the first to fully define the antibacterial mechanisms of LMEO2.

Materials and methods

Plant material and bacterial strains

Fruits of *L. mollis* were collected in summer 2018, in Wanzhou City, Chongqing Province, China, and identified by Prof. Bingyang Ding. *Escherichia coli* O157, *Escherichia coli* O104, *Escherichia coli* DH5α, *Salmonella* Enteritidis, *Listeria monocytogenes* J4045, *Listeria monocytogenes* Clip 11262, *Enterococcus faecalis* BM13, *Staphylococcus aureus* 204, and *Pseudomonas aeruginosa* 204 were provided by the Key Laboratory for Food Microbial Technology of Zhejiang Province (original bacterial strains were obtained from patients).

Essential oil extraction

Fresh samples of *L. mollis* fruits were grounded, meshed, and hydro-distilled for 5 h using a Clevenger-type apparatus. Extracted water-oil mixtures were separated into two distinct layers at 4°C. The EO supernatant was collected and sealed at 4°C in dark vials.

GC-MS analysis

LMEO2 was analysed through gas chromatography (TRACE GC Ultra) coupled with mass spectrometry (Agilent 5975B, Santa Clara, CA, USA). The GC was equipped with an Rtx-5 MS column (60 m long, 0.25 μm thick, and 0.25 mm inner diameter). Carrier gas was helium at 1.5 mL/min flow rate, and 1:10 split ratio. Samples were injected at 220°C with a split ratio of 1/40 over a 1-min interval. The column was maintained at 50°C for 2 min, increased at a rate of 3°C/min to 120°C for 2 min, and topping out at 250°C for 5 min. MS operating parameters were ionisation voltage of 70 eV, ion source temperature of 230°C, and electron multiplier energy of 1024 V. Retention times and mass spectra were used to identify LMEO2 components.

Determination of inhibition zone diameter (DIZ)

LMEO2 antibacterial activity was measured via DIZ experiments. Suspensions of nine bacterial strains which were mentioned earlier (100 μL, 1×10^7 CFU/mL) were cultured overnight, mixed with 10 mL of LB semisolid medium, and spread onto solid LB medium plate. Oxford cups containing 50 μL of LMEO2 were placed onto the plate surface, and incubated at 37°C for 24 h. DIZ was assessed using a Vernier calliper. Based on a previous study, DIZ values were classified as followings: zone diameters ≤ 8 mm, not sensitive (-); zone diameters between 8 - 14 mm, sensitive (+); and zone diameters between 14 - 20 mm, very sensitive (++) (Ponce *et al*., 2003).

Determination of minimal inhibitory concentration (MIC) and minimum bacterial concentration (MBC)

MIC and MBC were estimated following a previous study (Silva *et al*., 2011) with minor alterations. LMEO2 and citral was diluted to a range of concentrations in ethanol (0.01 to 100%), and 1 μL of each LMEO2 and citral concentration was assessed for its antibacterial activity through its addition to bacterial suspensions (1×10^7 CFU/mL) in LB media at 37°C for 24 h. The bacterial strains used in this assay were *Salmonella* Enteritidis, *E. coli* O157, and *E. coli* DH5α as they were the representative strains. MIC was assessed through the OD_{600}. 1 μL of ethanol served as solvent control. The lowest concentration of LMEO2 and citral without visible bacterial growth was defined as the MIC. Suspensions in concentrations above MIC were subcultured at 37°C for 24 h. MBC was defined as the lowest concentration at which a complete loss of bacterial colony growth occurred.

Time-kill assessments

Salmonella Enteritidis and *E. coli* O157 were selected to be further evaluated as representative strains. Time-kill assays were performed as described by Zhou *et al*. (2016). Bacterial suspensions were mixed with LMEO2 and citral at MIC and 2 × MIC in 96-well plates, and incubated at 37°C for 24 h. During incubation, samples were collected every 4 h. Each sample was spread onto LB agar medium, and incubated at 37°C for 24 h. Bacterial growth was expressed as log CFU/mL.

Alkaline phosphatase leakage assay

Cell wall damage was assessed by the alkaline phosphatase assay. Bacteria in the exponential growth phase were separated by centrifugation at 6,000 *g* for 8 min, and the supernatant discarded. The collected bacterial pellets were washed and resuspended in PBS (pH = 7.4) for three times. The suspensions were treated with LMEO2 and citral (control, at concentration of MIC and MBC), and incubated at 37°C for 4 h. AKP leakage was performed using the AKP kit (Jiancheng, China). Samples were detected on a microplate reader (Molecular Devices, SPECTRA MAX 190).

Relative electrical conductivity assay

Cell membrane permeability was assessed by the relative electrical conductivity assay (Diao *et al*., 2014) using Eq. 1:

$$REC\ (\%) = [(L2 - L1) / L0] \times 100 \quad \text{(Eq. 1)}$$

where, L0 = electrical conductivity of bacteria in 5% glucose that was heated in boiling water for 5 min as a control; L1 = conductivity of bacteria in 5% glucose exposed to MIC and MBC concentrations of LMEO2 and citral; while L2 = electric conductivity of samples following incubation for 12 h.

Nucleic acid assay

Nucleic acid assay was performed to investigate the integrity of cell membrane (Lv *et al*., 2011). Bacteria were washed in triplicate and resuspended in PBS following centrifugation at 6,000 *g* for 8 min. Collected cells were exposed to varying dilutions of LMEO2 and citral (at the concentration of MIC, MBC, and ethanol controls). Cells were incubated at 37°C, and the OD measured at 260 nm every 4 h.

Propidium iodide assay

PI assays were performed to detect cell membrane integrity (Novo *et al*., 2000) with minor modifications. Briefly, cells in exponential growth phase were resuspended in PBS in triplicate, and incubated in control, LMEO2, and citral (at concentration of MIC and MBC) for 4 h. Suspensions were maintained in the dark, and labelled with PI for 30 min. Fluorescent data were obtained using fluorescent microscopy (Leica DMI4000B, LC Ltd., Germany).

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Bacterial cells in exponential growth phase were treated with LMEO2 and citral (control, at concentration of MBC) overnight, and fixed in 2.5% glutaraldehyde at 4°C. Cells were washed three times in PBS, and dehydrated in a series of ethanol and absolute ethanol for 20 min. Samples were dehydrated, coated with platinum, and observed via SEM (Hitachi TM1000, HHTC Ltd., Japan). The samples for TEM were prepared as SEM. After absolute ethanol treatment, the samples were treated with embedding agent (spur) and acetone overnight at 70°C. Slices (70 - 90 nm) were obtained by ultrathin sectioning, and stained for 5 - 10 min. The stained slices were observed under TEM (Hitachi H-765, HHTC Ltd., Japan).

Statistical analysis

All experiments were performed in triplicate. Data were analysed using SPSS20. Statistical significance was determined when the *p*-value was less than 0.05.

Results and discussion

Essential oil characterisation

A total of 24 bioactive compounds accounting for 96.54% of the total LMEO2 were identified including citral, *cis*-citral, geraniol alcohol, linalool, and nerol, amongst others. LMEO2 were characterised by a significant predominance of citral (46.20%) and *cis*-citral (36.94%). This agreed with a previous study (Yang *et al*., 2014) which demonstrated that citral was the highest in LMEO2. The content of citral and *cis*-citral were slightly higher than that in the previous studies (27.49 and 23.57%, respectively). This discrepancy could have been due to different environmental conditions, sources, and planting regions of the samples (Zhan *et al*., 2016).

Antibacterial activity

The results of DIZ are shown in Table 1. The DIZ values of LMEO2 and citral varied from 8 to 14.9 mm, thus indicating that LMEO2 and citral exhibited strong antibacterial activity against Gram-negative bacteria. No activity of LMEO2 and citral against Gram-positive bacteria (*E. faecalis* BM13, *L. monocytogenes* J4045, *L. monocytogenes* Clip 11262,

and *S. aureus* 204) and one Gram-negative bacterium (*P. aeruginosa* 204) was observed. It can be concluded that Gram-positive bacteria were more resistant to LMEO2, which differed from previous studies. It is generally believed that Gram-negative cells should be more resistant to plant EOs because they possess a hydrophilic cell wall that can prevent the penetration of hydrophobic compounds of EOs. However, Burt found that carvacrol and thymol were able to cause disintegration of the outer membrane of Gram-negative bacteria, thus releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane (Calo *et al.*, 2015). This might be the mechanism of the results of LMEO2 against the tested bacterial strains observed in the present work. LMEO2 has also been used as a natural antibacterial agent due to its significant antibacterial activity (Eryiğit *et al*., 2014).

MIC is defined as the minimal concentration that inhibits cell growth, whilst MBC is defined as the minimal concentration that exerts bactericidal activity (Zhan *et al*., 2016). The MIC and MBC results are shown in Table 2, and *E. coli* O157 was most sensitive to LMEO2 and citral.

Based on these results, both of LMEO2 and citral demonstrated similar antibacterial activity against three strains of the tested bacteria. LMEO2 and citral showed different MIC and MBC to different bacterial strains. The chemical composition of LMEO2, its solubility in water, and the specific bacterial structures might have caused the discrepancy.

Table 1. Diameter of the inhibition zones following LMEO2 and citral treatments.

Strain	Sensitivity	
	LMEO2	Citral
Salmonella Enteritidis	++	++
E. coli O157	++	++
E. coli DH5α	+	+
E. coli O104	+	+
E. faecalis BM13	-	-
L. monocytogenes J4045	-	-
L. monocytogenes Clip 11262	-	-
S. aureus 204	-	-
P. aeruginosa 204	-	-

Table 2. The MIC and MBC of LMEO2 and citral against the tested bacteria.

Strain	MIC (%)		MBC (%)	
	LMEO2	Citral	LMEO2	Citral
Salmonella Enteritidis	0.05	0.05	0.1	0.1
E. coli O157	0.01	0.01	0.05	0.05
E. coli DH5α	0.01	0.05	0.1	0.1

MIC = minimum inhibitory concentration, MBC = minimum bactericidal concentration.

Time-kill assessments

E. coli O157 and *Salmonella* Enteritidis were selected for further experimentation. The effect of LMEO2 and citral on the growth of the bacterial are represented in Figure 1. The number of viable bacteria under the treatment of LMEO2 and citral at MIC decreased slightly in the first 4 h, and then stabilised. When compared with treatments at control and MIC, the loss of viability of *E. coli* O157 and *Salmonella* Enteritidis occurred most significantly at 2 × MIC. The number of viable cells decreased sharply in the first 12 h under the treatment of 2 × MIC, and then stabilised.

AKP leakage

AKP will not be detected unless the cell wall has been destroyed. Therefore, the leakage of AKP was used to detect the integrity of the cell wall in the

present work. The concentration of AKP in bacterial suspensions increased after treatment with LMEO2 and citral (Figure 2). Furthermore, when compared with treating samples with LMEO2 and citral, which are at MIC and control concentration, AKP increased most significantly with the treatment of LMEO2 and citral at MBC concentration. Therefore, the results demonstrated that LMEO2 and citral could act on bacterial cell walls by destroying bacterial structure, thus leading to the leakage of pericytoplasmic proteins.

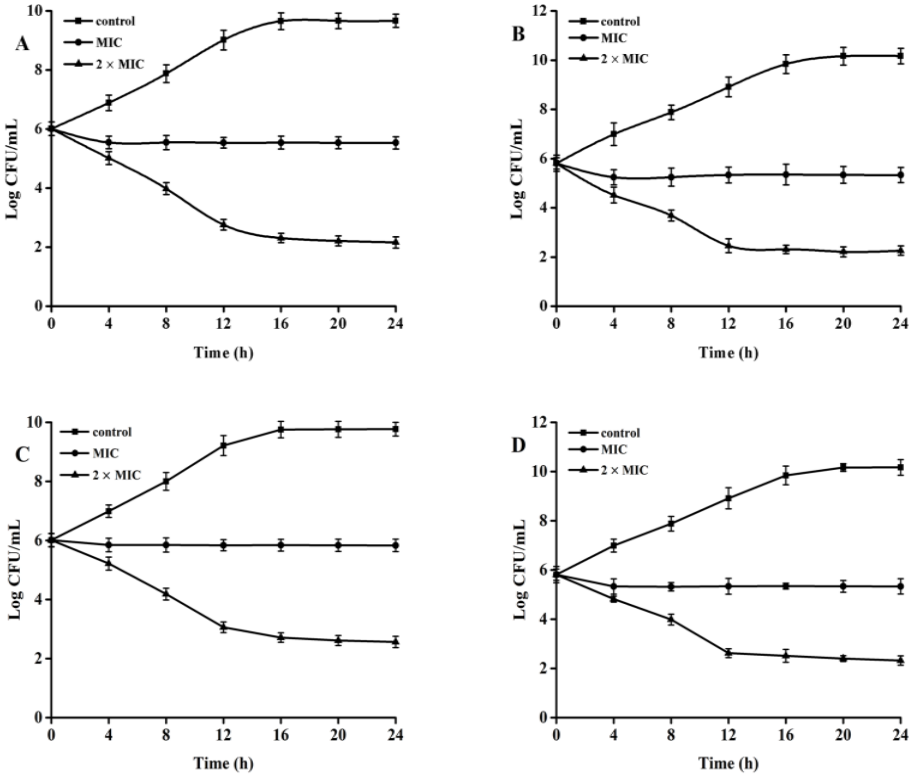

Figure 1. Effects of LMEO2 on the viability of *E. coli* O157 **(A)** and *Salmonella* Enteritidis **(B)**, and effects of citral on the viability of *E. coli* O157 **(C)** and *Salmonella* Enteritidis **(D)**. Error bars indicate the standard error of the mean.

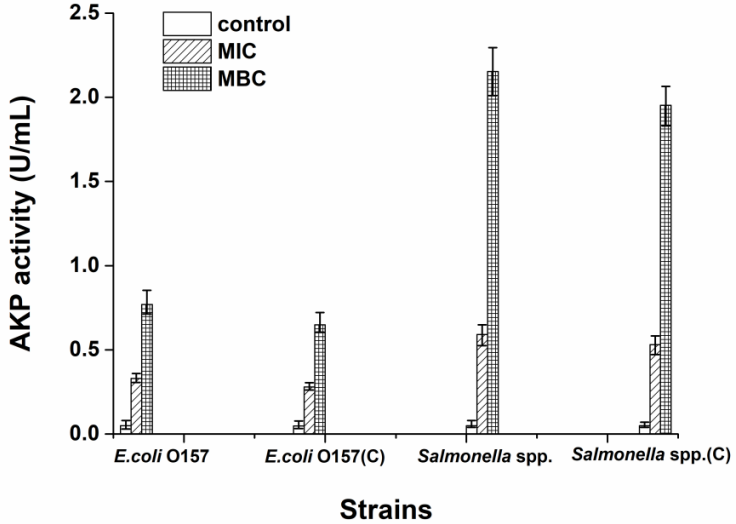

Figure 2. AKP leakage from *E. coli* O157 and *Salmonella* Enteritidis treated with different concentrations of LMEO2 and citral (represented by '(C)'). Data are mean of three replicates (± standard error). *p*-values of less than 5% ($p < 0.05$) were considered to be significant.

Cell membrane permeability

Relative electrical conductivity

The leakage of small electrolytes can lead to a rapid increase in relative electric conductivity (REC). Therefore, REC assays were performed to assess the changes in *E. coli* O157 and *Salmonella* Enteritidis membrane permeability. REC values increased with the change of LMEO2 and citral concentration. The increase in REC under the treatment of LMEO2 and citral was most obvious in the first 2 h. These results indicated that LMEO2 and citral could destroy the integrity of bacterial cell membranes, thus increasing the permeability. Furthermore, LMEO2 and citral destroyed the cell structure. This was consistent with the fact that minor perturbations to bacterial cells can detrimentally influence cell metabolism, thus resulting in bacterial death (Sharma *et al*., 2013).

Nucleic acid leakage

When cell membrane is destroyed, macromolecules, including nucleic acids, are released (Chen and Cooper, 2002). When treated with LMEO2 and citral (MIC and MBC), this led to a significant increase in OD_{260} of samples within 4 h. And after that, the values increased at a very slow rate. In comparison to control cells, the OD_{260} values of bacteria exposed to MBC were much higher. These results could have been due to cell membrane rupture and nucleic acid release in the presence of LMEO2, and the bacterial treated with citral exhibited slightly lower leakage degree when compared with LMEO2.

PI staining

PI is commonly used to evaluate the integrity of cell membranes due to its ability to stain DNA of dead cells. When compared to control cells which produced only background levels of red fluorescence (Figure 3B), bacteria treated with LMEO2 at MIC and MBC (Figure 3C and 3D) produced intense red PI staining. Therefore, the treatment of MIC LMEO2 led to the death of bacteria. Figures 3E and 3F revealed that the treatment of citral also led to the death of bacteria, and this might have been caused by the damage of cell walls.

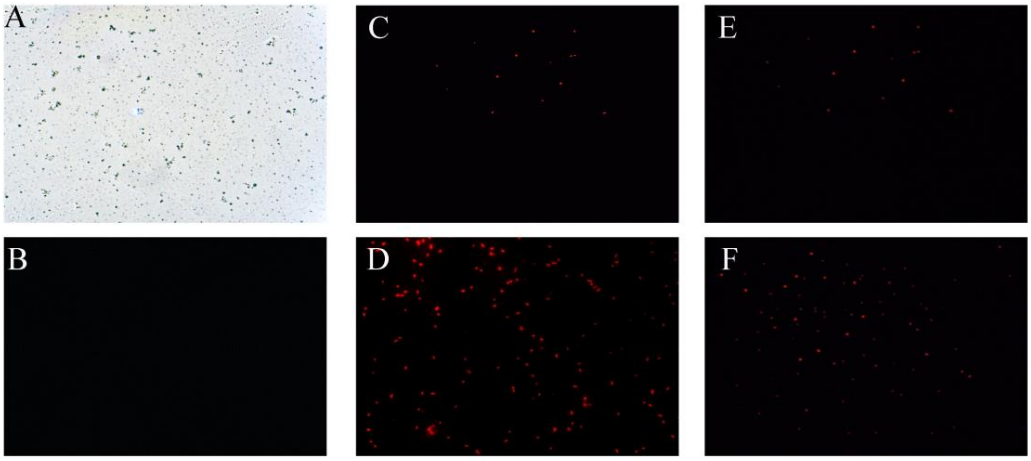

Figure 3. Fluorescent microscopy images of *E. coli* O157 treated with varying LMEO2 concentrations (control, MIC, and MBC). **(A)** Untreated bacteria (control) observed under an optical microscope; **(B)** control bacteria observed under a fluorescence microscope; **(C)** and **(D)** bacteria treated with LMEO2 (MIC and MBC, respectively) observed under a fluorescence microscope; **(E)** and **(F)** bacteria treated with citral (MIC and MBC, respectively) observed under a fluorescence microscope.

SEM and TEM

SEM was used to evaluate the morphological changes in treated bacteria. Both LMEO2 and citral led to severe adverse effects on the morphology of *E. coli* O157 and *Salmonella* Enteritidis. Control cells displayed characteristic morphologies. In contrast, cells treated with LMEO2 and citral at MIC possessed ruptured and rough surface membranes, and cell damage was visible, in which, cell leakage of the intracellular contents could be observed. These results were consistent with the ability of LMEO2 and citral to induce cell membrane permeability (Bajpai *et al*., 2009). TEM was performed to confirm the morphological alterations of LMEO2- and citral-treated cells. Untreated cells displayed regular rod-shaped structures, with consistent and intact cell

surfaces. In contrast, cells exposed to LMEO2 and citral MIC were deformed, and the cell walls were destroyed. Additionally, membrane boundaries were damaged, and the cells exhibited rough appearance. These data confirmed that the mechanism of action of LMEO2 and citral against pathogenic bacteria was the disruption of bacterial membrane integrity (Chauhan and Kang, 2014).

Conclusion

Food safety is a major global issue, and EOs have emerged as promising natural food preservatives. The present work revealed that LMEO2 was bactericidal to *E. coli* O157 and *Salmonella* Enteritidis through the cell wall and membrane disruption, as assessed through AKP, DNA, and RNA leakage assays, and confirmed through SEM and TEM imaging. The present work also proved that citral was the main bioactive component of LMEO2, and proposed that LMEO2 could be a promising candidate for a natural antibacterial food preservative that warrants future investigation for its benefits to food safety.

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