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# The Antibacterial of Limonene Against Gram Negative and Positive Bacteria

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**ABSTRACT**

*Foodborne disease outbreaks are prevalent worldwide, affecting human health which requires using medical products to heal. However, most medical contain nano-silver antimicrobial agents can kill bacteria and fungi, however and harms human organs based on several articles. Alternatively, limonene is a natural extract from citrus fruits and can be used as an antibacterial agent to combat these diseases. Its bio-material properties make it safe for skin contact and environmentally friendly, making it a promising candidate for addressing foodborne illnesses. This study investigates the susceptibility of limonene to the Gram-negative bacteria, Escherichia coli, and the Gram-positive bacteria, Bacillus Subtilis, by applying the minimum inhibitory concentration (MIC) method and disc diffusion method. The result of exterior ring diameter of inhibition stated mean value and standard deviation of Bacillus Subtilis at 1.133 ± 0.3682 is higher than Escherichia coli at1.067 ± 0.2055 indicated limonene is effectiveness against Bacillus Subtilis. Though the comparison limonene and nanoparticle silver has stated limonene is preferable antibacterial resistance due to the limonene produced 10 % higher than nanoparticle in comparison on the initial diameter inhibition zone against Escherichia coli through disc assay. The MIC method was applied to Bacillus Subtilis by diluting limonene with LB broth consecutively in each falcon tube and measuring optical density (OD) before and after incubation. As a result, only one petri dish has lower colonies at an optical density of 0.809 decreased from 0.834 which indicates that the minimum concentration dosage was 1 % (v/v). Therefore, these results indicate that limonene is bacteriostatic against Bacillus Subtilis in MIC.*

**Keywords:** Limonene, Escherichia coli, Bacillus Subtilis, nano-sliver

## 1. INTRODUCTION

Foodborne disease outbreaks are identified as a cause of multiple instances of a comparable illness across the world [1]. Food-borne pathogens are the major factor of foodborne illness and are common pathogens producing food safety issues to human health [2] [3]. Foodborne can be infected through the consumption of solid food containing bacteria including *Staphylococcus, Escherichia coli*, and *Escherichia* variants which are highly distributed in meat products [4], frozen food, and seafood, [5] that pose a significant public health hazard due to its high antibiotic resistance.

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Nano-sliver antibiotic agents are commonly used to overcome foodborne diseases and viruses as well. However, the production cost of nano-sliver antibacterial agents is high due to the depletion of mineral silver though applying a wide range of applications mostly digital electronic circuits can become a concerning issue. Several reviews of articles stated that the toxicity of applying nano-silver can raise health caution from human organs to active cells. [6] In recent studies, bioactive chemicals are found naturally in abundance at most plants' species and easily available for medicinal phytochemicals act as alternative ingredients to replace nano-silver antibiotic agents which are safer and more effective alternatives for the advancement of microbial resistance to synthetic medications. Limonene is one of the biochemicals dedicated to a major ingredient of fragrant plants and the most frequent terpene found in nature, especially in the essential oils from citrus fruits. D-limonene is generally accepted as safe to apply as a flavouring agent for consumption industries and sanitation or detergent products by well-known for its antimicrobial properties [7].

### 1.1 Theoretical Background

There are several paper reviews of are features mentioned on limonene addressed conducted experimentally in numerous investigations [7, 8, 9]. Most experiments were conducted on coating fruits for shelf-life extension through a biodegradable process with low antimicrobial resistance [7]. In contrast to antibiotics, essential oils are known to possess antimicrobial activity and have been evaluated mainly in liquid medium while the essential oils are highly volatile at room temperature which leads to the study of the efficiency of antimicrobial resistance against bacteria using limonene as an antimicrobial agent [7]. In this study, the aims are to identify the potential of limonene status bacteriostatic or bactericidal status against *Bacillus Subtilis* and *Escherichia coli*. The disc assay method is carried out to evaluate the antibacterial susceptibility against the two classes of the bacterium at control temperature by applying limonene along with selected solvents and determine the minimum inhibitory concentration (MIC) method on two classes of the bacteria at absorbance and isolation on an agar plate.

#### 1.1.1 MIC Method

Minimum inhibitory concentration (MIC) is a standard method to determine the susceptibility of microorganisms to antimicrobials for evaluating the performance compared to various methods of susceptibility testing. The MIC method is also utilized in diagnostics to confirm unusual resistance for delivering necessary results when compared to various methods including the disc diffusion method which is not ideal for determining the susceptibility of coagulase-negative staphylococci to teicoplanin. MIC also aims to find the lowest concentration of an antibacterial agent in mg/L or % (v/v) to prevent the observable growth of a test strain of an organism under closely regulated in vitro conditions. [10].

Moreover, the essential oil of Melaleuca alternifolia has a long history of functioning as an antiseptic focusing on the activity range of bacteria and fungi through numerous studies. Most essential oils composition generally consists of 50 per cent oxygenated monoterpenes and 50 per cent terpene hydrocarbons, with terpinen-4-ol as the main active component. 94 components have been identified as having a high level of notice in assessing the bioactivity of each essential oil chemical composition. The antimicrobial efficiency of a chemical is defined in terms of minimum inhibitory concentration (MIC), which is the lowest concentration compound capable of suppressing the growth of the challenged organism. For example, the disc diffusion assay method applied solvent or agent to a paper disc in the centre point on an agar plate seeded with the test microorganism or bacteria. However, the essential oil is unsuitable for testing due to low solubility easily cause movement at the agar plate [11]. While the broth and agar dilution methods are extensively used to establish the MIC of tea trees and various essential oils the ability to compare results from different research is limited due to discrepancies in test methodologies and criteria [12].

## 2. MATERIAL AND METHODS

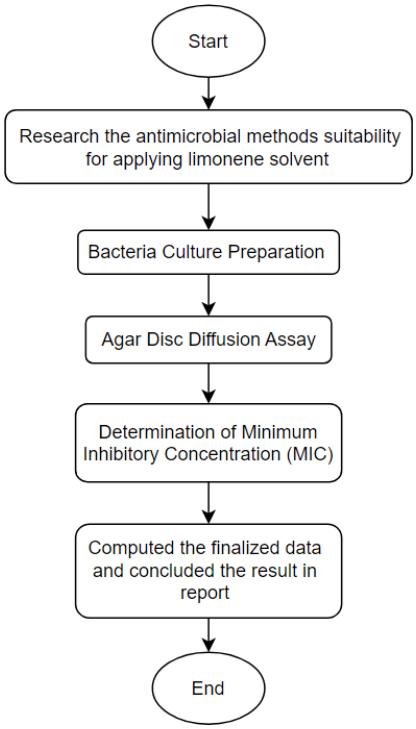



Figure 1 The flow chart of the antibacterial susceptibility process.

The experimental process is summarised in a flow chart illustrated in Figure 1, where it initials from bacteria culture preparation to disc diffusion assay and proceeds to the determination of the MIC method.

### 2.1 Bacteria Culture Preparation

The Luria Bertani broth (LB) was prepared for *Escherichia coli* and *Bacillus Subtilis* growth and media was used for the disc diffusion method. The LB solution is prepared in a glass bottle through the autoclaved process. The bacteria culture was prepared by using previous centrifuge stocks of *Escherichia coli* and *Bacillus Subtilis* and LB broth. A 100 µL solution of *Escherichia coli* was inoculated into the falcon tube alongside adding 500 µL of LB broth solution. The culture was placed in an incubator at 200 rpm, 30°C overnight according to the 0.5 McFarland standard, the bacteria cultures of *Escherichia coli* and *Bacillus Subtilis* were diluted with 5 ml of LB broth for measuring the absorbance at OD0.8±0.1 through the spectrophotometer [13].

### 2.2 Agar Disc Diffusion Assay

The autoclaved paper discs and solvents were prepared. 500 µL of *Escherichia coli* suspension used on bacteria lawing on LB agar. Moreover, the paper discs were dripped into each solvent in sequence from limonene, ethanol to the antibiotic, Kanamycin for placing the marked segments on the petri dish and placed in a shaking incubator at 37 °C overnight for promoting inoculum growth. Next day, all the triplicates will be examined on the presence of a clear zone on the limonene and antibiotic segment which will be computed the diameter in centimetres by using equations (1), equation (2), and (3) and recorded in tabulated data. The procedures were repeated for *Bacillus Subtilis* culture.

To compute the diameter of the clear zone, equation (1) will be applied.

$$D_F = D_{IC} - D_{PD} \quad (1)$$

The initial diameter of the paper disc, $D_{PD}$= 0.6 cm (measured in scale)
The initial diameter of the clear zone, $D_{IC}$
The final exterior ring of diameter of the clear zone, $D_F$

To compute the mean value, and standard deviation by applying equations (2) and (3)

$$\bar{x} = \frac{\sum_{i=1}^{N} x_i}{N} \quad (2)$$

$$\sigma = \sqrt{\frac{\sum_{i=1}^{N} x_i}{N} - (\bar{x})^2} \quad (3)$$

### 2.3 Determination of Minimum Inhibitory Concentration (MIC)

In the MIC section, the method performed using the broth dilution method is determining the lowest concentration of an antimicrobial agent and examining the bacterial colons on a series of agar plates through broth dilution containing dilutions of the antimicrobial agent. Referred to Table 1, the ratio of limonene concentrations and LB broth was set accordingly on each falcon tube for this study.

**Table 1** The parameter used to perform MIC

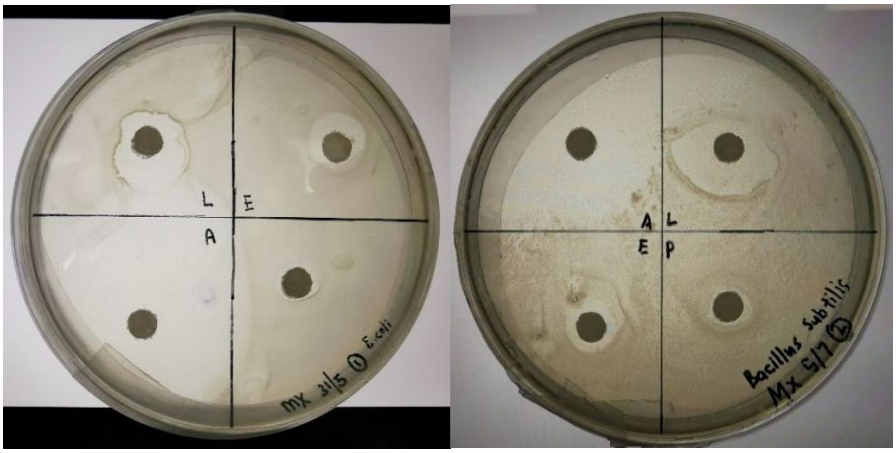
| No. | Description |
|---|---|
| A | Pure 10 ml Limonene + Pure 1 ml OD0.8 *Bacillus Subtilis* |
| B | Diluted 1 ml Bacteria suspension from Tube A with 9 ml pure LB Broth solution |
| C | Diluted 1 ml Bacteria suspension from Tube B with 9 ml pure LB Broth solution |
| D | Diluted 1 ml Bacteria suspension from Tube C with 9 ml pure LB Broth solution |

The LB broth and limonene suspension solution are required to mix to become homogenized for improved immersion. Moreover, 1 ml of OD0.8±0.1 of *Bacillus Subtilis* was added into the falcon tube A to become a base limonene bacteria suspension A, following the limonene bacteria suspension A will be added from falcon tube B to C and C to D through each consecutive dilution of LB broth. Each limonene bacteria suspension has measured the absorbance (OD) of *Bacillus Subtilis* recorded according to the sequence while using the spectrophotometer. This process is the three-tube categorization method which determines the susceptibility of anaerobes bacteria, *Bacillus Subtilis* and determines the minimum concentration of limonene for inhibition of the *Bacillus Subtilis* inoculum through incubation [14]. The limonene bacteria suspensions were placed in the shaking incubator at 200 rpm, 37°C overnight. Proceed to the next day, the absorbance of each limonene bacteria suspension was measured, and an additional 500 µL of limonene bacteria suspensions were inoculated on the TSB agar, and the limonene bacteria suspensions were placed in the incubator at 37°C overnight. The next day, the isolated *Bacillus Subtilis* colonies from the limonene bacteria suspensions will be examined and recorded.

## 3. RESULTS AND DISCUSSION

The disc diffusion assay method is used to test a conventional antibiotic, this involves creating a lawn of bacteria on an agar plate for the antibiotic disc to occur through incubation at 30°C overnight [9]. The optical density (OD) of *E. coli* and *Bacillus Subtilis* was required adjusted from OD1.0 to OD0.8±0.1 at 600mn necessary to estimate biomass in microbial and mammalian cell processes [15]. The spectrophotometer light scattered by a culture sample will depend on the concentration of cells, the species, and the strain of the microbe, and the growth calibration must be performed for accurate measurement of cell density which is a relevant parameter to monitor for performing the disc diffusion assay [15]. This method is relevant can cause antibiotic disc to diffuse into the agar to create a concentration gradient for measuring the zone of inhibition overnight to determine the effectiveness of the solvents against the bacteria resistance [16].

### 3.1 Limonene Susceptibility Testing Against *Escherichia Coli* and *Bacillus Subtilis*




| Short Terms | Full Description |
|---|---|
| L | Limonene |
| E | Ethanol |
| A | Antibiotic, Kanamycin |
| P | Paper disc |

**Figure 2.** The inhibition zone of *Escherichia coli* and *Bacillus Subtilis* with list of indications.

Based on Figure 2 displayed the reaction occurred of *Escherichia coli* and *Bacillus Subtilis* on these triplicates. The paper disc indicated as sterilized paper for negative control while the ethanol segment indicated to terminate the bacteria at concentration between of 80% to 85% for positive control [17].

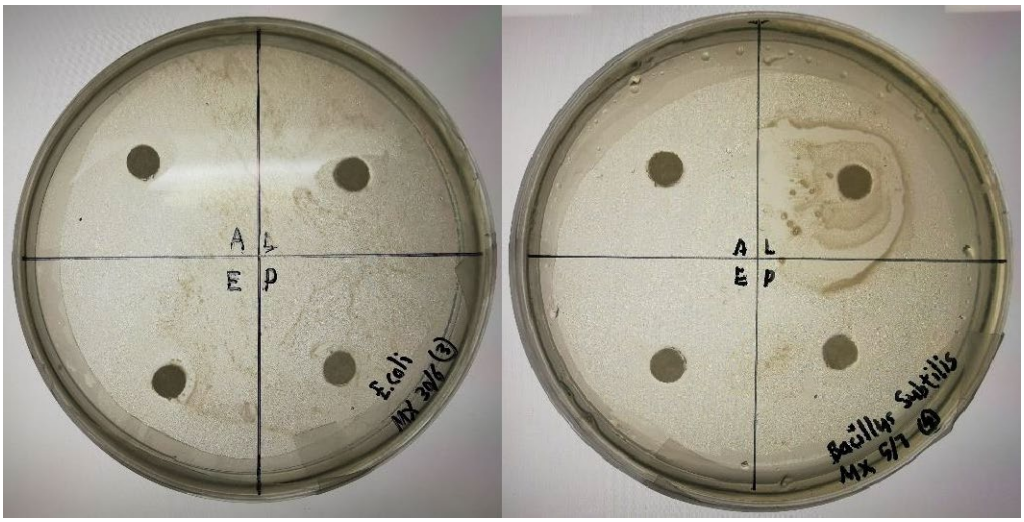



**Figure 3.** The clear zone does not occur in *Escherichia coli* and *Bacillus Subtilis*.

In addition, there are certain triplicates with diffused limonene unable to show antibacterial resistance against Escherichia coli and Bacillus Subtilis in Figure 3 which can conclude that limonene is inconsistent in occurring antibacterial resistance to the two classes of bacteria. Based on another research from Yılmaz & Karavana, 2020, also encountered similarities in the results where orange limonene has more effective against Escherichia coli in 24 hours while the antimicrobial effect can be considered as proliferation or nonproliferation in the assigned segment under the triplicates which also expressed as contact inhibition [18].

### 3.2 Disc Diffusion Assay

**Table 2** The mean value and standard deviation of *Escherichia coli* and *Bacillus Subtilis*

| Bacteria | Mean ± Standard Deviation (cm), $D_F$ |
|---|---|
| *Escherichia coli* | 1.067 ± 0.2055 |
| *Bacillus Subtilis* | 1.133 ± 0.3682 |

Based on the previous procedure on diffusion method where the tabulated data based on the final exterior ring diameter of inhibition zone paper disc referred to in Table 2. The tabulated data concludes that the mean and standard deviation of *Bacillus Subtilis* at 1.133 is higher than *Escherichia coli* 5.8 ± 44 % percent range that indicates that limonene at higher efficient antibacterial resistance to *Bacillus Subtilis* compared to *Escherichia coli*. This data can be validated by examining the clear zone produced by *Bacillus Subtilis* and *Escherichia coli*, both limonene segments referred to Figure 2. According to Lu Zhen Xiang's research, the mean value and standard deviation value of *Bacillus Subtilis* at 1.133 ± 0.3682 cm using the disc diffusion method are in range to the crude bacteriocin method on *Bacillus Subtilis* is at 11 mm (1.1 cm) which verified that the bacteriocins of Gram-positive bacteria has an excellent antibacterial effect leading to conclude that *Bacillus Subtilis* has effective of antibacterial resistance against limonene [19]. While the mean value and standard deviation value of *Escherichia coli* 1.067 ± 0.2055 cm using the disc diffusion method are also in range to the crude bacteriocin method on *Escherichia coli* at 10mm (1.0 cm) which defined that *Escherichia coli* has antibacterial effects that might function through bacteriocins and lead to autolysis where the process that breakdown part of cell by self-produced enzymes [19].

**Table 3** The mean value and standard deviation of initial diameter of inhibition zone produced by *Escherichia coli* and *Bacillus Subtilis*

| Bacteria | Mean ± Standard Deviation (cm), $D_{IC}$ |
|---|---|
| *Escherichia coli* | 1.667 ± 0.2054 |
| *Bacillus Subtilis* | 1.8 ± 0.8110 |

Based on Table 3 shown the tabulated data on the initial diameter of clear zone include the measurement of paper disc. By comparing the values of mean and standard deviation between *Escherichia coli* and *Bacillus Subtilis* still indicates that *Bacillus Subtilis* is higher than E*scherichia coli* by 7.38±74.67 percent. According to a researcher, Loo's research on using nano-particle silver implied that nano-particle silver has stable efficient antibacterial resistance against *Escherichia coli* at 15mm (1.5 cm) [20]. In this scenario, the comparison of limonene and nano-particle silver indicates that limonene is at higher producing diameter inhibition zone against *Escherichia coli* than using nano-silver by a range difference at 10 % even though the measurement of paper disc is included.

### 3.3 Dilution Method and Selective Agar

**Table 4** The optical density variation and the number of colonies grown on *Bacillus Subtilis*

| No. | Antibiotic | Optical Density (OD)at 600 nm *Bacillus Subtilis* | | Number of colonies |
|---|---|---|---|---|
| | | Before | After | |
| A | Limonene (10 ml) | 0.146 | 0.912 | TMTC |
| B | Dilution of 9 ml LB Broth from Limonene A (1 ml) | 0.834 | 0.809 | 2 |
| C | Dilution of 9 ml LB Broth from Limonene B (1 ml) | 0.617 | 0.809 | >200 |
| D | Dilution of 9 ml LB Broth from Limonene C (1 ml) | 0.727 | 0.809 | >20 |

Based on Table 4 can be observed that the A* limonene has the highest number of colonies produced on the TSB agar and the characteristic of *Bacillus Subtilis* colonies were attached to form the flat, larger quantity of irregular colonies which is verified as a too many to count [21]. While the B* diluted limonene produced the lowest colonies on TSB which has suppressed the bacteria growth. While the C* diluted limonene produced approximately 200 colonies on TSB and the characteristic of *Bacillus Subtilis* colonies has distance spacing between one colony to another. Lastly, the D* diluted limonene has approximately 20 colonies and the colonies' characteristic of *Bacillus Subtilis* has a light colour separating each space. The characteristics of *Bacillus Subtilis* colonies. Based on the observation in all cases, the concentration of limonene at 1 ml will activate antibacterial resistance against *Bacillus Subtilis*.

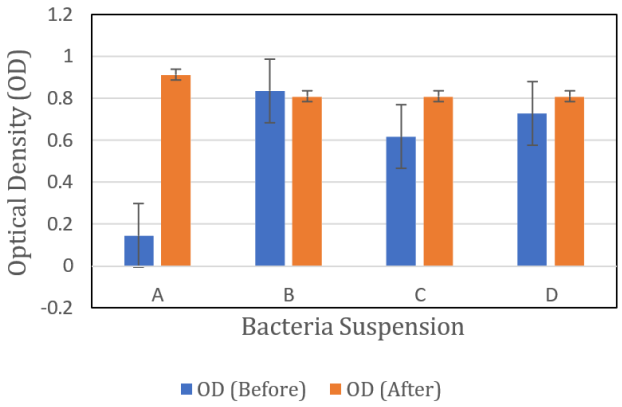



**Figure 4** The graph of relativity on optical density of *Bacillus Subtilis*.

Referring to Figure 4 the bar chart displayed the relativity OD of *Bacillus Subtilis* before and after incubation. In the A* section can be observed that before OD at 0.146 after the incubation at 24 hours, the OD increased to 0.912 which indicates that *Bacillus Subtilis* has promoted growth. This situation has a similarity to Han Ying Jie's research, where the membrane conductivity of *L. monocytogenes* increased when treated with limonene [22]. While the C* section showed an increment in OD from 0.617 to 0.809 and the D* section also showed an increment in OD from 0.727 to 0.809. Lastly, the B* section shows a slight decrease in OD from 0.834 to 0.809 which indicates that *Bacillus Subtilis* has shown suspended growth. The hypothesis on the observation indicates limonene is bacteriostatic which is defined as that limonene can only suppress the growth of bacteria at a minimal concentration dosage of limonene at 1 ml of 10 ml (10% v/v). In general terms, limonene is indicated as less susceptible to *Bacillus Subtilis*. The MIC values from the previous studies have displayed a large differential range through the variation of methods applied. In this scenario, the comparison of the OD results is difficult due to the lack of implying the proper standard method for the determination of antibacterial activity of applying limonene to other solvents [20].

## 4. CONCLUSION

In a nutshell, the findings suggest that limonene is not a stable source of alternative antibiotic chemicals to fight against gram-negative bacteria, *Escherichia coli* and positive bacteria, *Bacillus Subtilis* due to recent studies that limonene is an essential oil which has lower solubility and density resulting limonene unable to dissolve with bacteria suspension. Through analyzation in the disc diffusion assay, the limonene was analyzed by measurement of the clear zone or inhibition zone which indicates that *Bacillus Subtilis* has the highest diameter clear zone (cm) at a standard deviation and mean value of 1.133 ± 0.3682. Therefore, limonene is defined as effective against *Bacillus Subtilis* in antibacterial properties. However, the MIC values obtained in the current study indicate that limonene is bacteriostatic at a minimal concentration dosage of 1 % (v/v) with single broth dilution which has displayed the *Bacillus Subtilis* optical density (OD) decreased by approximately 3 % overnight incubation while the *Bacillus Subtilis* colonies shown at petri dish B displayed 2 colonies produced on TSB agar. In a nutshell, limonene is identified as a bacteriostatic agent and is more efficient in antibacterial resistance compared to nano-particle silver against *Escherichia coli* through analysis of the comparison of the diameter of the inhibition zone. This research proves that limonene has the moderate capability to replace nano-silver in modern medicine due to has good antimicrobial resistance against *Escherichia* bacteria. Limonene is a natural bio-chemical which has lower oral and dermal toxicity, which is safe for humans to apply in the medical field and insect-repellent products are eco-friendly to the environment. Hence, further research on limonene is recommended by enhancing the solubility of limonene to improve antibacterial effect consistency on the MIC method and disc assay.

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