

PHYSICOCHEMICAL PROPERTIES, GC-MS ANALYSIS, AND ANTIBACTERIAL ASSAY OF *Citrus limon* PEELS ESSENTIAL OIL AGAINST ANTIBIOTIC RESISTANT *Escherichia Coli*

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ABSTRACT

Essential oils (EOs) produced naturally by plants have been used as antimicrobial agents despite the majority of compounds have not been studied. The study was conducted to assess the effect of EOs found in *Citrus limon* peels against antibiotic resistance of *E. coli* by quantifying the specific gravity, refractive index, and GC-MS. The result of GC-MS presented that D-limonene, α -citral, and β -citral are the major constituents of *C. limon* EO, presenting 56.85; 13.24; and 10.40% of the oil content, respectively. Tests on the essential oil of *C. limon* confirm its potential antibacterial activity. This assertion was supported by the MIC and MBC values obtained at 250 μ g/mL and 500 μ g/mL, respectively. Meanwhile, tetracycline showed identical MIC and MBC at 125 μ g/mL. The interaction between essential oil and tetracycline was evaluated using a checkerboard assay. Moreover, *C. limon* peels essential oil had a synergistic to decrease biofilm formation in bacterial tested. In addition, the combination of *C. limon* peels essential oil and tetracycline illustrated potential activities to lysis the cells and inhibit the efflux pumps of *E. coli*. The result suggests the combination of *C. limon* peels essential oil and tetracycline could be used to eradicate antibiotic-resistant *E. coli*.

Keywords: Citrus Limon, GC-MS, Antimicrobial, Biofilm, Membrane Permeability, Efflux Pump.

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INTRODUCTION

The commitment to ensuring food safety is necessary, due to this concern reaching prominence in middle-income countries around the world.¹ Therefore, multiple food industries vie to enhance product safety. Meanwhile, the majority of foodborne infections are caused by *Campylobacter*, *Salmonella sp*, and *Escherichia coli* which are known as the most common types of bacteria that cause food-borne illnesses.² It is believed that people have applying the EO for industrial markets (perfumes and flavors) for ages, however, there has been a focus on their putative antibacterial properties.^{3,4,5} Citrus is economically beneficial for preventing infectious organisms.⁶ The EOs of *Citrus sp*. have been reported for their pharmacological activities, such as antioxidant and anticancer.⁶ Moreover, other studies revealed *Citrus* EO activity against foodborne pathogens; *Listeria spp*⁴, *Salmonella spp*, *Escherichia coli*³, *Campylobacter jejuni*, *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus*. This study assessed the peel's chemical constituents for multiple antibacterial resistance mechanisms, such as antibiofilm, efflux pump inhibitor, membrane permeability activities as well as the synergistic effect of *C. limon* EOs with tetracycline.

EXPERIMENTAL

Materials

The materials such as tetracycline, phosphate buffer saline (PBS), Tablets of crystal violet, anhydrous sodium sulfate, Dimethyl sulfide (DMSO), and microbial agar were purchased from several notorious pharmaceutical companies, such as Sigma-Aldrich, Merck, Becton, and Dickinson for analytical purposes.

Bacterial Strains

The bacterial resistance was obtained by the MERO Foundation, Bali, Indonesia.

Essential Oil Extraction

Citrus limon was collected from Medan, North Sumatera, Indonesia. The procedures refer to the previous study⁷ with slight modification. The essential oils from *C. limon* peels were extracted using hydrodistillation in a Clevenger apparatus and modified for 6 hours, with distilled water.⁷

Determination of the Physicochemical Properties of *Citrus limon* Essential Oil

The determination of *C. limon* essential oil is divided into two tests, there are the specific gravity and refractive index, which are benefits to illustrate the purity and quality of the EO. A pycnometer in 1-ml volume is used to calculate the specific gravity, whereas an Abbe Refractometer is applied for counting the refractive index.

Chemical Compositions Analysis of Essential Oil

Analysis of the chemical compositions of *C. limon* essential oil using GC-MS.⁸ Briefly, the data were collected and determined by referring to the National Institute of Standards and Technology (NIST) WebBook, to obtain the peak area of each chromatogram for identifying and calculating.

Determination Antibacterial Activity

Antibacterial activity was determined by using the microdilution method to reach the minimal inhibitory concentration (MIC) by several modifications to previous studies.^{9,10} Briefly, bacterial suspensions were adjusted to 0.5 McFarland and diluted with 0.9% sodium chloride to yield 1×10^6 CFU/mL. Furthermore, the bacterial suspension was then added to 96-well microplates containing twofold dilutions of *C. limon* essential oil and 24 hours of incubation at 37 °C.

Checkerboard Assay

This test was conducted using the checkerboard method^{8,11} with several modifications. Briefly, *C. limon* essential oil was diluted twofold with BHI along the x-axis of microplates, while the y-axis for antibiotics a similarly diluted. Each well was then filled with a bacterial suspension containing approximately 1×10^6 CFU/mL and stored for at least 24 hours at 37 °C.

Anti-biofilm Formation Assay

Antibiofilm formation was tested by using 96-well microplates.⁸ Briefly, the wells were filled with sample and bacteria suspension, then incubated at 37 °C for 24-48 hours, while the control consisted of untreated wells. After one day, rinse the wells with PBS and incubate with 200 ml of crystal violet (0.3% in DMSO) added selectively. The degradation of biofilm was subsequently measured at 560 nm.

Loss of 260 nm Absorbing Material

The overnight culture of the bacterial was cleaned and resuspended in 0.9% sodium chloride solution, and the concentration was made at 5×10^8 CFU/mL, by combining *C. limon* EO and Tetracycline at MIC, and synergy concentration, while the control was given no treatment. Each sample was saved in an incubator for a day at 37 °C. The optical density measured by the UV-Vis Spectrophotometer was 260 nm. This assay was taken thrice. This test was conducted with several modifications compared to the previous study.⁸

Bacteriolysis Activity

A bacteriolysis test was conducted as stated by the previous study.^{12,8} Briefly, *E. coli* bacteria from an overnight culture were suspended in normal saline. After obtaining the final concentration at 0.5 McFarland, the synergistic combination of *C. limon* essential oil and tetracycline was added to the bacterial suspensions and cultured in an incubator at 37 °C. Tetracycline and DMSO were used for control. The measurement by

using a UV-Vis spectrophotometer, the absorbance of the supernatant at 260 nm (OD260) and 590 nm (OD590). This assay was taken thrice.

Quantify the Total Amount of Ethidium Bromide (EtBr)

E. coli were cultivated in 10 ml of Sodium Chloride until they reached the mid-log phase, which can be detected at 600 nm. Based on the MIC and FICI of *C. limon* EO, 50 μ L of *C. limon* EO was added to a 96-well black microplate to create the sample for analyzing EPI. 100 μ L of *E. coli* suspension was added, and the final concentration was calculated to be 0.4 mg/L. Furthermore, the 96-well was saved in an incubator at 37 °C for 30 minutes. In addition, 50 mL of EtBr was applied to the microplates, and the absorbance of the bacterial EPIs was detected. The fluorescence plate reader was used to conduct the EtBr accumulation assay (wavelength excitation of 530 nm and emission of 605 nm), and the absorbance was measured four different times (0, 5, 15, and 45 minutes). The results are calculated by the value of the Relative Fluorescent Unit (RFU). The method was conducted as previously described with several modifications.¹³

RESULTS AND DISCUSSION

Identifications, Yields, and Chemical Profiles of *Citrus limon* Essential Oil

In this study, *C. limon* was identified at Medanense Herbarium Plant Systematics Laboratory (MEDA), Medan, Indonesia. As a result, the values of yield, specific gravity, and refractive index were 0.9%; 0.853; and 1.4632, respectively which indicate the quality of the EO obtained.¹⁴ Table-1 shows around 40 compounds were detected. Monoterpene was found as the most abundant compound with a percentage area of 60.36%. As mentioned by a previous study, the constituent of *C. limon* essential oil was limonene at 43.07¹⁵; 68.7¹⁶, and 90.41%¹⁷, this varies depending on plant variety and geographical region.¹⁸

Table-1: Chemical Constituents of *Citrus limon* EO identified by GC-MS

No.	Determined Constituents	Area (%)	RT (Min)
Monoterpene			
1	1R- α -Pinene	0.4	6.469
2	β -Pinene	0.7	7.225
3	B-myrcene	2.14	7.377
4	D-Limonene	56.85	8.183
5	γ -Terpinene	0.06	8.561
6	Neo-allo-ocimene	0.21	9.607
Oxygenated Monoterpene			
1	Linalool	1.83	9.179
2	Cis-Limonene oxide	1.04	9.746
3	Citronellal	0.19	9.998
4	Isoneral	0.25	10.137
5	3,6-Octadienal, 3,7-dimethyl-	0.50	10.414
6	I-4-terpineol	0.20	10.502
7	L- α -Terpineol	1.47	10.729
8	Cis-Geraniol	3.33	11.170
9	β -Citral	10.40	11.384
10	Lemonol	2.49	11.523
11	α -Citral	13.24	11.813
12	Nerol	0.14	12.922
13	Trans-2-Decenol	0.15	13.653
14	α -Citral	0.27	21.114
15	Nerolic Acid	0.21	22.236
Sesquiterpene			
1	α -Trans-Bergamotene	0.65	13.981
2	β -Sesquiphellanderene	0.05	14.182
3	β -Cis-Farnesene	0.07	14.661
4	Beta-Bisabolene	0.86	14.926
Oxygenated Sesquiterpene			
1	Trans-Farnesol	0.14	12.115

2	Caryophyllene oxide	0.04	15.972
3	α -Bisabolol	0.09	17.119
Non-Terpene Derivatives			
1	Ocatanal	0.31	7.692
2	1,1-Dimethyl-decyl-mercaptan	0.18	8.776
3	Nonyl chloroacetate	0.16	10.301
4	Tridecanal	0.25	12.267
5	Geraniol formate	0.25	13.199
6	Farnesyl acetate	0.79	20.811
Monoterpene		60.36	
Oxygenated Monoterpene		35.71	
Sesquiterpene		1.63	
Oxygenated Sesquiterpene		0.27	
Non-Terpene Derivatives		1.94	
Total Identified		100%	

Antibacterial Activity

According to the study, *C. limon* EO exhibited significant antibacterial activity for combating bacterial resistance of *E. coli* strains. This finding was in agreement with a previous study that reported that terpene derivatives found in *Citrus* EO, including limonene, nerol, and myrcene were known to inhibit both gram bacteria (*Staphylococcus aureus* and *E. coli*).¹⁹

Checkerboard Test

The checkerboard test resulted in a synergistic effect between *C. limon* EO and tetracycline. The majority of *C. limon* EO components are known to diffuse in bacterial membranes, causing cytoplasmic loss, and modification of ion transports.²⁰ Therefore, *C. limon* EO and tetracycline function synergistically against the bacteria.²¹ (Table-2)

Table-2: The Result of the Checkerboard Test Between *C. limon* EO and Tetracycline

<i>Citrus</i> EOs- Tetracycline	MIC k ($\mu\text{g/mL}$)		MIC m ($\mu\text{g/mL}$)	FIC Index	Interaction
<i>Citrus limon</i>	250		7,8	0.5	Synergy
Tetracycline	125		62,5		

Note: k = alone; m = combination

Antibiofilm Formation

Microorganisms were capable of forming biofilms.^{22,23} As shown in Fig.-1, this result indicated that less concentration of *C. limon* EO allowed to inhibit biofilm formation by twofold more than *C. limon* EO alone. Previous studies also supported this result by mentioning that limonoids adequate to inhibit cell signaling of quorum sensing and biofilm.^{23,24}

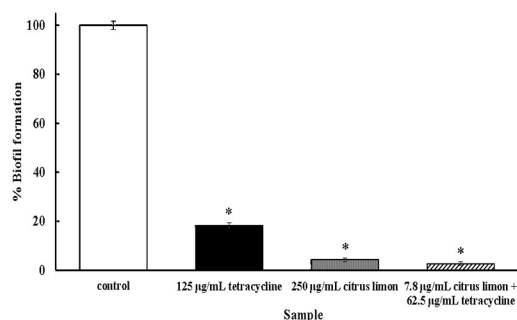


Fig.-1: The Effect of Essential Oils of *Citrus limon* and Tetracycline in Biofilm Degradation. *P<0.05 Significant Different with Respective Control

Loss of 260 nm Absorbing Material

In Fig.-2, it can be seen that the combination of *C. limon* EO and tetracycline disrupted *E. coli* cell leakage compared to other samples. Previous study supports this result, by mentioning that essential oil consists of multiple compounds enabled to impair membrane structure and cause a loss of membrane integrity.^{25,26}

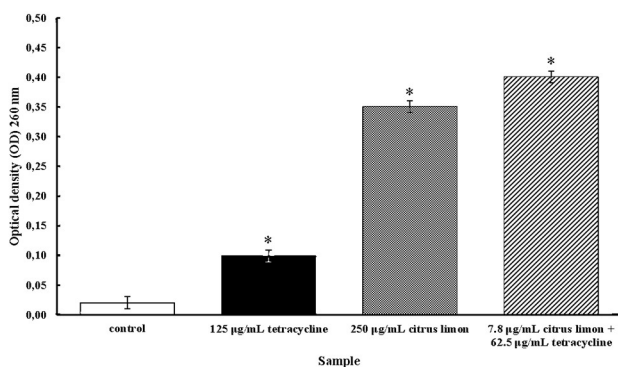


Fig.-2: Optical Density (OD) 260 nm Bacterial Resistant of *E. coli* Treated with Essential Oils of *Citrus limon* and Tetracycline. *P<0.05 Significant Different with Respective Control

Membrane Permeability Activity

According to the results, the mixture of *C. limon* essential oil and tetracycline was able to lyse *E. coli* cells by absorbing more than half of the crystal violet (55%) compared to *C. limon* essential oil and control (Fig. 3). In this part, the increase in crystal violet absorption is likely attributable to the expansion of essential oil-treated bacterial cells.²⁷

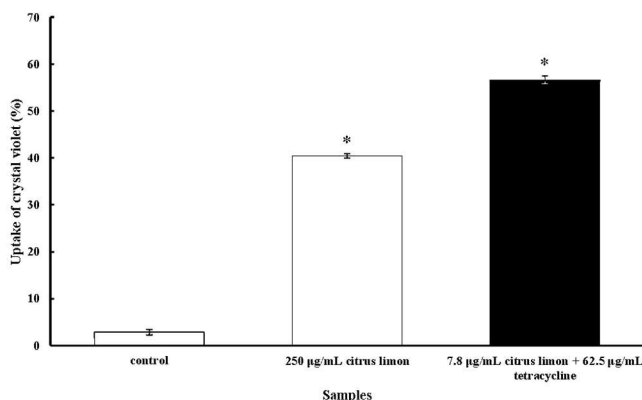


Fig.-3: The Uptake of Crystal Violet by *E. coli* after Treatment with Essential Oils of *Citrus limon* and Tetracycline. *P<0.05 Significant Different with Respective Control

Quantification of Intracellular EtBr Concentration

The results in Fig.-4 can be concluded that the combination of *Citrus limon* essential oil showed the ability to block multidrug efflux pump in a clinical isolate of *E. coli*, which indicates their potential compounds for a wider range of pump-inhibiting activities against multidrug-resistant pathogens.

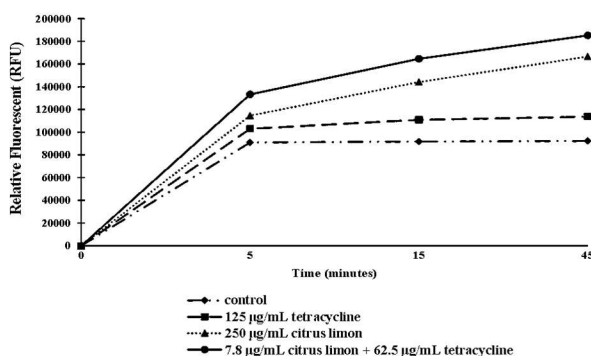


Fig.-4: EtBr Efflux Assay of *Escherichia coli* after Treatment with Essential Oils of *Citrus limon* and Tetracycline

Terpene derivatives were also reported as significant efflux pump inhibitors (EPIs) in a wide range of bacterial strains.²⁸ The spread of infection by the bacterial resistance of *E. coli* has been expanding

worldwide and caused foodborne outbreaks for the last five years.²⁹ Regarding this condition, there is an urgent to seek an alternative approach to overcome the outbreaks. Utilizing natural antibacterial agents and antibiotics with a synergistic effect is a prominent method for combating this.

CONCLUSION

The essential oil of *C. limon* has antibiotic activity against gram-negative microorganisms. In addition, multiple mechanisms of *C. limon* essential oil to inhibit tested bacterial strains have been reported, including inhibiting biofilm formation, disrupting membrane permeability, and producing efflux pump inhibitors to inactivate efflux pumps in most resistant *E. coli* strains. Therefore, to evaluate the bioavailability, efficacy, and toxicity of *C. limon* essential oil combined with antibiotics, additional in vivo studies are urgently required.

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CONFLICT OF INTERESTS

There is no conflict of interest, according to the authors.

AUTHOR CONTRIBUTIONS

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