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Research article

Kinetics Study of Antibacterial Activity of Cajuput Oil (*Melaleuca cajuputi*) on *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*

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Abstract

The kinetics of antibacterial activity of cajuput oil (*Melaleuca cajuputi*) to *Escherichia coli*, *Bacillus cereus*, and *Staphylococcus aureus* were investigated. The aim of this study was to determine the reaction orders of cajuput oil as an antibacterial agent. The extraction of cajuput oil was conducted by water-steam distillation. The yield was 0.88%. GC-MS analysis showed that the cajuput oil contained β-ocimene (19.35%), 1.8-cineole (17.67%), limonene (12.09%), β-caryophyllene (9.51%), γ-terpinene (8.93%), 2-β-pinene (8.85%), α-terpinolene (4.96%), α-humulene (4.10%), α-terpineol (2.83%), and p-cymene (2.33%). The extract showed antibacterial activity to *E. coli*, *B. cereus*, and *S. aureus*, with the reaction orders of 0.4460, 0.8235 and 0.6928, respectively.

Keywords

cajuput oil;
antibacterial;
Escherichia coli;
Bacillus cereus;
Staphylococcus aureus;
kinetics;
Melaleuca cajuputi

1. Introduction

Plants are the sources of medicinal compounds that have been used by humans for a long time [1]. Plants are used in this way because they have various components, such as essential oils (EOs), which can have significant potencies as medicinal ingredients [1, 2]. EOs can be defined as plant secondary metabolites that are volatile and provide either tastes or smells [3]. These secondary metabolites are classified as terpenoids producing from the pathway of isoprenoid. They are produced and secreted from specific issues of plants. Terpenoid compounds can damage the cell walls and mitochondria of bacteria [4, 5]; thus, some terpenoid compounds found in essential oils are prospective antibacterial agents.

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The search for new antibacterial compounds that can overcome the disease caused by *Escherichia coli*, *Staphylococcus aureus,* and *Bacillus cereus* is necessary. All types of these bacteria are detrimental to human beings as they adversely affect food quality and safety [6, 7]. *Staphylococcus aureus* and *B. cereus* can cause food poisoning and other diseases such as skin and systemic infection [5]. *Bacillus cereus* can also cause pneumonia, bacteremia, and meningitis in humans with impaired immune systems [8-10]. While *E. coli* (gram-negative) presents a pathotype that may be responsible for extraintestinal infections of the urinary tract and other diseases related to food poisoning [5].

One of the sources of prospective essential oils is cajuput oil, which is cultivated massively in Indonesia. Cajuput oil is widely used as a traditional medicine to relieve flatulence, nausea, colds, and itchiness [11, 12]. Even though the oil has been widely used, there are a few research reports on antibacterial activity of cajuput oil. Studies of cajuput oil have been limited to the measurement of its bacterial inhibition zone at one concentration [1, 3, 13, 14]. Therefore, this research aims to determine the effect of varying cajuput oil concentration on its inhibition activity against three bacteria, i.e. *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*. Chemical kinetics study was conducted by determining the reaction orders. The order of reaction is known to represent the significant effect of the essential oil concentration to the inhibition of the bacterial activities [15]. In this study, the reaction order values, as indicators of the effect of essential oil concentration on inhibitory activity on bacteria, were validated by a statistical test.

2. Materials and Methods

2.1 Materials

The materials used in this research were cajuput oil (obtained from the Kelang islands - Maluku, Indonesia), *Escherichia coli* ATCC 14579^T, *Staphylococcus aureus* ATCC 25925^T, *Bacillus cereus* ATCC 35218^T, Amoxicillin, tween 20 (Merck), anhydrous $MgSO_4$ (≥99.5%, Merck), distilled water and nutrient agar (Himedia). All chemicals used were analytical grade.

2.2. Isolation of cajuput oil

A total of 2.5 kg of cajuput leaves were soaked into a water-steam distillation chamber that was filled with 10 l of water. The distillation was conducted for 4-6 h. The distillation product was a two-phase liquid, which was then separated in a separating funnel and followed by the addition of anhydrous $MgSO_4$ powder to remove the remaining water in the oil layer.

2.3. GC-MS analysis of cajuput oil

The content of cajuput oil was analyzed by a Gas Chromatography-Mass Spectrometry (GC-MS, SHIMADZU QP-5000). The type of column used was a Rastek DB-5MS (0.25 mm x 30 m); Helium was used as the gas carrier with a 42 ml/min flow rate. The temperature of the injector and detector was 250°C. The column temperature was started at 80°C and held for 2 min, and then was raised by 5°C/min to 250°C. Meanwhile, the mass spectrometer conditions were 70 eV of ionization energy, split ratio of 25, and detection area of 40-500 m/z. The parameters were setup to comply with the MS index data of the Wiley library.

2.4. Antibacterial activity testing

The disk-diffusion agar method was used to determine the antimicrobial activity of the cajuput oil. A suspension of bacterial cells (1×10^5 CFU/ml) was spread on a solid nutrient agar (NA) medium. Filter paper disks (diameter 6 mm) were soaked in various concentrations of cajuput oil (100%, 75%, 50%, 25%). The variations in concentration of cajuput oil were made by diluting cajuput oil with tween oil to give the desire concentration in v/v. Amoxicillin, which was used as a positive control and tween oil as a negative one, were then placed on the NA media. All disks were placed at a temperature of 4°C for 2 h, followed by incubation at a temperature of 37°C for 24 h. The inhibition zone diameters were measured and expressed in millimeters at the various times of 12 h, 18 h, and 24 h. All of these treatments were repeated three times.

2.5. Data processing and analysis

The inhibition zone measurement results were then displayed in the graphs to show a comparison of the inhibition activity of each bacterial test. Rate of inhibition zone formation was determined from the average data of inhibition zone per incubation period for each bacterial concentration. The rate of the formation of inhibition zones and the concentrations obtained were then used as data to analyze chemical kinetics. The relationship between antibacterial activity and concentration (dose or total content) [16-20] is described by equation (1).

$$ZoI \approx [A] \tag{1}$$

ZoI is the zone of inhibition (ZoI) which was formed during reaction. Meanwhile, A is the concentration of A. Decomposition of A during reaction is described by equation (2).

$$\frac{d(ZoI)}{dt} \approx -\frac{d[A]}{dt} \tag{2}$$

Therefore,

$$r_{ZoI} \approx r_{[A]} \tag{3}$$

Thus, mathematics will be applicable:

$$r_{ZoI} \approx K\, r_{[A]} \tag{4}$$

and because

$$r_{[A]} = k\,[A]^n \tag{5}$$

then:

$$r_{ZoI} = K.k\,[A]^n \tag{6}$$

which can be simplified to:

$$r_{ZoI} = k'\,[A]^n \tag{7}$$

Where $k' = K.k$

If both sides are applied against the logarithm, then the following was obtained:

$$\ln r_{ZoI} = \ln k' + \ln\,[A]^n \tag{8}$$

Linear regression of equation (8) by plotting ln [A] as X and ln r_{ZoI} as Y, would find the slope as the order of reaction. The reaction orders obtained were then compared using the one-way ANOVA statistical test to compare the results. The test results were determined by the following hypothesis criteria:

H_o is acceptable if $F_{score} \leq F_{tab\ (\alpha = 0.01)}$

H_a is acceptable if jika $F_{score} > F_{tab\ (\alpha = 0{,}01)}$,

With the following test criteria:

H_o is accepted if all group variants have the same value

H_a is accepted if there is at least one group variant with different values

3. Results and Discussion

3.1. Isolation and characterization of cajuput oil

The isolation of cajuput oil was performed by water-steam distillation. During the distillation process, water vapor flowed and penetrated the skin tissues of the cajuput plants. Essential oils were taken through the process of hydro diffusion [21]. The oil-water mixture diffused through the membranes during osmosis. The membranes on materials' surface were enlarged and further evaporation of steam volatile components occurred, with the components then flowing into the condenser. The results of the distillation process were two layers; one of water, the other of oil. The oil layer, which contained the essential oil of cajuput which, was then separated from the water layer.

The remaining water in the cajuput oil was then removed by the addition of anhydrous $MgSO_4$. Finally, a clear water-free cajuput oil yield of 0.88%, was obtained, a yield that was in accordance with Idrus *et al.* [22], who reported that the yield of cajuput oil isolated by the steam distillation method ranged from 0.33 to 1.25%. The process's low results might have occurred due to several influencing factors, such as cultivation techniques, post-harvest handling, and distillation process conditions [23]. The GC-MS chromatogram of cajuput oil is shown in Figure 1. Meanwhile, the constituent components along with retention times and % areas are listed in Table 1.

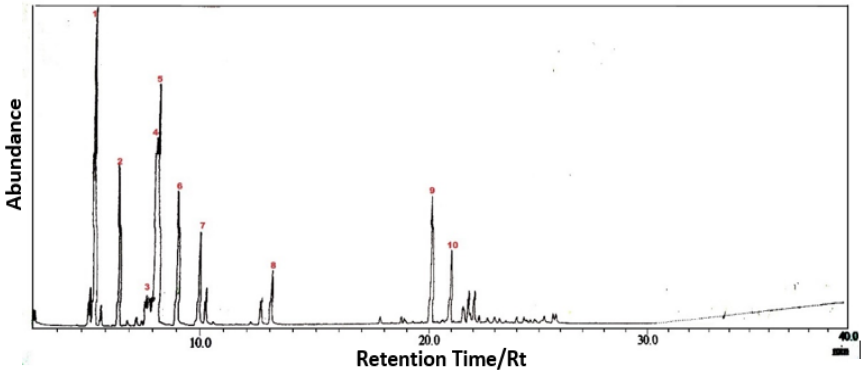

Figure 1. Chromatogram of cajuput oil from gas chromatography

Table 1. The constituent components, retention time and % area of cajuput oil from mass spectrometry

No.	Compounds	Retention time (min)	%Area
1.	β-ocimene	5.565	19.35
2.	2-β-pinene	6.575	8.84
3.	p-cimene	7.883	2.33
4.	1.8-cineole	8.127	17.67
5.	limonene	8.229	12.09
6.	γ-terpinene	9.053	8.93
7.	α-terpinolene	9.954	4.96
8.	α-terpineol	13.038	2.83
9.	β-caryophillene	20.121	9.51
10.	α-humulene	20.962	4.10

Three bacteria (*E. coli, S. aureus,* and *B. cereus*) were used to determine the effect of various concentrations of cajuput oil as an antibacterial agent. Nutrient agar (NA) was chosen as a medium because it was the most commonly used for bacterial cultivation [24]. The disk-diffusion method was applied because of its low price and high flexibility [25]. The clear zones that formed as an indication of the antibacterial inhibition of cajuput oil on *E. coli*, *S. aureus,* and *B. cereus* were presented in Figure 2. On the other hand, the clear zone diameters of varied concentrations for the bacteria test were displayed in Table 2. The measurement results revealed the increasing diameters of the inhibition zones over time. The inhibition categories based on the different concentrations of cajuput essential oil applied to the bacteria after incubation period are listed in Table 3. The classification of antibacterial inhibitory effects were: insensitive (diameter ≤ 8.0 mm), moderately sensitive (8.0 <diameter <14.0 mm), sensitive (14.0 <diameter < 20.0 mm), and extremely sensitive (diameter ≥ 20.0 mm) [26]. Table 2 showed that the inhibition zone increased when a higher concentration was used. This finding indicated that the inhibition zone diameter was dependent on the cajuput oil concentration and the incubation period. The obtained data were in accordance with the results of antibacterial tests reported by Musta *et al.* [20] and Bereksi *et al.* [27]. Compared with the positive control groups, the cajuput oil was less effective as an antibacterial against *B. cereus*. The positive controls produced the clear zone diameter, which was greater than the diameter of the clear zone formed with the use of cajuput oil on *B. cereus* at all concentrations. The results of each bacterial test's clear zone for every time interval were used for determination of the formation rate of the clear zone, as presented in Table 4.

The data of the formation rate of a clear zone from several concentrations for each bacterial test was then used to analyze the chemical kinetics, as shown in Figure 3. Antibacterial reactions exhibited the orders of reaction, k' and r^2, respectively for *E. coli*: 0.4460, 2.3622, and 0.9928; *S. aureus*: 0.8235, 0.0909 and 0.9026; and *B. cereus*: 0.6928, 1.1753, and 0.9715. Different reaction orders might produce different curves eventually. All of the reaction orders obtained were in the range of 0 and 1. A zero order reaction indicates that the change in concentration does not affect the rate of reaction; and an order of 1 means that the concentration has a linear effect on the rate of the reaction [28]. Based on this statement, variation of the cajuput oil concentration affects the rate of formation of the inhibition zone. However, the effect is not high (not linear). The differences in reaction orders showed that the clear zone formation rates were *E. coli* < *S. aureus* < *B. cereus*, but based on the results of statistical tests with the one-way ANOVA test, it was shown that F_{score} for testing on *E. coli*, *S. aureus*, and *B. cereus* were 2.87, 2.10, and 4.42, respectively, all of which were

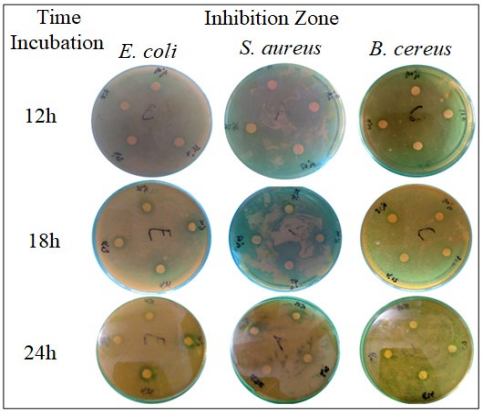

Figure 2. The clear zone of each bacterial test during a specific time interval of the incubation period

Table 2. Inhibition activities of cajuput oil on *E. coli, B. cereus*, and *S. aureus*

No.	Bacteria	Treatment	Zone of Inhibition Diameter (mm)		
			12 h	18 h	24 h
1.	*E. coli*	100%	12.53±0.17	13.73±1.03	14.77±0.68
		75%	11.17±0.51	13.13±0.17	14.07±1.08
		50%	8.97±0.85	9.73±1.25	13.23±5.30
		25%	7.17±0.51	7.23±0.73	7.87±0.23
		Tween Oil	0.00±0.00	0.00±0.00	0.00±0.00
		Amoxicilin 2%	8.07±0.30	8.3±1.57	9.19±0.17
2.	*B. cereus*	100%	11.97±0.65	12.53±0.50	12.77±0.68
		75%	11.07±0.68	12.43±0.75	12.63±0.58
		50%	7.33±0.89	7.83±0.41	8.63±0.35
		25%	2.43±0.42	6.73±0.23	7.83±0.40
		Tween Oil	0.00±0.00	0.00±0.00	0.00±0.00
		Amoxicilin 2%	14.63±0.43	18.43±1.82	19.97±0.91
3.	*S. aureus*	100%	5.63±5.23	12.77±1.08	13.17±3.19
		75%	0.00±0.00	9.33±0.70	9.73±3.39
		50%	0.00±0.00	8.53±1.82	8.63±1.88
		25%	0.00±0.00	3.01±0.96	7.63±0.85
		Tween Oil	0.00±0.00	0.00±0.00	0.00±0.00
		Amoxicilin 2%	0.00±0.00	2.43±0.42	5.97±1.34

Table 3. Different levels of inhibitory activity of each varied concentration on *E. coli, B. cereus*, and *S. aureus*

No.	Bacteria	Concentration	Category of Inhibition
1.	*E. coli*	100%	Sensitive
		75%	Sensitive
		50%	Moderately Sensitive
		25%	Insensitive
2	*B. cereus*	100%	Moderately Sensitive
		75%	Moderately Sensitive
		50%	Moderately Sensitive
		25%	Insensitive
3.	*S. aureus*	100%	Moderately Sensitive
		75%	Moderate Sensitive
		50%	Moderately Sensitive
		25%	Insensitive

Table 4. Inhibition zone formation rate of various concentrations of cajuput oil on *E. coli*, *S. aureus* and *B. cereus*

No.	Treatment	Inhibition Zone Formation Rate (mm/h)		
		E. coli	*S. aureus*	*B. cereus*
1.	100%	0.8066	0.5758	0.7418
2.	75%	0.7481	0.3074	0.7131
3.	50%	0.6127	0.2773	0.4677
4.	25%	0.4417	0.1616	0.3011
5.	Amoxicilin 2%	0.5077	0.1279	0.8960
6.	Tween Oil	0.0000	0.0000	0.0000

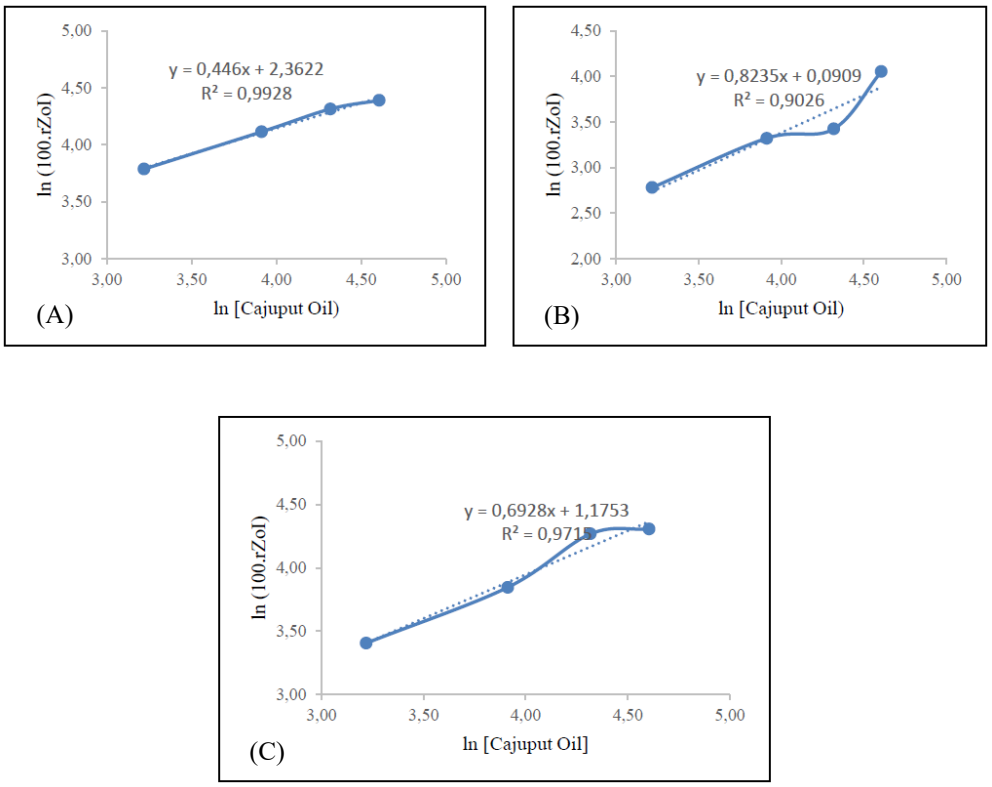

Figure 3. Analysis of chemical kinetics antibacterial activity of cajuput oil on *E. coli* (A), *S. aureus* (B), and *B. cereus* (C) in regression with plot ln [cajuput oil] vs ln (100.rZoI)

smaller than F_{tab} ($\alpha = 0.01$) = 7.59. These results indicated that there was no significant difference in the effect of variations in the concentration of cajuput oil on the average formation rate of the zone of inhibition for all tested bacteria. In other words, all of the concentrations used resulted in a relatively similar formation rate of the zone of inhibition and, therefore, the use of cajuput oil at low concentrations as an antibacterial against *E. coli*, *S. aureus*, and *B. cereus* is an efficient choice. Swamy *et al*. [29] reported that even at 1/100 dilution, some essential oils are capable to inhibit microbial activity.

4. Conclusions

Cajuput oil has varying inhibitory effects on *E. coli*, *B. cereus,* and *S. aureus*. The formed zone of inhibition was an outcome dependent on the cajuput oil concentration and the incubation period. The power of activity of cajuput oil on *E. coli*, *S. aureus* and *B. cereus* was indicated by the reaction orders of 0.4460, 0.8235, and 0.6928, respectively. The one-way ANOVA statistical test showed that all concentrations gave relatively the same formation rate of the zone of inhibition for all tested bacteria.

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