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RESEARCH

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Essential Oils and their Active Constituents Effective against Non-growing *Mycobacterium intracellulare*

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Abstract

Background *Mycobacterium intracellulare* (*M. intracellulare*) is a common, slow-growing opportunistic pathogen that can cause chronic pulmonary and extrapulmonary infections. Despite its rising incidence, standard treatments are often ineffective in eradicating *M. intracellulare*, leading to prolonged treatment and high recurrence rates, likely due to persistence of non-growing bacteria. Although essential oils are known for their antimicrobial properties, their effects on *M. intracellulare*, particularly in its non-growing phase, have not been well studied.

Methods We screened 151 essential oils to assess their antimicrobial activity against stationary-phase non-growing *M. intracellulare*. Essential oils with significant activity were further evaluated at different concentrations by MIC and drug exposure tests.

Results Thirty-four essential oils were found to have activity at 5000 μg/mL, with 18 showing effectiveness at 1250 μg/mL. Six essential oils, Ajwain, Oregano, Palmarosa, Thyme, Mountain Savory, and Litsea Cubeba had the highest activity, achieving 100% bacterial clearance after one day exposure. Carvacrol, the key active component of Ajwain, Oregano, Thyme, Mountain Savory, eradicated stationary-phase bacteria at 310 μg/mL concentration within one day, while citronellol, the active component of Palmarosa, at 630 μg/mL achieved complete clearance after three day exposure.

Conclusions We have newly identified several essential oils, including Ajwain, Oregano, Thyme, Mountain Savory, Palmarosa, and Litsea Cubeba and their active components such as carvacrol and citronellol, to have promising activity against *M. intracellulare,* and these findings may have implications for developing improved treatments for *M. intracellulare* infections.

Keywords *Mycobacterium intracellulare*, Stationary phase, Essential oils, Antimicrobial activity, Carvacrol, Citronellol

Introduction

M. intracellulare is a slow-growing, non-tuberculous mycobacterium (NTM) that, along with *M. avium*, forms the *Mycobacterium avium* complex (MAC). These bacteria are commonly found in soil and water sources, with frequent exposure occurring through daily activities such as using showerheads [1]. MAC is the most common pathogen among NTM species [2], primarily causing chronic lung disease in humans, it can also lead to extrapulmonary diseases such as lymphadenitis in children and disseminated MAC disease in

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immunocompromised individuals [3], with an increasing incidence worldwide [4]. In MAC-related pulmonary disease (MAC-PD), *M. intracellulare* is the main pathogen in mainland China [5]. According to the 2020 ATS/ERS/ESCMID/IDSA Clinical Practice Guideline [6], the current treatment regimen for MAC-PD patients is a three-drug regimen including a macrolide, namely clarithromycin or azithromycin combined with rifampicin and ethambutol. MAC pulmonary disease patients who are macrolide-sensitive receive treatment for at least 12 months after culture conversion. The prolonged treatment is due to the presence of non-growing bacterial persisters, against which current drugs are not very effective. For patients with cavitary or advanced/severe bronchiectasis or macrolide-resistant MAC lung disease, parenteral amikacin or streptomycin is recommended in the initial treatment regimen. However, after clinical use of the recommended standard regimen, the culture conversion rate is unsatisfactory, between 45 and 70%, and the recurrence rate can be as high as 60% [1]. Moreover, the resistance rate to macrolides can reach 9.2%−12% [7]. The treatment of *M. intracellulare* is particularly challenging due to their inherent resistance mechanisms, such as waxy cell wall, efflux or acquired antibiotic resistance. While susceptibility to macrolides and amikacin shows good correlation with clinical outcomes, susceptibility to rifampin and ethambutol do not demonstrate a strong correlation [1]. Although new drugs, such as bedaquiline and repurposed clofazimine, have shown promising results in some clinical studies [8], their efficacy and the combined effects with other antibiotics remain to be developed and validated. Currently, the optimal treatment regimen for patients with different conditions has not been established, and there is a lack of effective medications [9]. Existing drugs are prone to resistance, and relapse is common with prolonged treatment. Therefore, identifying new drugs with good activity against *M. intracellulare* is crucial for developing better treatment options.

Natural products from nature can be classified into plant-based (such as herbal medicines and essential oils), animal-based, microorganism-based, and mineral-based according to the sources. These natural products play important roles in various medical fields. For example, aspirin [10] derived from willow bark has anti-inflammatory, antipyretic, and heart disease-preventing effects. Paclitaxel [11], derived from Taxus tree bark, plays a significant role in the treatment of breast cancers. Allicin [12], derived from garlic, has antibacterial and antiviral properties. These natural products not only provide valuable treatments but also inspire further exploration of new therapeutic agents.

Essential oils (EOs) are mixtures of aromatic compounds extracted from plants, typically including terpenes, aldehydes, ketones, alcohols, and esters. Traditionally, they are used not only in perfumery, cosmetics and food preparation, but also widely applied in anti-inflammatory, antioxidant, and antibacterial treatments [13]. Numerous studies have shown that various active components within EOs exhibit antibacterial activity against a range of Gram-positive and Gram-negative bacteria, as well as fungi, including methicillin-resistant *Staphylococcus aureus* [14], *Streptococcus mutans* [15], *Salmonella enterica* [16], *Escherichia coli* [17], *Candida albicans* [18] and *Borrelia burgdorferi* [19]. For mycobacteria, most studies on EOs have focused on *M. tuberculosis* [20] and rapidly growing non-tuberculous mycobacteria, such as *M. abscessus* [21]. However, there has been limited research on the activity of EOs against *M. intracellulare*. Currently, only *Juniperus communis*, and *Helichrysum italicum* (Immortelle) have been reported to inhibit the adhesion and biofilm formation of mycobacteria [22, 23]. However, so far, no study on activity of EOs against non-growing mycobacteria, which are thought to be underlying the prolonged treatment and post-treatment relapse, has been reported. Like previous studies [24, 25] that used stationary-phase bacterial cultures as a model for non-growing persisters, in this study we assessed a large panel of EOs and their active components for activity against non-growing as well as growing *M. intracellulare*. We identified 34 new EOs that have not previously reported to have activity against *M. intracellulare*, among which 6 EOs, Ajwain, Oregano, Palmarosa, Thyme, Mountain Savory and Litsea Cubeba had the highest activity.

Materials and methods

Bacterial strain, culture media and culture conditions

The *M. intracellulare* 1576 strain was cultured in liquid 7H9 medium with 10% OADC at 37 °C in a shaking incubator at 200 rpm for 14 days to obtain a stationary-phase bacterial suspension. This bacterial suspension was used directly for EOs screening and subsequent experiments without dilution or resuspension in medium or buffer.

Antibiotics and EOs

The antibiotics used in this study (azithromycin, moxifloxacin hydrochloride, clarithromycin, rifampicin, and clofazimine) were purchased from Macklin (Shanghai, China), rifabutin was purchased from Yuanye Bio-Technology Co., Ltd (Shanghai, China), and ethambutol was purchased from Aladdin (Shanghai, China). Each antibiotic was prepared at an initial concentration of 5 mg/ml, aliquoted, and stored at −20 °C. Moxifloxacin hydrochloride was dissolved in ddH_2O and filtered through a

membrane, while the other antibiotics were dissolved in Dimethyl Sulfoxide (DMSO). The 151 EOs were purchased from Fabulous Frannie (California, USA), and each oil was initially in its pure 100% form. We prepared a 20% concentration by diluting it with DMSO (100μL of 100% EOs were mixed with 400μL DMSO), followed by further dilution with stationary phase bacterial solution. The isolated compounds (citronellol, linalool, eugenol, and thymol) from EOs were purchased from Aladdin, allicin and geraniol from Yuanye, and carvacrol and cinnamaldehyde from Acmec Biochemical Co., Ltd (Shanghai, China).

Screening of EOs for their activity against stationary phase *M. intracellulare*

The stationary phase bacterial culture was used to screen a panel of 151 EOs for activity against *M. intracellulare* at a concentration of 5000 μg/mL. The stationary phase culture and the EOs were added to a 96-well plate, which was then incubated at 37 °C without shaking for different times. On the 1st, 4th, and 7th day, a 96-well plate replicator was used to transfer the exposed cultures to 7H11 plates containing 10% OADC. These plates were then further incubated for 10 days to observe bacterial growth. The EOs that exhibited activity in the first round of screening were subjected to a second round of re-screening at 5000 μg/mL, 2500 μg/mL, and 1250 μg/mL, following the same procedure. Clarithromycin, rifampicin, and ethambutol at 50 μM served as controls.

Antimicrobial susceptibility test

To test the effectiveness of the EOs in inhibiting the growth of *M. intracellulare*, we determined the MIC (Minimum Inhibitory Concentration) using the microdilution method recommended by CLSI (Clinical and Laboratory Standards Institute) [26]. In brief, the EOs and their active components (except Thymol) were serially diluted two-fold within a concentration range of 10,000 μg/mL to 78 μg/mL, while the powdered Thymol was initially prepared at a concentration of 5000 μg/mL and serially diluted two-fold from 64 μg/mL to 0.03 μg/mL in the experiment. The test was conducted in CAMHB medium containing 5% OADC, with the bacterial suspension calibrated using a McFarland turbidity meter. Standard antibiotic controls, as well as negative and positive controls, were included. The 96-well plates were incubated at 37 °C without shaking for 7–10 days, until bacterial growth in the wells could be observed with the naked eye.

Validation of active EOs and their main chemical ingredients by CFU count

Based on the screening results, it is necessary to validate the results by drug exposure experiments with stationary-phase cultures exposed to EOs and their active components, followed by CFU (colony-forming unit) count. The stationary-phase cultures were transferred to 1.5 mL Eppendorf tubes, and the EOs and potential active components were tested at 1250 μg/mL. The control antibiotics (Azithromycin, Moxifloxacin, Clarithromycin, Clofazimine, Rifabutin, Rifampicin, and Ethambutol) were used at 50 μM. Controls without antibiotics, EOs, or active components, as well as a control with 1250 μg/mL DMSO, were included. On days 1, 3, 7, and 14, the tubes were centrifuged at 8000 rpm for 5 min, the supernatant was removed, and the pellets were resuspended in PBS. After a series of dilutions, 10 μL of the bacterial suspension was spotted onto 7H11 plates containing 10% OADC. The plates were incubated at 37 °C for about 2 weeks, when the colonies were counted. This procedure was repeated three times.

Results

Screening of EOs for activity against stationary phase *M. intracellulare*

We screened 151 EOs (5000 μg/mL) (Supplementary Table 1) and identified 34 active EOs (Table 1) to have activity against stationary phase *M. intracellulare* after 7 days of exposure, including All Spice, Ajwain, Copaiba Balsam, Cinnamon Bark, Cilantro, Clove, Cinnamon, Cassia, Geranium, Ho Wood, Patchouli, Pine Needle, Oregano, Litsea Cubeba, Palmarosa, Thyme, Protect, Muscle Ice, Moody Girl, Happy Feet, Garlic, Lemon Myrtle, Elemi, Clove Bud, May Chang, Germ Fighter, Spikenard, Mountain Savory, Rose Wood, Onion, Woods, Spice, MBS, and Sandalwood. In the second round of screening at a lower concentration of 1250 μg/mL, 5 EOs (Ajwain, Oregano, Palmarosa, Thyme, Mountain Savory) were effective against stationary phase *M. intracellulare* after just one day of exposure (Table 1). By the 7th day, 18 EOs at 1250 μg/mL (Onion, Sandalwood, All Spice, Ajwain, Copaiba Balsam, Cinnamon Bark, Cilantro, Clove, Cinnamon, Cassia, Oregano, Litsea cubeba, Palmarosa, Thyme, Garlic, Clove bud, Germ fighter, Mountain savory) showed no bacterial growth (Table 1). In contrast, the bacteria treated with the control antibiotics Clarithromycin, Rifampicin and Ethambutol still had bacterial growth.

MICs of the active EOs, antibiotics and selected main ingredients

The MICs were determined for the 34 active EOs (which showed inhibitory effects at a concentration of 5000 μg/mL) and control antibiotics (clarithromycin, azithromycin, moxifloxacin, clofazimine, rifampicin, rifabutin, ethambutol), and the results are presented in Table 2. The active components of the 18 EOs effective

Table 1 Antimicrobial activity of 34 active EOs against stationary-phase *M. intracellulare* at varying concentrations

EOs	Bacterial survival after 1, 4 and 7 days of treatment with EOs								
	5000 µg/mL			2500 µg/mL			1250 µg/mL		
	Day1	Day4	Day7	Day1	Day4	Day7	Day1	Day4	Day7
Ajwain	-	-	-	-	-	-	-	-	-
Oregano	-	-	-	-	-	-	-	-	-
Palmarosa	-	-	-	-	-	-	-	-	-
Thyme	-	-	-	-	-	-	-	-	-
Mountain savory	-	-	-	-	-	-	-	-	-
Germ fighter	-	-	-	-	-	-	+	-	-
Clove bud	-	-	-	-	-	-	+	-	-
Cassia	-	-	-	-	-	-	+	-	-
Cilantro	-	-	-	-	-	-	+	-	-
Clove	-	-	-	-	-	-	+	-	-
Copaiba Balsam	-	-	-	-	-	-	+	-	-
Sandalwood	-	-	-	-	-	-	+	-	-
Cinnamon	-	-	-	+	-	-	+	-	-
All Spice	-	-	-	-	-	-	+	+	-
Litsea cubeba	-	-	-	-	-	-	+	+	-
Cinnamon Bark	-	-	-	-	-	-	+	+	-
Garlic	+	-	-	+	-	-	+	+	-
Onion	+	-	-	+	-	-	+	+	-
Spice	-	-	-	-	-	-	+	+	+
Rose wood	-	-	-	-	-	-	+	+	+
Protect	-	-	-	-	-	-	+	+	+
Geranium	-	-	-	-	-	-	+	+	+
MBS	-	-	-	+	-	-	+	+	+
Spikenard	-	-	-	+	-	-	+	+	+
May chang	-	-	-	+	-	-	+	+	+
Lemon myrtle	-	-	-	+	-	-	+	+	+
Ho wood	-	-	-	+	-	-	+	+	+
Patchouli	-	-	-	+	-	-	+	+	+
Pine needle	-	-	-	+	-	-	+	+	+
Muscle ice	-	-	-	+	+	+	+	+	+
Moody girl	-	-	-	+	+	+	+	+	+
Happy feet	-	-	-	+	+	+	+	+	+
Elemi	-	-	-	+	+	+	+	+	+
Woods	+	-	-	+	+	+	+	+	+

Stationary phase *M. intracellulare* was incubated with different concentrations of EOs in 96-well plates, and after 1, 4, and 7 days of incubation, the bacterial suspension was transferred to 7H11 plates using a 96-well replicator and observed for bacterial growth. "+" means there is bacterial growth, "-" means there is no bacterial growth. Treating stationary-phase bacteria with Ajwain, Oregano, Palmarosa, Thyme, and Mountain savory at 1250 µg/mL for one day resulted in complete elimination of the bacteria

at a concentration of 1250 µg/mL are summarized in Supplementary Table 2. Among the EOs, Sandalwood, Copaiba Balsam, Oregano, and Patchouli showed relatively strong inhibitory effects with an MIC of 630 µg/mL. Ajwain, Cinnamon Bark, Clove, Cinnamon, Cassia, Palmarosa, Thyme, Clove Bud, Mountain Savory, MBS, Geraniol, and Cinnamaldehyde also inhibited the growth of *M. intracellulare* with an MIC of 1250 µg/mL. The MIC for All Spice, Cilantro, Germ Fighter, Spice, Litsea Cubeba, and Eugenol was 2500 µg/mL. We selected EO active components Thymol, Eugenol, Carvacrol, Geraniol, Linalool, Citronellol, and Cinnamaldehyde for further experiments. Among the selected EO components, Carvacrol exhibited the strongest inhibitory effect against *M. intracellulare* with an MIC of 310

Table 2 MICs of active EOs and their main components against *M. intracellulare*

EOs	MIC
Sandalwood	630 µg/mL
Copaiba Balsam	630 µg/mL
Oregano	630 µg/mL
Patchouli	630 µg/mL
MBS	1250 µg/mL
Thyme	1250 µg/mL
Palmarosa	1250 µg/mL
Clove bud	1250 µg/mL
Mountain savory	1250 µg/mL
Clove	1250 µg/mL
Cinnamon	1250 µg/mL
Cassia	1250 µg/mL
Cinnamon Bark	1250 µg/mL
Ajwain	1250 µg/mL
All Spice	2500 µg/mL
Cilantro	2500 µg/mL
Germ fighter	2500 µg/mL
Spice	2500 µg/mL
Litsea cubeba	2500 µg/mL
Garlic	5000 µg/mL
Rose wood	5000 µg/mL
Geranium	5000 µg/mL
Protect	5000 µg/mL
Elemi	5000 µg/mL
May chang	10,000 µg/mL
Spikenard	10,000 µg/mL
Muscle ice	10,000 µg/mL
Moody girl	10,000 µg/mL
Happy feet	10,000 µg/mL
Lemon myrtle	10,000 µg/mL
Ho wood	10,000 µg/mL
Pine needdle	> 10,000 µg/mL
Onion	> 10,000 µg/mL
Woods	> 10,000 µg/mL
Selected main ingredients	
Thymol*	> 64 µg/mL (0.0064%)
Carvacrol	310 µg/mL
Geraniol	1250 µg/mL
Cinnamaldehyde	1250 µg/mL
Eugenol	2500 µg/mL
Citronellol	5000 µg/mL
Linalool	10,000 µg/mL
Antibiotics	
Rifabutin	0.125 µg/mL
Clofazimine	0.25 µg/mL
Moxifloxacin	1 µg/mL
Clarithromycin	1 µg/mL
Rifampicin	1 µg/mL
Ethambutol	2 µg/mL
Azithromycin	8 µg/mL

Table 2 (continued)

* The initial concentration of powdered Thymol is 5 mg/mL, and its MIC was determined using concentrations from 64 µg/mL to 0.03 µg/mL. The remaining EOs and active compounds are in liquid form, and the concentration range used for MIC determination is from 1% (10,000 µg/mL) to 0.0078% (78 µg/mL)

µg/mL whereas other EO components were less active with MICs between 1250–10000 µg/mL (Table 2).

Comparison of the active EOs and selected active components with common antibiotics for activity against stationary phase *M. intracellulare*

Stationary phase *M. intracellulare* is very difficult to kill. Common antibiotics such as clarithromycin, rifampicin, ethambutol, azithromycin, and others like moxifloxacin, clofazimine, and rifabutin showed weak activity against stationary phase *M. intracellulare* at a concentration of 50 µM (Fig. 1). According to the CFU results (Table 3), at a concentration of 1250 µg/mL, Ajwain, Oregano, Palmarosa, Thyme, Mountain Savory, and Litsea Cubeba achieved a 100% clearance of stationary-phase bacteria after just 1 day of treatment. Sandalwood, Clove Bud, Cilantro, Clove, Cinnamon, and Cassia also achieved 100% clearance of *M. intracellulare* after three days of treatment. After 7 days of exposure, Cinnamon Bark, All Spice, and Garlic were able to fully clear the bacteria as well. However, after up to 2 weeks of treatment, Germ Fighter, Onion, and Copaiba Balsam still had some remaining bacteria, although Onion EO was able to reduce the bacterial count by three orders of magnitude (Table 3).

Carvacrol and citronellol as highly active EO components against stationary-phase *M. intracellulare*

Among the selected active components, Carvacrol had an MIC of 310 µg/mL and Citronellol had an MIC of 5000 µg/mL (Table 2). Only Carvacrol and Citronellol at a concentration of 630 µg/mL were able to completely eradicate stationary phase *M. intracellulare* (Table 3, Fig. 1). Even better, carvacrol at 310 µg/mL was able to completely eliminate the bacteria after 1 day of exposure, while Citronellol reduced the bacterial count by five orders of magnitude after 1 day of exposure and achieved complete clearance after 3 days (Table 3, Fig. 1). In contrast, Allicin, Geraniol, Linalool, Eugenol, Cinnamaldehyde, and Thymol, which could reduce some bacterial counts, did not achieve complete eradication (Table 3, Fig. 1).

Discussion

So far, there is limited research on essential oil activity against *M. intracellulare*. Currently, reports indicate that Juniper and Immortelle essential oils exhibit

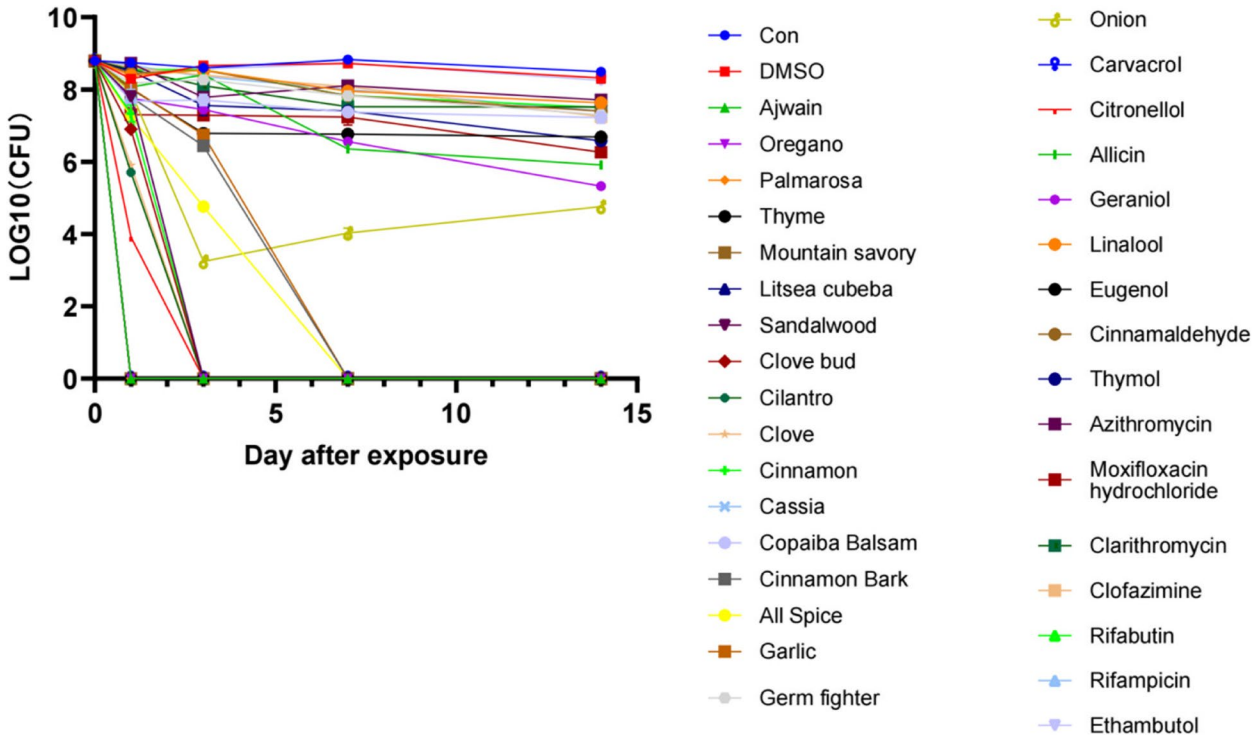

Fig. 1 Activity of commonly used antibiotics, active EOs and active ingredients against stationary phase *M. intracellulare*. Ajwain, Oregano, Palmarosa, Thyme, Mountain savory and Litsea cubeba could eradicate all stationary phase cells after 1-day exposure. Sandalwood, Clove bud, Cilantro, Clove, Cinnamon and Cassia could eradicate all stationary phase cells after 3-day exposure. Cinnamon Bark, All Spice and Garlic could eradicate all stationary phase cells after 7-day exposure. EOs, their active ingredients, and antibiotics were used at 1250 μg/mL, 630 μg/mL, and 50 μM, respectively

antimicrobial, anti-adhesion, and anti-biofilm activities against *M. intracellulare* [22, 23], while citrus oils [27] can also inhibit its growth. Apart from these, there are no other reports on the activity of EOs against *M. intracellulare*. Our study expands on these findings by identifying new EOs that are highly active against *M. intracellulare*. In this study, we identified 34 EOs and specific active components with potent activity against stationary-phase non-growing *M. intracellulare*. Among them, Ajwain, Oregano, Palmarosa, Thyme, Mountain Savory, and Litsea Cubeba EOs are the top 5 highly active EOs that achieved complete bacterial clearance within one day at 1250 μg/mL concentration. The EOs Clove Bud, Clove, Sandalwood, Cilantro, Cinnamon, and Cassia were able to clear all stationary-phase bacteria within three days at 1250 μg/mL. Cinnamon Bark, All Spice, and Garlic at 1250 μg/mL achieved complete bacterial clearance after seven days of treatment. Among the active components, carvacrol and citronellol displayed the strongest bactericidal effects, with carvacrol achieving complete eradication at a concentration as low as 310 μg/mL after one day of exposure. These findings highlight the potential for certain EOs and their constituents to act as potential effective agents against non-growing *M. intracellulare*, which are often resistant to standard antibiotic treatments.

Our results are consistent with prior studies that have demonstrated the antibacterial properties of EOs, such as Oregano [28] and Thyme [21], which contain carvacrol [29] and thymol [30], known for their efficacy against various bacterial pathogens. It has been reported that the EOs Ajwain, Oregano, Palmarosa, Thyme, Litsea cubeba, Clove Bud, Clove, Cinnamon, Cassia, Cinnamon Bark, and Garlic exhibit activity against Mycobacteria [21, 28, 30–35]. However, this is the first time that Mountain Savory, Sandalwood, Cilantro, and All Spice EOs have been identified as having activity against Mycobacteria. Moreover, except for Garlic EO, the activity of these 14 EOs against *M. intracellulare* is reported here for the first time. Previous studies have reported that Ajwain EO effectively inhibited the growth of *M. tuberculosis* [30], with an MIC of 30 μg/mL. The MIC of Ajwain EO against *M. intracellulare* is 1250 μg/mL. Also, we previously found that Palmarosa EO at a concentration of 1250 μg/mL, Thyme EO at 5000 μg/mL, and Cinnamon EO at 630 μg/mL had antibacterial effects against *M. abscessus*

Table 3 CFU count of stationary-phase *M. intracellulare* exposed to antibiotics, EOs and their active components

Treatments	CFU count (CFU/mL)			
	Day 1	Day 3	Day 7	Day 14
Control (no treatment)	$5.63 \pm 1.02X10^8$	$4.07 \pm 0.87X10^8$	$6.73 \pm 0.51X10^8$	$3.13 \pm 0.61X10^8$
DMSO (1250 μg/mL)	$2.12 \pm 0.20X10^8$	$4.73 \pm 1.10X10^8$	$5.30 \pm 0.43X10^8$	$2.20 \pm 0.78X10^8$
EOs (1250 μg/mL)				
Ajwain	0	0	0	0
Oregano	0	0	0	0
Palmarosa	0	0	0	0
Thyme	0	0	0	0
Mountain savory	0	0	0	0
Litsea cubeba	0	0	0	0
Sandalwood	$6.47 \pm 1.25X10^7$	0	0	0
Clove bud	$8.03 \pm 0.50X10^6$	0	0	0
Cilantro	$5.10 \pm 0.39X10^5$	0	0	0
Clove	$8.10 \pm 0.70X10^5$	0	0	0
Cinnamon	$2.40 \pm 1.11X10^7$	0	0	0
Cassia	$6.90 \pm 2.85X10^7$	0	0	0
Cinnamon Bark	$6.13 \pm 0.83X10^7$	$2.90 \pm 0.79X10^6$	0	0
All Spice	$1.87 \pm 0.51X10^7$	$5.83 \pm 0.47X10^4$	0	0
Garlic	$1.10 \pm 0.10X10^8$	$5.66 \pm 1.33X10^6$	0	0
Germ fighter	$1.57 \pm 0.50X10^8$	$1.93 \pm 0.51X10^8$	$6.67 \pm 0.55X10^7$	$2.07 \pm 0.50X10^7$
Onion	$8.80 \pm 0.92X10^7$	$1.76 \pm 0.15X10^3$	$1.11 \pm 3.52X10^4$	$5.83 \pm 0.75X10^4$
Copaiba Balsam	$7.11 \pm 5.80X10^7$	$5.40 \pm 1.80X10^7$	$2.43 \pm 0.57X10^7$	$1.77 \pm 0.58X10^7$
Active ingredients				
Carvacrol#	0	0	0	0
Citronellol*	$8.40 \pm 1.04X10^3$	0	0	0
Allicin*	$1.18 \pm 0.03X10^8$	$2.56 \pm 0.45X10^8$	$2.30 \pm 0.20X10^6$	$8.23 \pm 0.87X10^5$
Geraniol*	$5.67 \pm 0.42X10^7$	$2.83 \pm 0.67X10^7$	$3.63 \pm 0.42X10^6$	$2.13 \pm 0.32X10^5$
Linalool*	$2.50 \pm 0.60X10^8$	$3.50 \pm 0.78X10^8$	$9.13 \pm 0.61X10^7$	$4.33 \pm 0.96X10^7$
Eugenol*	$1.05 \pm 0.14X10^8$	$6.20 \pm 0.82X10^6$	$5.87 \pm 0.40X10^6$	$5.00 \pm 1.30X10^6$
Cinnamaldehyde*	$2.87 \pm 0.76X10^8$	$3.53 \pm 0.93X10^8$	$6.73 \pm 0.42X10^7$	$2.67 \pm 1.04X10^7$
Thymol*	$3.19 \pm 0.36X10^8$	$5.33 \pm 0.45X10^7$	$2.53 \pm 0.25X10^7$	$3.83 \pm 0.76X10^6$
Antibiotics				
Azithromycin	$5.40 \pm 0.36X10^8$	$6.06 \pm 0.25X10^7$	$1.28 \pm 0.03X10^8$	$5.13 \pm 0.35X10^7$
Moxifloxacin	$2.13 \pm 0.90X10^7$	$1.97 \pm 0.31X10^7$	$1.83 \pm 0.87X10^7$	$1.90 \pm 0.56X10^6$
Clarithromycin	$3.63 \pm 0.74X10^8$	$1.30 \pm 0.35X10^8$	$3.40 \pm 0.75X10^7$	$3.47 \pm 1.50X10^7$
Clofazimine	$4.07 \pm 0.49X10^8$	$2.53 \pm 0.32X10^8$	$1.21 \pm 0.12X10^8$	$1.87 \pm 0.64X10^7$
Rifabutin	$3.63 \pm 0.91X10^8$	$3.63 \pm 0.83X10^8$	$6.93 \pm 0.97X10^7$	$3.26 \pm 0.74X10^7$
Rifampicin	$4.70 \pm 0.46X10^8$	$2.33 \pm 0.65X10^8$	$1.00 \pm 0.13X10^8$	$2.53 \pm 0.25X10^7$
Ethambutol	$3.47 \pm 1.21X10^8$	$3.73 \pm 0.50X10^8$	$5.33 \pm 0.51X10^8$	$1.83 \pm 0.25X10^8$

[#] The concentration of the active component is 310 μg/mL

[*] The concentration of the active component is 630 μg/mL

The concentration of antibiotics used was 50 μM, active EOs were used at 1250 μg/mL, and their active components were used at 630 μg/mL or 310 μg/mL. Ajwain, Oregano, Palmarosa, Thyme, Mountain savory, and Litsea cubeba EOs at 1250 μg/mL for 1 day could reduce the bacterial count to zero. Sandalwood, Clove bud, Cilantro, Clove, Cinnamon, and Cassia EOs at 1250 μg/mL for 3 days could achieve the same result. The active compound Carvacrol at 310 μg/mL for 1 day could reduce the bacterial count to zero, while Citronellol at 630 μg/mL for 3 days could do the same

[16]. In our study, Thyme EO showed a better activity against *M. intracellulare*, with an MIC of 1250 μg/mL. Sandalwood EO was able to inhibit growing-phase *M. intracellulare* at a concentration of 630 μg/mL. The main active component of Cinnamon and Cinnamon Bark, cinnamaldehyde, is the primary compound that inhibited

the growth of *M. tuberculosis*, with effects comparable to the first-line drug ethambutol [33]. However, in this study, cinnamaldehyde did not appear to have significant activity against *M. intracellulare*. Additionally, we newly discovered that Geraniol, the main active component of Palmarosa EO, has no effect on our strain, while another component, Citronellol, exhibits inhibitory activity against *M. intracellulare*, completely clearing stationary-phase bacteria within three days at a concentration of 630 μg/mL. Although Carvacrol has previously been reported to have activity against *M. tuberculosis* and rapidly growing drug-resistant mycobacteria, its activity against the slow-growing *M. intracellulare* is reported here for the first time.

In this study, the active EOs we identified to have activity against *M. intracellulare* have previously been reported to exhibit various biological and pharmacological properties. Ajwain not only has certain effects on the respiratory, cardiovascular, urinary, reproductive, and gastrointestinal systems, but also possesses antiparasitic, antibacterial, antifungal, and antitoxin activities [27]. Oregano can be used to treat respiratory and gastrointestinal diseases and also serves as an antimicrobial agent [36]. Thyme has pharmacological effects including antioxidant, anti-inflammatory, immunomodulatory, antibacterial, antitussive, and anticancer activities [37]. Palmarosa has antibacterial, antifungal, and mycotoxin-reducing effects [14, 21, 38]. Litsea cubeba has broad-spectrum antibacterial and antioxidant properties [39] and Mountain Savory exhibits antibacterial and insecticidal activities [18, 40]. Further in vivo animal studies are warranted to investigate the potential of these active EOs for the treatment of *M. intracellulare* infections in the future.

The antibacterial mechanisms of EOs may be related to the disruption of cell membranes, interference with energy metabolism, inhibition of cell wall synthesis, suppression of key enzyme activities, and antioxidant effects [41–43]. For the EOs with strong activity in our study, we selected several active components for testing: Thymol and Carvacrol from Ajwain, Oregano, Thyme, and Mountain Savory, Geraniol from Palmarosa and Litsea cubeba, and Citronellol from Palmarosa. From the CFU results, it can be seen that Thymol and Geraniol had poor activity against *M. intracellulare* in the stationary phase, while Carvacrol and Citronellol were able to clear *M. intracellulare* in a short time during the stationary phase. The antibacterial mechanism of Carvacrol may be related to the inhibition of cell wall enzymes, disruption of the bacterial cell wall and membrane, increased permeability, and leakage of cytoplasm, all of which interfere with bacterial viability [44]. The antibacterial mechanism of Citronellol may involve the disruption of bacterial cell wall integrity [45], inducing oxidative stress in the cells, and triggering lipid peroxidation of the cell membrane [46]. However, future studies are needed to address the detailed mechanisms of action of active EOs and their active components against *M. intracellulare*.

Although we identified several essential oils (EOs) and their active components with strong activity against stationary-phase *M. intracellulare*, our study has some limitations that require further investigation. EOs are complex mixtures, and the precise interactions among their components, as well as their individual contributions to the observed antimicrobial effects, remain unclear. Additionally, the exact mechanisms of action, particularly against stationary-phase bacteria, are not fully understood, and the potential for synergistic effects with conventional antibiotics remains unexplored. While our in vitro results are promising, the therapeutic potential of these EOs has not been validated in animal models, and their potential toxicity and safety, optimal dosage, and pharmacokinetics need to be evaluated in vivo. Addressing these limitations will be critical for advancing the therapeutic application of EOs in combating *M. intracellulare* infections.

In conclusion, we have identified several new EOs including Ajwain, Oregano, Thyme, Mountain Savory, Palmarosa, and Litsea Cubeba and their active components such as carvacrol and citronellol to have potent activity against *M. intracellulare*. Further research is needed to investigate the in vivo activity and safety of these EOs, their pharmacological and biological properties, as well as their potential for combination with existing treatment regimens for more effective treatment of *M. intracellulare* infections.

Supplementary Information

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Supplementary Material 1.

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Not applicable.

Authors' contributions
Xiuzhi Jiang drafted the initial manuscript and collected the data, while Dan Cao, Bihan Xu,Yanghui Xiang and Tiantian Wu analyzed the data and made contributions to the experimental methodology. Xin Yuan provided guidance on the use of plotting software. Ying Zhang conceived the study and made substantial revisions. All authors reviewed and approved the final version of the manuscript.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors agreed to publication of this paper in this journal.

Competing interests
The authors declare no competing interests.

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