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# Research Article
# Antimicrobial Evaluation of *Melaleuca alternifolia* and *Melaleuca citrina* Essential Oils Against *Listeria monocytogenes* and *Escherichia coli* Applied in Disinfection

Lam Ngoc Ngan, Pham Thiet Trinh, Tran Ngoc Quy and Do Tan Khang

Institute of Food and Biotechnology, Can Tho University, Xuan Khanh, Ninh Kieu, Can Tho, Vietnam

## Abstract

**Background and Objective:** Bacterial contamination and consecutive infections had been highly concerned. Contamination has increasingly become prevalent which occurred in food, processing-food factories and laboratories resulting in severe consequences, more importantly, many dangerous diseases for humans. Thus, the investigation of natural compounds which are non-antibiotics, could inhibit the growth of pathogens and restrict the side effects to apply in disinfections as well as preventing bacterial contamination is imperative. The intention of this research was to study the antimicrobial activity of *Melaleuca* essential oils (consisting of *Melaleuca alternifolia* and *Melaleuca citrina* oils) which was also called tea tree oil (TTO), grounded on the chemical components. **Materials and Methods:** The bioactivity of TTO was evaluated by the combination of several methods including agar well diffusion and broth micro-dilution to determine minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs). The compositions of *Melaleuca* essential oils were identified by GC-MS analysis. **Results:** There were sixteen and twelve components obtained in *M. alternifolia* and *M. citrina* oils. The main compounds of *M. alternifolia* and *M. citrina* were eucalyptol (51.88 and 76.55%) and terpene-4-ol (30.08 and 4.93%), respectively. The *Melaleuca* oils showed high antibacterial activity which was assessed with two bacterial species representing Gram-positive and Gram-negative bacteria, *Listeria monocytogenes* and *Escherichia coli*. The antimicrobial efficacies were recorded at high values in *L. monocytogenes* with inhibition zones (13.67 and 16.00 mm), MICs were 0.2 and 1.25%, *E. coli* with inhibition zones (12.33 and 12.67 mm), MICs were 0.3 and 3.0% for *M. alternifolia* and *M. citrina*, respectively. **Conclusion:** The bioactivity of *Melaleuca* essential oils was proven that be effective in resisting both Gram-positive and Gram-negative bacteria. Thus, the TTO could be examined as a potential selection substitution for antibiotics.

Key words: Melaleuca oil, tea tree oil (TTO), antibacterial activity, *Melaleuca alternifolia*, *Melaleuca citrina*, *Listeria monocytogenes*, *Escherichia coli*

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Corresponding Author: Do Tan Khang, Institute of Food and Biotechnology, Can Tho University, Xuan Khanh, Ninh Kieu, Can Tho, Vietnam



Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The infection has become increasingly prevalent due to the complication of pathogenic growth. It should be concerned accompanying with pragmatic evidence related to the severe consequences caused by pathogenic infection. Over the period of 33 years from 1980 to 2013, the world witnessed 12.102 explosions involving human infectious diseases which were recorded at the figure of 215, with a total number of just over 44 million cases throughout 219 countries. Apart from this period, WHO's investigation on a global scale conducted in the year 2008 indicated that there were over 12 million cases corresponding to deaths, making 21% of the total mortal circumstances. In recent years, the pathogenic contamination recorded in food has been problematic and associated with foodborne diseases caused by pathogens that received a significant examination by governments and international organizations, such as WHO[1]. According to the evaluation of WHO performed in 2015 involving food-contaminating status, it was clearly presented that there were 10% of surveyed participants were foodborne patients, caused by plenty of hazardous compounds originating from bacteria, viruses, parasites and chemical preservatives. The result indicated that the consumption of non-hygienic and harmful food threatened human life and recorded 600 million victims of foodborne disease annually, among which, 56 million mortal cases were revealed, the mortality accounted for 9.3%[2]. Bacteria was considered one of the most fundamental pathogens found in diseases related to food toxicity, typically *Listeria monocytogenes* and *Escherichia coli* were examined as two dominant pathogenic bacteria[3]. Some previous research revealed that *Listeria monocytogenes* and *Escherichia coli* have been involved in distinct food-contaminated outbreaks[4,5].

According to the report of Camargo *et al.*[6], *L. monocytogenes* is a Gram-positive bacterium, one of the most ubiquitous foodborne pathogens, the factor causing food-processing environment contaminations and persisting on the surfaces of the equipment, utensils, floors and drains as biofilms, eventually creating cross-contamination in final products. It was highly convincing that *L. monocytogenes*, the main cause of Listeriosis has been recognized as severe contamination in humans resulting in 1,591 cases of food-infecting patient in European. Comparatively, this figure in death cases was detected in the US which was nearly 225[3]. Besides, *L. monocytogenes* also has the ability to infect the laboratory, which was described as pseudo-outbreaks caused by contaminated laboratory culture media[7]. It was apparently clear that bacteria had been being evolved in unpredictable ways resulting in antibiotic resistance[8] and complicatedly occurred with the high frequency in current years[9]. Current solutions seem to be insufficiently effective in inhibiting bacterial growth owing to the antibiotic-resistant phenomenon in bacteria. The investigation in France revealed that there were 61 *L. monocytogenes* strains that showed multi-antibiotics resistance, which had been examined by clinical trials with more than 23 antibiotics[10]. In addition, two cases of multidrug-resistant (MDR) *L. monocytogenes* strains were globally reported including prosthetic valve endocarditis in a Swiss patient at the University Hospital in Zurich, Switzerland[11] and meningitis in a Greek neonate who developed 21 days after birth[12]. In recent years, many Chilean scientists have detected some antibiotic-resistant genes of *L. monocytogenes* strains isolated from ready-to-eat foods[13]. *E. coli* is a Gram-negative bacterium, a popular pathogen that is most commonly found inhabiting the lower intestinal tract of warm-blooded animals, including humans and causing some diseases related to the gastrointestinal tract, digestive systems and severe consequences in some special cases. *E. coli* comprises a large group of bacteria, among which, Shiga Toxin-Producing *E. coli* (STEC) could be a potential risk of diarrhea. The year 2011 witnessed 4,000 cases of infection and recorded 50 deaths caused by *E. coli*, which was assessed within 16 countries[3]. There are some strains of *E. coli* that can exist in the environment and spread their pathogen to food, soil and water in particular ways[14]. It was noticeable that *E. coli* have a broad spectrum in antibiotic resistance meaning that they can resist plenty of antibiotics. According to the research of Bong *et al.*[15], many experiments related to antibiotic activity were evaluated in *E. coli* with 20 frequently used antibiotics after which recorded 16 types were resisted, accounting for 80% of the total amount of antibiotics. Typically, *E. coli* showed the highest antibiotic resistance to ampicillin (82.79%) and trimethoprim-sulfamethoxazole (40.86%)[16].

Antibiotic utilization and its side effects should not be underestimated due to the fact that there is much research showing the detrimental impacts related to human health including diarrhea, pseudomembranous colitis and rose risk of consequent diseases were caused by the imbalance of beneficial microorganism systems according to partial immune deficiency[17].The promising alternatives to antibiotics in inhibiting bacterial infection are natural products extracted from plants including some essential plant oils which have been broadly studied all over the world. The antimicrobial activity of many essential oils from plants was researched such as Basil, Oregano, Thyme[18] and some medicinal plants including *Acorus calamus*, *Allium sativum*, *Mucuna pruriens* and *Sesamum indicum*[19]. Besides these types of plant extracts,

the essential oils from tea tree oil (TTO) or *Melaleuca alternifolia* (*M. alternifolia*) and *Melaleuca citrina* (*M. citrina*) were considered as potential plant oil in antibacterial activity.

There is much research that investigated the antimicrobial activity and application of TTO. In this article, the term TTO will refer to the oils of *M. alternifolia* and *M. citrina*. *Melaleuca alternifolia* (Maiden and Betche) Cheel and *Melaleuca citrina* (Curtis) Dum. Cours. also called "red bottle brushes" belong to the genus *Melaleuca*, family Myrtaceae. They are indigenous plants in Australia, particularly in Queensland and New South Wales but are now cultivated all over the world[20,21].

The antimicrobial properties of TTO were evaluated on planktonic and biofilm-forming *Streptococcus mutans* and proved that it has some beneficial properties as an anti-cariogenic agent that can be used against *S. mutans*[22]. Most of the previous studies revealed that the essential oil extracted from *M. alternifolia* and *M. citrina* have a lot of compounds having high antimicrobial ability. According to the report of Carson *et al*.[23], *M. alternifolia* oil has 14 main components in which terpinen-4-ol obtains the highest composition, approximately, 40.1%. Terpinen-4-ol is a potent bactericidal agent[24] that possesses antifungal properties[25]. A previous investigation detected twenty-four components contained in the oil of *M. citrina*, which mainly were 1,8-cineole (61.2%), α-pinene (13.4%) and β-pinene (4.7%)[26]. This research investigated the tea tree oil distillation process according to the different times to select the optimized extraction time and evaluates the antibacterial activity of the oils against two bacterial species, *L. monocytogenes* and *E. coli.*

## MATERIALS AND METHODS

**Study area:** This research was conducted over the period of 6 months, from May to October, 2022.

**Plant materials:** The *Melaleuca alternifolia* (Maiden and Betche) Cheel. and *Melaleuca citrina* (Curtis) Dum. Cours. plant materials were collected from Long An and Hau Giang Province, Vietnam, respectively. The taxonomic identification of these plant samples was verified by a plant taxonomist, Phung T.H., MSc of the Botanical Laboratory, Department of Biology, School of Education, Can Tho University, Vietnam. Voucher specimens [Ma15092022-LA0001 and Mc20092022-HG0018] were deposited at Can Tho University, Vietnam.

**Extraction of essential oil using steam distillation:** The oil extraction used the steam distillation method, which was performed at the Laboratory of Organic Chemistry, Department of Chemistry, School of Education, Can Tho University, Vietnam. Fresh leaves and twigs (850 g each) of *Melaleuca alternifolia* and *Melaleuca citrina* samples were used as ingredients combined with 3 L of distilled water to conduct the oil extraction experiment. The ingredients were contained in a round-bottom flask of distillation apparatus which was fairly and tightly packed to fit completely. The steam was allowed to move through the raw materials due to the leaving of several spaces. There is a flow of water drained into the condenser, allowing the oil to flow into the tube. When the steam distillation process was initiated, the water was heated by heating the mantle resulting in the vapor passing through the samples. During the essential oil extraction, the temperature needed to be reached the boiling point of TTO which is 165°C and stabilized until the end of this process. After the oil distillation was finished, the essential oil was collected, followed by performing the "liquid-liquid extraction" technique. The obtained TTO was contained in a sealed amber glass sample tube and preserved at 4°C within the refrigerator[26,27].

The essential oil collected from the steam distillation process was separated by liquid-liquid extraction technique. The distilled solution contained three layers after adding diethyl ether, including diethyl ether, TTO and from top to bottom, respectively. This is because the density of each substance is not similar, which were 0.713 g $mL^{-1}$ (diethyl ether), 0.885-0.906 g $mL^{-1}$ (essential oil) and 1.000 g $mL^{-1}$ (water)[27]. After extracting with diethyl ether, there was a small amount of water in this solution and anhydrous sodium sulfate was added to eliminate the water.

The yield of TTO extraction (% v/w) was calculated according to the following formula[28]:

$$\text{Essential oil productivity (\%)} = \frac{\text{Volume of extracted oil (mL)}}{\text{Fresh weight of plant material (g)}} \times 100$$

**Gas Chromatography-Mass Spectrometry (GC-MS) analysis:** The GC-MS analysis of the oils was performed on a GC/MS Thermo Machine, Thermo Xcalibur software, NIST MS Search 2.0 Mass Spectral Library with a DB-5 capillary column (0.32 mm×30 m×0.25) and the carrier gas was used in this system which was Helium. The temperature of the sample injection on the column was 240°C. The temperature program commenced at 50°C and was maintained within 1 min. In the first stage, the temperature increased at the rate of 2°C/min to 70°C, subsequently, it was adjusted to rise to 150°C, with a speed of 5 min. In the third stage, the temperature rose 10 min and reached a peak of 230°C[29].

**Antibacterial assay:** Two bacterial species were used to investigate the antibacterial activity of tea tree oil which are Gram-positive bacteria: *Listeria monocytogenes* obtained from the Department of Microbiology, VNU HCMC-University of Science and Gram-negative bacteria: *Escherichia coli* (ATCC 25922) collected from Department of Molecular Biology, Biotechnology Research and Development Institute, Can Tho University. In this study, the antimicrobial ability of both types of *Melaleuca* oils was evaluated by several following methods including agar well diffusion associated with the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the microdilution method.

**Agar well diffusion:** According to the research of Valgas *et al.*[30], the agar disc diffusion method was used to assess the antimicrobial activity of TTO with a modification related to the nutrient broth medium. The bacteria were cultured in tubes containing 20 mL of Luria-Bertani (LB) broth and incubated overnight in the warm condition of 37°C. The bacterial inoculum measured the optical density at the wavelength of 600 nm ($OD_{600}$) and standardized with sodium chloride 0.85% solution to obtain turbidity which was comparable to the figure of McFarland 0.5 standard ($1.0\times10^8$) CFU $mL^{-1}$. The microbial suspension was consistently spread with sterile cotton swabs on the surface of the Petri dish containing LB agar. Two series of essential oil serial dilutions were prepared including a series that yielded concentrations of 10, 20, 30% (v/v) and the other performed at 25, 50 and 75% (v/v) which were diluted by Dimethyl Sulfoxide (DMSO) 15% solution. Amoxicillin (5 mg $mL^{-1}$) and DMSO 15% solution was used as positive and negative control, respectively. There were 5 wells with a diameter of 7 mm cut in the agar surface of each 90 mm Petri dish with 20 mm between each other. About 50 µL of natural extracts at different concentrations, positive and negative control were added in each well. Subsequently, the plates were incubated at 37°C for 24 hrs and the diameter of the inhibitory zone was measured in mm. These experiments were triplicated.

**Determination of MICs:** The MIC is defined as the lowest concentration of antimicrobial agent that prevents the visible growth of microorganisms[31]. In other words, the testing samples with certain concentrations prepared in tubes, among which, the concentration would be determined as MIC if its turbidity was visualized by unaided eyes compared to the turbidity of control samples after incubation for 24 hrs[32]. The microdilution assay using the 96-well microplates method combined with OD measurement was applied to evaluate the antibacterial activity and determine the MIC of investigated samples against 2 strains of bacteria (*L. monocytogenes* and *E. coli*)[33]. The bacterial cultures were similarly prepared and adjusted the turbidity according to the McFarland 0.5 standard as the description above. The bacterial inoculum was diluted at the ratio of 1:1 of each inoculum in fresh LB broth to utilize in microdilution experiments. The oil extract solution was prepared in a series of densely ascending concentrations from 0.1 to 10% (v/v) and applied to the microplate, followed by the addition of bacterial culture. Eventually, each well had 200 µL as the total volume. The microplates were measured at the OD value at the wavelength of 600 nm by using a spectrophotometer (Thermo Scientific Multiskan GO) and incubated at 37°C for 24 hrs. Afterward, the OD value was measured again to assess bacterial growth. To indicate only the bacterial turbidity, the total OD value did not contain the OD of the background (LB fresh). After 24 hrs of incubation, if the difference between this OD value and the figure measured at 0 hr was lower than the change in OD value recorded in wells containing only bacterial culture without oil extracts, it was determined that the bacterial growth was prevented.

**Determination of MBCs:** The MBC is determined as the least concentration of antimicrobial substances required to kill microorganisms[30]. The experiments determining the MBCs were continuously conducted after the MICs of tested samples had been detected. Many tested samples having concentrations which were the same and higher than the MICs, were selected to perform experiments related to MBCs. The determination of MBCs was tested by sub-culturing the broth dilution on agar plates without a test antimicrobial agent. The sterile LB agar was prepared in Petri dishes and 50 µL of each distinct concentration sample was spread on the surface of the nutrient agar by using a glass spreading rod. Additionally, the experiment could be conducted by drop plate methods of counting viable bacteria[34]. The Petri plates were incubated at the relevant condition of 37°C for 24 hrs. Subsequently, the bacterial growth in the Petri dish was observed via the appearance of colonies and the MBCs were determined as the lowest concentration of the series without the colony formation.

**Statistical analysis:** The data were expressed as Mean±Standard deviation. One-way ANOVA Test was used to analyze the difference between groups of data. The $p<0.05$ was considered statistically significant. All statistical tests of variation were performed with Minitab Software Version 18 (Pennsylvania, USA).

## RESULTS AND DISCUSSION

**Productivity of essential oil extraction:** The productivity of both kinds of *Melaleuca* oils extraction by steam distillation was evaluated in several different stages. Generally, the yield of *M. alternifolia* oil extraction was highly greater than the figure of *M. citrina*. As shown in Table 1, the *M. alternifolia* oil volume rose as the extraction time increased and reached the highest value at 120 min. However, the figures related to the yield of oil extraction at two different times, 100 and 120 min, were not significant. Thus, the time was examined as relevant for the extraction process, which was 100 min, which could avoid the time and energy consumption in case of conducting distillation within 120 min. The average productivity of *M. alternifolia* extraction was 2.84±0.12% which was higher than that the yield had been recorded in the previous study, in the range of 1 to 2% of the weight of fresh plant ingredients[21]. There were some reasons that could influence the yield of essential oil extraction, typically the moisture content of the raw ingredients (leaves and twigs) and the temperature that the mixture of ingredients and steam were heated[27]. In addition, the extraction time could be a factor affecting productivity. If the distillation process was conducted in a short time which was not long enough to vaporize most of the oil content in raw material, the yield of extraction would be low and the utilization of material would not be effective. This is the reason why our research performs the experiment to evaluate the yield of essential oil extraction according to distinct times to select the relevant time which could optimize the extraction process.

Table 2 demonstrated the yield of *M. citrina* distillation and 100 min were selected as the suitable extraction time which had been clarified above. The extraction productivity of *M. citrina* oil was 0.57±0.03%, quite low in general, which was nearly similar to the findings of previous investigation related to the percentage yields of *M. citrina*, approximately 0.7% (leaves) and 0.5% (stems)[35].

**GC-MS analysis:** The GC-MS analysis result of *Melaleuca* oils illustrated that there were 16 peaks in the sample of *M. alternifolia* oil while, the *M. citrina* oil had 12 peaks recorded (Fig. 1 and 2). The number of peaks were identified equal to the quantities of constituents contained in the oil. As shown in Table 3, there were two main components with high percentages including eucalyptol (51.88%) and 1-terpene-4-ol (22.71%) obtained in *M. alternifolia* oil. The 1-terpene-4-ol and α-Terpineol were isomers of terpene-4-ol[36], therefore, the percentage of terpene-4-ol calculated in *M. alternifolia* oil was 30.08% which achieved the required standard of ISO 4730:2004. This result was similar to the figure recorded in the previous research of Chi *et al.*[28]. The terpene-4-ol was assessed as the key component which greatly contributed to the antibacterial activity of the essential oil[23]. Besides, the other compounds obtained in the oil such as α-Terpineol and Globulol were also accepted with a certain amount following the ISO 4730:2004 standard.

The components of *M. citrina* oil were different from *M. alternifolia*. The main compounds of *M. citrina* oil were eucalyptol (76.55%) and α-pinene (7.78%), which were higher than the figure recognized in *M. alternifolia* oil. However, the important component greatly attributed to the antimicrobial ability was terpene-4-ol, accounting for the small percent of 4.93%. This might generally influence the effectiveness of *M. citrina* oil in resisting microbials. The distinction related to the extraction productivity and components of oils might be involved in the different genetics and geographical conditions in particular places where the tea tree had been cultivated[26].

Table 1: Yield of *M. alternifolia* oil steam distillation in different extraction time

| Batch | Raw ingredient (g) | Extraction time (min) | Oil volume (mL) | Yield (%) |
|---|---|---|---|---|
| 1 | 850 | 60 | 9.03±0.25$^{c}$ | 1.06±0.03$^{c}$ |
| 2 | 850 | 80 | 16.33±1.53$^{b}$ | 1.92±0.18$^{b}$ |
| 3 | 850 | 100 | 24.17±1.04$^{a}$ | 2.84±0.12$^{a}$ |
| 4 | 850 | 120 | 24.77±0.68$^{a}$ | 2.91±0.08$^{a}$ |

Values are means of triplicate determination±standard deviations. The volume and yield of essential oil values within each column followed by different lowercase letters are statistically significantly different ($p<0.05$)

Table 2: Yield of *M. citrina* oil steam distillation in different extraction time

| Batch | Raw ingredient (g) | Extraction time (min) | Oil volume (mL) | Yield (%) |
|---|---|---|---|---|
| 1 | 850 | 60 | 1.23±0.25$^{c}$ | 0.15±0.03$^{c}$ |
| 2 | 850 | 80 | 2.43±0.25$^{b}$ | 0.29±0.03$^{b}$ |
| 3 | 850 | 100 | 4.83±0.21$^{a}$ | 0.57±0.03$^{a}$ |
| 4 | 850 | 120 | 5.27±0.25$^{a}$ | 0.62±0.03$^{a}$ |

Values are means of triplicate determination±standard deviations. The volume and yield of essential oil values within each column followed by different lowercase letters are statistically significantly different ($p<0.05$)

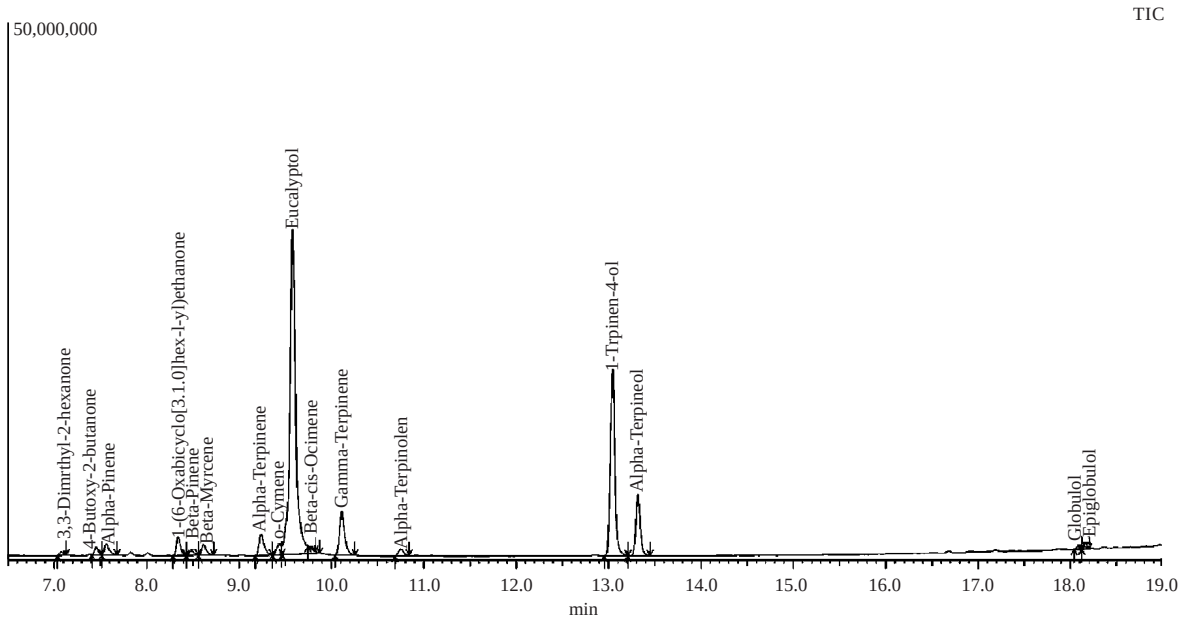


Fig. 1: Chromatogram of *M. alternifolia* oil from GC-MS analysis

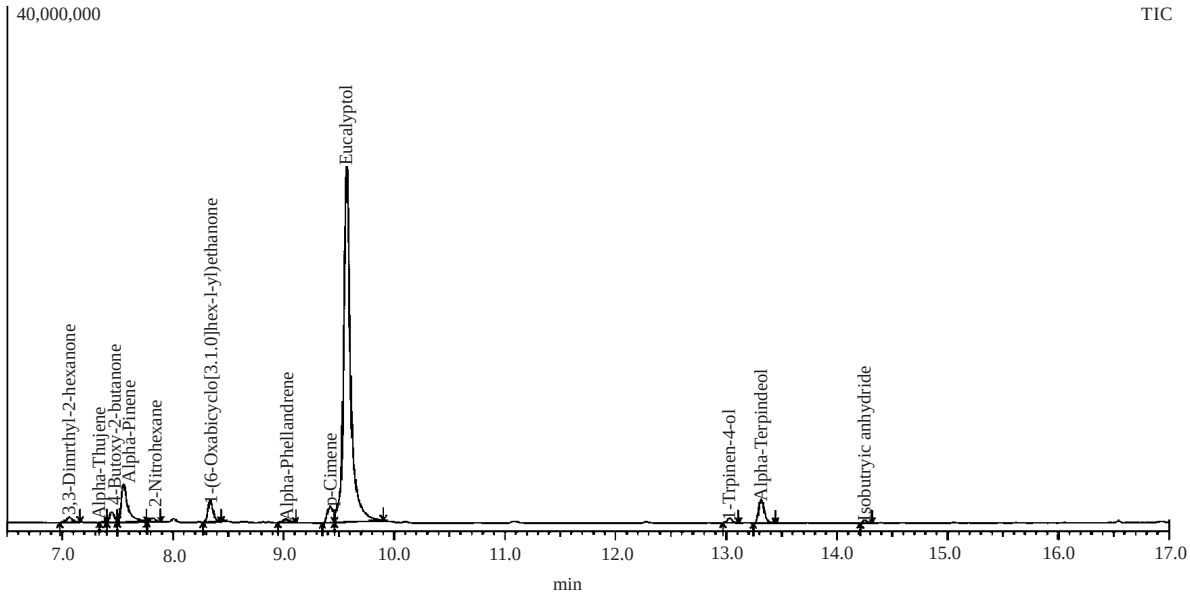


Fig. 2: Chromatogram of *M. citrina* oil from GC-MS analysis

**Antibacterial activity by agar well diffusion:** The experimental figures reported in Table 4 proved that both types of *Melaleuca* oils expressed the antibacterial activity against *L. monocytogenes* (Gram-positive bacteria) and *E. coli* (Gram-negative bacteria) via the diameter of inhibition zone observed in the Petri dishes (Fig. 3). The obtained results detected that the antimicrobial ability of essential oil was proportional to its concentration meaning that the inhibitory zone expanded according to the increase in oil concentration. It was apparently seen that *L. monocytogenes* had a higher susceptibility to the oil than *E. coli* with a larger inhibition zone in different concentrations. Among them, the highest diameters of inhibitory zone recorded in *L. monocytogenes* at the concentration of 75% were 16.00±1.00 mm and 13.67±0.58 mm for *M. alternifolia* and *M. citrina*, respectively while the figure evaluated in *E. coli* were 12.67±1.16 mm and

Table 3: GC-MS analysis of *Melaleuca* oils

| | *M. alternifolia* | | *M. citrina* | |
|---|---|---|---|---|
| Component | [a]RT | Area (%) | [a]RT | Area (%) |
| 3,3-Dimethyl-2-hexanone | 7.077 | 0.32 | 7.066 | 1.01 |
| α-Thujene | - | - | 7.365 | 0.16 |
| 4-Butoxy-2-butanone | 7.456 | 0.73 | 7.447 | 1.58 |
| α-Pinene | 7.564 | 1.35 | 7.555 | 7.78 |
| 1-(6-Oxabicyclo [3.1.0] hex-1-yl) ethanone | 8.343 | 2.04 | 8.338 | 3.39 |
| 2-Nitrohexane | - | - | 7.819 | 0.58 |
| β-Pinene | 8.485 | 0.59 | - | - |
| α-Terpinene | 9.240 | 2.97 | - | - |
| α -Phellandrene | - | - | 9.020 | 0.67 |
| p-Cymene | - | - | 9.424 | 2.93 |
| o-Cymene | 9.430 | 1.34 | - | - |
| Eucalyptol | 9.582 | 51.88 | 9.571 | 76.55 |
| β-cis-Ocimene | 9.775 | 0.09 | - | - |
| γ-Terpinene | 10.111 | 5.92 | - | - |
| α-Terpinolene | 10.753 | 0.93 | - | - |
| 1-Terpinen-4-ol | 13.046 | 22.71 | 13.031 | 0.78 |
| α-Terpineol | 13.318 | 7.37 | 13.316 | 4.15 |
| β-Myrcene | 8.615 | 1.31 | - | - |
| Globulol | 18.080 | 0.25 | - | - |
| Epiglobulol | 18.159 | 0.20 | - | - |
| Isobutryic anhydride | - | - | 14.254 | 0.43 |

[a]RT: Retention time

Table 4: Antimicrobial activity of both *Melaleuca* oils tested by agar well diffusion

| | *L. monocytogenes* (+) Gram positive bacteria | | *E. coli* (-) Gram negative bacteria | |
|---|---|---|---|---|
| Concentration (%) (v/v) | *M. alternifolia* oil | *M. citrina* oil | *M. alternifolia* oil | *M. citrina* oil |
| Control (+) | 15.67±0.58$^{ab}$ | | 11.67±0.58$^{a}$ | |
| Control (-) | - | | - | |
| 10% | 5.00±1.73$^{f}$ | 1.67±0.58$^{g}$ | 3.67±0.58$^{ef}$ | 2.00±0.00$^{f}$ |
| 20% | 8.33±0.58$^{de}$ | 3.67±0.58$^{fg}$ | 8.00±1.73$^{cd}$ | 2.67±0.58$^{f}$ |
| 30% | 10.00±1.00$^{d}$ | 8.00±1.00$^{de}$ | 11.00±1.00$^{abc}$ | 4.33±0.58$^{ef}$ |
| 25% | 9.33±0.58$^{d}$ | 6.00±1.00$^{ef}$ | 8.33±1.16$^{bcd}$ | 4.00±1.00$^{ef}$ |
| 50% | 13.00±1.73$^{bc}$ | 10.50±0.50$^{cd}$ | 11.33±1.53$^{ab}$ | 6.17±1.04$^{de}$ |
| 75% | 16.00±1.00$^{a}$ | 13.67±0.58$^{ab}$ | 12.67±1.16$^{a}$ | 12.33±1.16$^{a}$ |

Values are means of triplicate determination±standard deviations, (v/v) demonstrates for the dilution based on the volume unit used to measure the substances. Inhibition zone diameter values within each column followed by different low case letters are significantly different in statistics ($p<0.05$)

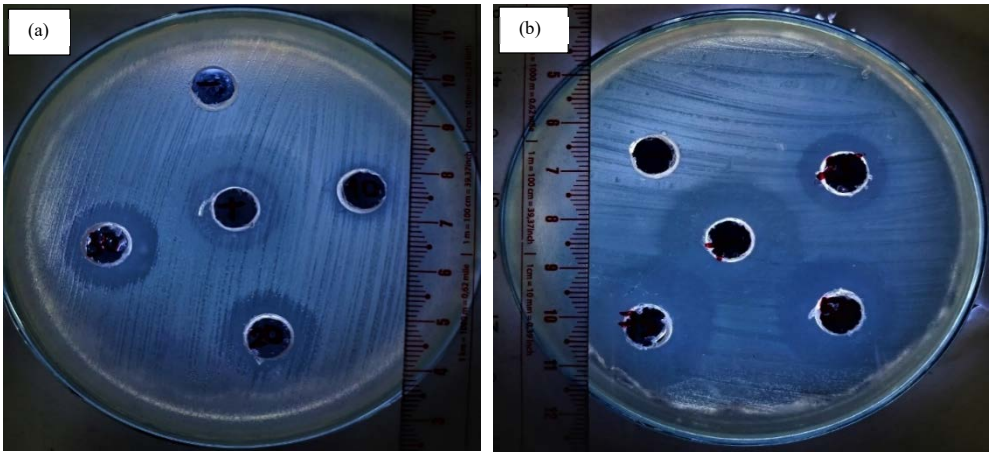


Fig. 3: Inhibition zones observed in the Petri dishes, (a) 10, 20 and 30% and (b) Petri dish performed the antibacterial activity at the concentration of 25, 50 and 75%

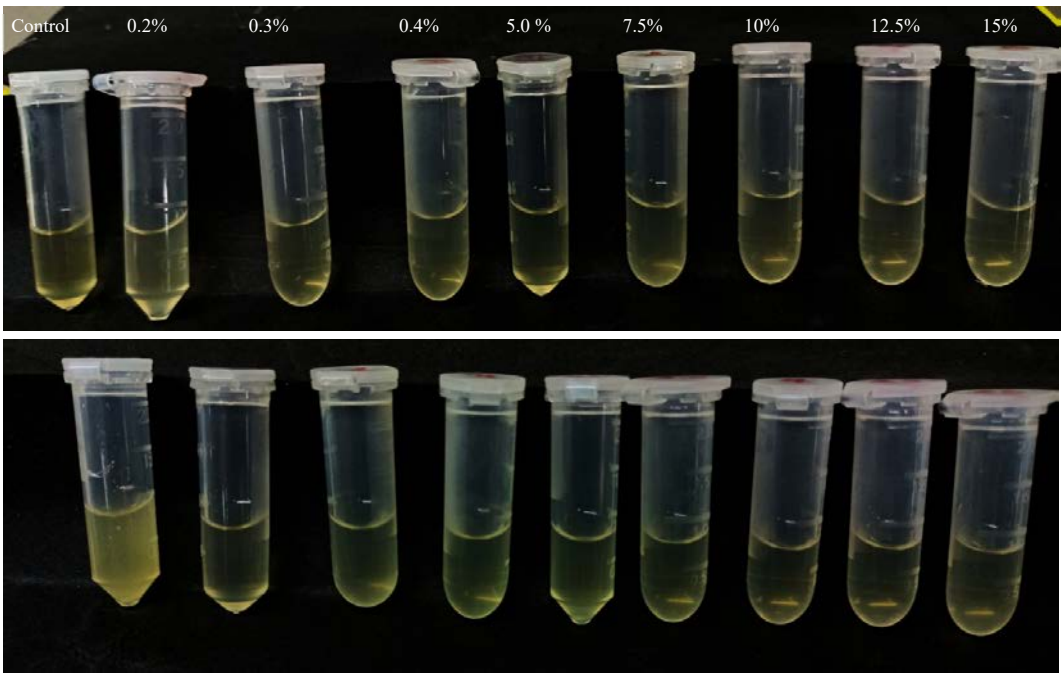


Fig. 4: Turbidity of testing samples observed in *L. monocytogenes* before and after incubation of 24 hrs

12.33±1.16 mm. The value of the inhibitory zone measured at 50% and 75% of *M. alternifolia* oil against two strains of bacteria were not significant to the data observed at positive control (Amoxicillin (5mg $mL^{-1}$)) (15.67±0.58 mm and 11.67±0.58 mm for *L. monocytogenes* and *E. coli*, respectively). It was convinced that the *M. alternifolia* oil at the concentration of 50 and 75% had an antibacterial activity which was similar to positive control. In the case of *M. citrina* oil, the diameter of inhibitory zones was mostly smaller than the figure of *M. alternifolia* oil at many concentrations (Table 3) and insignificant in comparison with the inhibitory zone of positive control at the level of 75%. Thus, the antimicrobial activity of *M. alternifolia* oil was dominant compared to *M. citrina* oil due to the difference related to the constituents obtained in each kind of essential oil. Furthermore, the essential oil was extracted from different organs of the same plant which could indicate the distinct levels of antimicrobial activity. The *Melaleuca* oils in this research mainly extracted from leaves and twigs showed differences in antibacterial expression, which was lower than the data recognized in the oils distilled from flowers[37].

This could be examined that both *Melaleuca* oils indicated the broad spectrum of antibacterial activity against both Gram-positive and the Gram-negative bacteria[38]. Particularly, the experimental data of *Melaleuca* oils (Table 3) delineated that the inhibitory effect on Gram-positive bacteria was higher than on Gram-negative bacteria, which agreed with the results of some previously published investigations[39].

**Determination of MICs and MBCs:** The MIC of *M. alternifolia* oil against *L. monocytogenes* was 0.2%, which was comparable to the study of Oussalah *et al.*[40] and Liu *et al.*[41], lower than the MICs recorded in *E. coli*, was 0.3%, insignificant to the baseline MICs (0.25-0.5%) revealed in previous research[42]. The *M. alternifolia* oil had MICs ≤4% (v/v) in resisting both bacteria above and was assessed as the high antibacterial activity[40]. The MICs recorded in experiments with *M. citrina* oil were greater than the data in *M. alternifolia* oil, which were 3.0 and 1.25% for *E. coli* and *L. monocytogenes*, respectively. The turbidity of testing samples in different concentrations was shown in Fig. 4 and 5. It was evidently seen that the turbidity of MICs could be observed by unaided eyes compared to the control. The experimental results related to MICs in this investigation proved that the tea tree oil enabled to inhibition a wide range of pathogenic microorganisms at low levels of concentration[23].

Current research detected the MBCs of *M. alternifolia* oil against both *E. coli* and *L. monocytogenes* whereas, these figures on *M. citrina* have not been recorded yet. The *M. alternifolia* oil nearly killed the viability of *L. monocytogenes* at 12.5% (v/v) mean while, the MBCs in *E. coli* was higher, 47.5% with the modes of action described in the article of Carson *et al.*[23]. This result demonstrated that

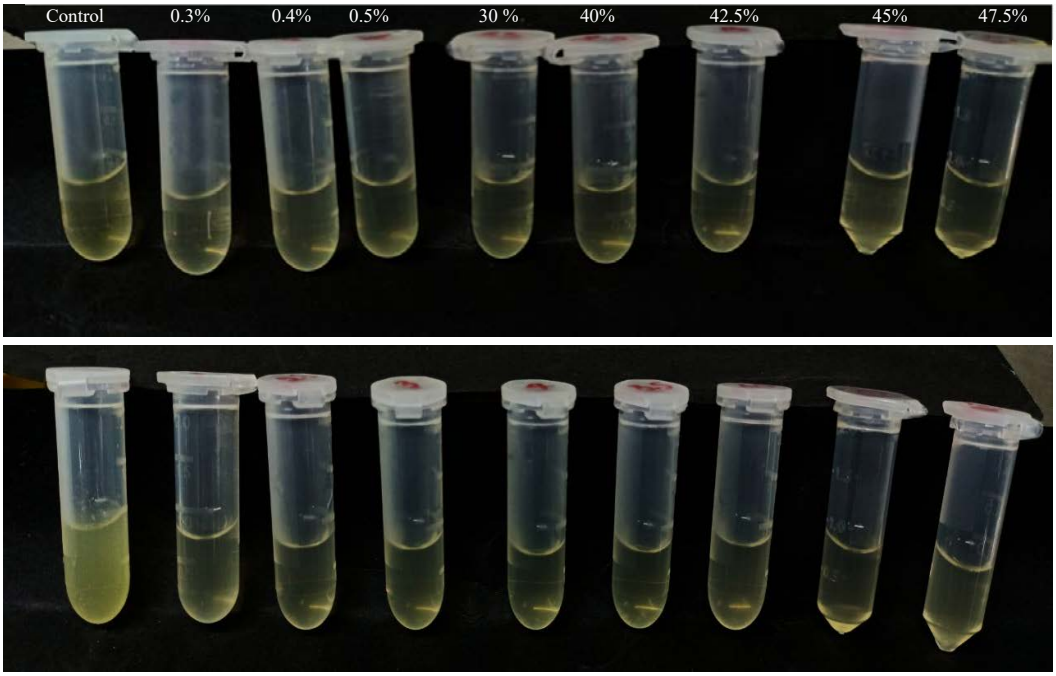


Fig. 5: Turbidity of testing samples observed in *E. coli* before and after incubation of 24 hrs

the Gram-negative bacteria (*E. coli* as an example) was difficult to destroy its cells in comparison with Gram-positive bacteria, which was explained by the study of Jones[43], grounded on the difference in membrane structure between Gram-positive and Gram-negative bacteria. To be specific, the membrane of Gram-positive bacteria was structured by a single outer membrane outside covering a cell wall while, Gram-negative bacteria had three layers containing an inner membrane, a cell wall and an outer membrane, posing an additional barrier to molecule entry.

## CONCLUSION

The investigation evaluated the extraction of both *M. alternifolia* and *M. citrina* oil to optimize the distillation time. It was easily seen that the *Melaleuca* oils richly included antibacterial components which enabled to prevent the growth of both Gram-positive and Gram-negative bacteria, such as *L. monocytogenes* and *E. coli*. Due to the difference in the compositions of each kind of essential oil, the bioactivity is also distinct between *M. alternifolia* and *M. citrina* oil, typically the percentages of some major compounds indicating the high antibacterial ability as terpene-4-ol, α-Terpineol and eucalyptol. Furthermore, the findings of this study expressed the potential of applying essential oils as natural antibacterial agent's *in vitro* conditions which might limit some side effects and could be an alternative to antibiotics. It is necessary to restrict the antibiotics-resistance or multi-drug resistance which has increasingly occurred in microorganisms, particularly bacteria.

## SIGNIFICANCE STATEMENT

This study convinced that the essential oils extracted from both kinds of *Melaleuca* species, especially *Melaleuca alternifolia*, have effective antimicrobial activity even with some dangerous-diseased bacteria as *L. monocytogenes* and *E. coli*, which could be applied to disinfect in scope of laboratories and processed factories to restrict the risk of bacterial contamination. Furthermore, the *Melaleuca* essential oils are examined as promising alternatives for antibiotics in usage of antibacterial as well as prevent side effects for consumers. Besides, utilization of the essential oils as well as natural plant extracts, also contributes to the environmental protection due to their friendly effects which would be beneficial for our lives.

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