Biotransformation of Compounds in Laja Gowah Oil (*Alpinia malaccensis* (Burm.f) Roscoe) by *Aspergillus niger* and Its Antibacterial Activity

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Abstract. Laja gowah (Alpinia malaccen is a Zingiberaceae plant which has been used as traditional medicine by Indonesians. Research on the biotransformation of the compound content of laja gowah oil with *Aspergillus niger* has been carried out. The biotransformation process was carried out at room temperature with various incubation times of 24, 48, 72, and 96 hours. Analysis of compound content of laja gowah oil and biotransformation products using Gas Chromatography - Mass Spectrometer. Analysis of the biotransformation process was carried out using Thin Layer Chromatography using four types of solvents with different polarity. The compound content of laja gowah oil includes methyl cinnamic compounds, eucalyptol, camphor, and beta-pinene. Antibacterial test of the biotransformation product was carried out on *Escherichia coli* and *Staphylococcus aureus* bacteria through the agar diffusion method. The optimal incubation time for biotransformation of laja gowah oil was 72 hours to produce methyl cinnamate (96.98%). Biotransformation with Aspergillus niger does not produce new derivative products, increases levels of methyl cinnamic compounds, so that it can be used as a method of improving the quality of laja gowah oil. Based on the results obtained, the transformation product containing higher methyl cinnamate inhibits the growth of *Staphylococcus aureus*.

INTRODUCTION

Laja gowah or forest galangal (*Alpinia malaccensis* (Burm.f.) Roscoe) is a plant from the Zingiberaceae family that is related to white galangal and red galangal. Laja gowah has been used as a medicinal plant by the people of Indonesia. The rhizome of laja gowah is used as a medicine for ulcers, wound medicine, stomachache medicine, and strong medicine. Laja gowah fruit can be eaten and used as a cooking spice or dried as tea. Laja gowah oil was isolated from the rhizome of the laja gowah plant by means of the steam distillation method. Laja gowah oil is one of Indonesia's newly developed essential oil commodities with promising business prospects.

Laja gowah oil contains compounds of the hydrocarbons, alcohols, ketones, esters, ether, and carboxylic acids. Methyl cinnamate is the main component of laja gowah which is included in the ester compound with the molecular formula $C_{10}H_{10}O_2$ and a molecular weight of 162.185 g/gmol. Methyl cinnamate is obtained from plant biological processes and can also be synthesized from cinnamic acid compounds through the esterification process with methanol compounds. Methyl cinnamate has antibacterial activity [1], antipyretic, analgesic, and anti-inflammatory [2]. Methyl cinnamate is used as an active ingredient in the cosmetics, medicine and food industries. The development of methyl cinnamic isolation from laja gowah plants continues to be developed to meet market needs. Riyanto et al (2012)

reported that it has succeeded in isolating 98% methyl cinnamate from laja gowah oil through the spinning band distillation column method [3]. However, this method requires high pressure and heat so it requires high energy.

One of the ways of derivatization of chemical compounds that is safe and environmentally friendly is biotransformation. Biotransformation is the modification of chemical compounds by utilizing extracellular enzymes produced by microorganisms. Biotransformation reactions are selective and specific in changing existing substrates [4]. One of the microorganisms that can be used in biotransformation is *Aspergillus niger* [5]. *A. niger* is a type of fungus that produces extracellular enzymes with high activity and is easy to maintain. In addition, *A. niger* is easy to obtain from nature and can be cultivated on simple growth media on a laboratory scale [6]. *A. niger* can secrete the enzymes cellulase, chitinase, α-amylase, glucoamylase, catalase, pectinase, lipase, lactase, invertase, and protease. [7]. The enzymes of *A. niger* are widely used for the pharmaceutical, food, and biotransformation purposes of organic compounds [8].

The use of *A. niger* as a biotransformation agent is reported to be able to produce new derivative compounds from the substrate and increase the main compound content of essential oils. The existence of a carbon skeleton in the terpenoid group structure can be a source of substrate for *A. niger*. *A. niger* is a versatile fungus capable of enzymatically transforming various types of terpenoid compounds [9]. The metabolite products produced from *A. niger* have a high yield. Types of reactions that can be carried out by A. niger include hydroxylation, oxidation of various functional groups, reduction of double bonds, demethylation, hydrolysis of epoxides, ring splitting, and conjugation [9].

In this study, the biotransformation of laja gowah oil was carried out using *A. niger* with variations in incubation time to determine the relationship between the growth phase of *A. niger* and the metabolite products produced. Identification of the product of the compound transformation reaction in laja gowah oil using the Gas Chromatography-Mass Spectrophotometer. The transformed derivative can be detected from the mass spectrum obtained, while the derived compound content can be determined by the percent area value on the chromatogram. The purpose of this study was to determine the effect of incubation time of laja gowah by *A. niger* and to determine its potential as an antibacterial.

MATERIAL AND METHODS

Material and Instruments

The materials used include laja gowah oil from CV. SESMU Essential Oil Tangerang, *Aspergillus niger* isolate (ATCC 6275 from the Surabaya Health Laboratory Center), nutrient broth media (Merck), n-hexane (Merck), Potato Dextrose Agar (Merck) media, potato infusion, aquades (PT. Brataco), methanol (Merck), ethyl acetate (Merck), dichloromethane (Merck), and dextrose (Merck), Mueller Hinton agar (Merck) media, bacterial isolates from the collection of STIKES Microbiology Laboratory, Anwar Medika Hospital (Escherichia coli, Listeria monocytogenes, Neisseria gonorrhoeae, Staphylococcus aureus). The instruments used in the research included rotary shaker (IKA), autoclave, (GEA) laminar air flow (WINA), vacuum rotary evaporator (IKA), micro pipette, Gas Chromatography - Mass Spectrometer (GC-MS, Agilent 19091S-433, column HP-5MS 5% Phenyl Methyl Silox), and beaker equipment (Iwaki Pyrex).

Culture Preparation and Aspergillus niger Growth Curve

Method of culture preparation for *A. niger* referred to in reports of Purkan et al. (2015), *A. niger* isolates were cultured with Potato Dextrose Agar (PDA) media which was prepared by dissolving 20 grams of PDA media with 1000 mL of distilled water. Furthermore, PDA media was sterilized by autoclaving at a temperature of 121 ºC for 15 minutes and sterile PDA media as much as 5 mL was poured into the test tube [10]. A total of 1 ose culture of *A. niger* was streaked in a zig zag manner on oblique tube PDA media using a sharp implanted needle. Then the *A. niger* culture was incubated at 30 ºC for 3 days. The incubation process adjusted to the *A. niger* growth curve which entered the log phase on the third day [11]. *A. niger* growth curve was made by inoculating 10 mL of *A. niger* starter solution in 100 mL of Potato Dextrose Broth (PDB) liquid medium, then shaking it at 125 rpm at 30 ˚C. Measurement of the growth profile of *A. niger* based on dry mycelium weight observed every 24 hours. *A. niger* growth profile was observed for 10 days. The growth curve was obtained from plotting the relationship between dry mycelium weight (Y axis) and incubation time (X axis).

The Biotransformation of Laja Gowah Oil by *Aspergillus niger*

The biotransformation process of laja gowah oil using Potato Dextrose Broth (PDB) media is made from a solution of potato infusion and dextrose diluted with distilled water, then PDB media is sterilized. A total of 1 ose of *A. niger* culture was put on PDB media and incubated at a temperature of 30 ºC for 3 days with an agitation speed of 125 rpm. The biotransformation process is carried out by adding laja gowah oil (0.1% v / v) to the PDB medium. The biotransformation process was carried out with time variations of 24, 48, 72, and 96 hours. The biotransformation mixture suspension that was incubated at a temperature of 30 ºC with an agitation speed of 125 rpm was extracted using n-hexane solvent. The isolates of the biotransformed compounds were obtained by separating the n-hexane solvent with a vacuum rotary evaporator.

Characterization of biotransformation products of lajah gowah oil

The observation of the biotransformation process in laja gowah oil was identified using a thin layer chromatography method. The concentrated extract of the biotransformation results was spotted on the TLC plate and eluted using four solvents with different polarity values (n-hexane, dichloromethane, ethyl acetate, and methanol). TLC stain analysis was observed under a UV lamp at λ 366 nm. The Retardation Factor (Rf) values at each variation of the incubation time were compared. The change in the Rf value indicates a transformation of the compound in the red gowah steamer oil. The identification of functional group changes at each incubation time was analyzed using the resulting IR spectrum characteristics. Identification of biotransformed compounds was identified by GC-MS.

Antibacterial test

The isolates of the test bacteria were cultured with Nutrient Broth (NB) media for 24 hours. A total of 15 mL of the sterilized Mueller Hinton Agar medium was poured into a petri dish. After the media has solidified, take a sterilized cotton swab and put it in a test tube containing a suspension of the test bacteria. Then the cotton bud is rubbed over the entire surface so that it is evenly distributed on the petri dish. In MHA media that has been inoculated with bacteria, discs were made from discs that have been soaked in laja gowah oil, laja gowah oil which has been fermented for 24 hours, 48 hours, 72 hours, 96 hours. DMSO 15% was used as a negative control and ciprofloxacin solution as a positive control, then the agar medium was incubated for 24 hours at 37 °C. The diameter of the inhibition zone formed was measured using a ruler. The antibacterial test was repeated three times.

RESULT AND DISCUSSIONS

Aspergillus niger Growth Curve

The *A. niger* growth curve provides information about the growth phase of *A. niger* which is associated with the incubation time of laja gowah oil biotransformation. Figure 1 shows the growth curve of A. niger which was observed for 10 days. Based on the resulting data, the initial phase of adaptation (lag) occurs on day 0-1, in this phase the cell adjusts to the enzyme environment to break down the substrate. The next accelerated phase (logarithmic) occurs on days 1-3, because in this phase the cells begin to divide and become more active. The exponential phase occurs on days 4-5, in this phase the cells can divide optimally because of the availability of many nutrients, so that cell activity increases [6]. At the beginning of the exponential phase extracellular enzymes are produced. On days 5-7, there is a stationary phase seen from the rest of the dry cell period. In this phase, the number of cells that grow is relatively the same as the number of dead cells. The profile in the stationary phase is a straight horizontal line and there are many secondary metabolites. Furthermore, on days 8-10 is the phase of death of *A. niger* which can be seen from the decrease in dry cell mass. In the death phase, nutrients have begun to decrease and there are other metabolites that are toxic and can inhibit cell growth, so that the growth rate of *A. niger* begins to decline [12].

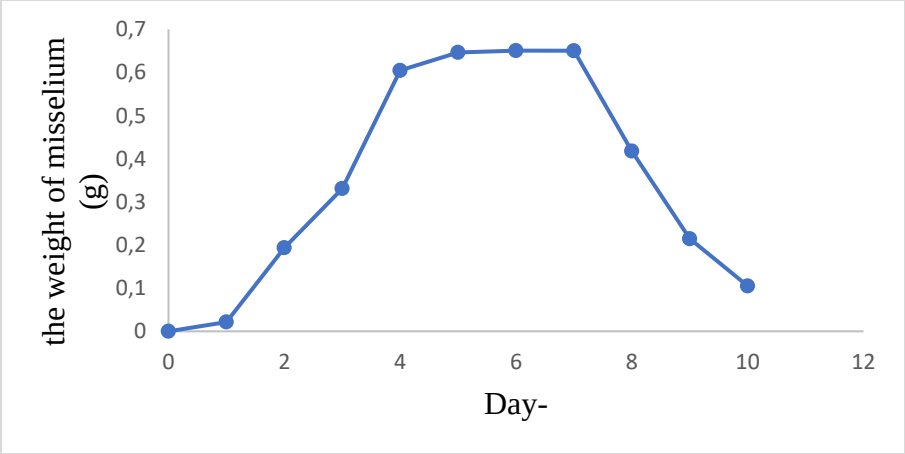

FIGURE 1. The growth curve of *Aspergillus niger*

Characterization of Laja Gowah Oil

The chromatogram of laja gowah oil used as a substrate in the biotransformation by Aspergilus niger is shown in Figure 2, there are 7 peaks indicating that they contain 7 chemical compounds. The methyl cinnamic content isolated from the rhizome of laja gowah oil is around 44 - 80% [13]. Table 1 shows that the methyl cinnamate content laja in gowah oil has met the international quality standard (> 70%) that is 90%.

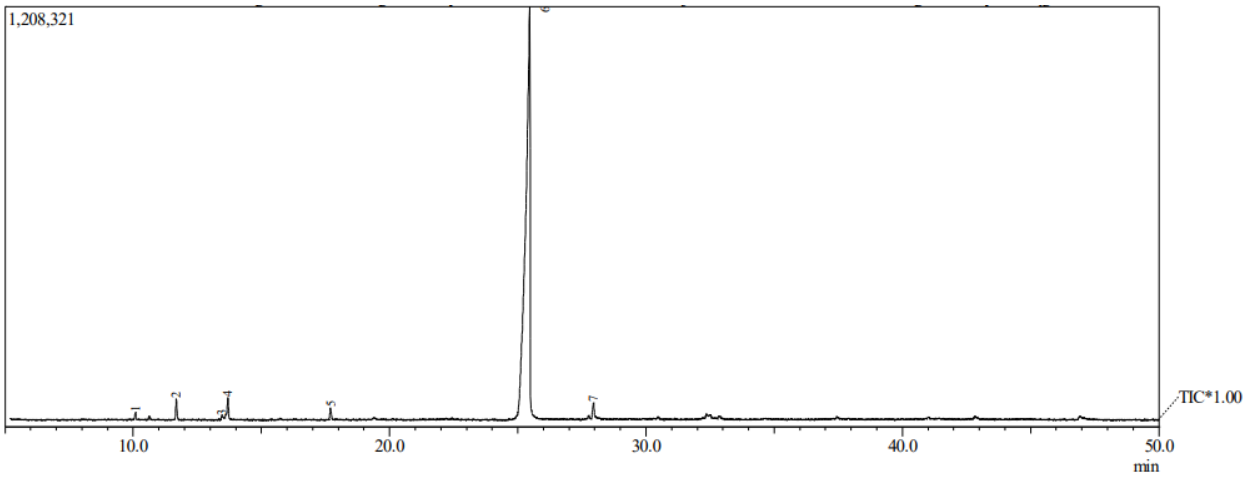

FIGURE 2. Total Ionic Chromatogram Laja Gowah Oil

TABLE 1. The content of laja gowah oil compounds

Peak	Compound Name	Area%	SI	m/z
1	Trans-ocimene	0.55	86	136, 121, 105, 93, 79, 67, 43, 41, 27
2	Beta-pinene	1.34	91	136,121, 107, 93, 79, 69, 53, 41, 27
3	Para cymene	0.94	92	134, 119, 103, 91, 77, 65, 51, 39, 27
4	Eucalyptol	1.50	88	154, 139, 121, 108, 81, 69, 43, 41, 27
5	Camphor	0.88	87	152, 136, 121, 108, 95, 81, 69, 55, 41, 27
6	Methyl cinnamate	93.35	96	162, 147, 131, 117, 103, 91, 77, 63, 51, 39, 27
7	Decane	1.44	90	142, 113, 99, 85, 71, 57, 43, 41, 27

The Biotransformation of Laja Gowah Oil

Biotransformation of laja gowah oil by *A. niger* begins in the logarithmic phase (the third day of the *A. niger* incubation period) because it is in this phase that cell division occurs and its activity becomes more active. The biotransformation process of laja gowah oil was carried out with four variations of the incubation time, namely 24 hours, 48 hours, 72 hours, and 96 hours. The biotransformation process of laja gowaj oil was determined by thin layer chromtography using four solvents of different polarity. Based on the results obtained in Table 2, The Rf value did not change significantly, there was a slight decrease in the Rf value when the biotransformation process began. Only one stain is produced, this indicates that there has not been any biotransformation process that has resulted in new derivative products. It is necessary to analyze the product using a mass spectrophotometer to confirm the content of the compounds in the biotransformation product of laja gowah oil. Anwar et al (2018) reported that the biotransformation product of geraniol with *A. niger* was only formed on the 7th day after the entry of geraniol compounds [14]. Based on this study, the biotransformation product was formed on the 10th day of the growth curve of *A. niger*.

TABLE 2. The Rf values of the biotransformation products

Incubation time (h)	n-hexane	dicloromethane	Ethyl acetate	ethanol
0	0.17	0.72	0.84	0.75
24	0.16	0.69	0.83	0.70
48	0.12	0.69	0.83	0.70
72	0.16	0.69	0.83	0.70
96	0.14	0.70	0.82	0.69

Analysis of the formation of the biotransformation product of laja gowah oil was also carried out using the FT-IR instrument to observe the changes in functional groups that occurred. Based on the results of functional group analysis using FT-IR (Figure 3), there was no significant difference between the infrared spectra of laja gowah oil and biotransformation products at various incubation times. The infrared spectrum of the biotransformation product is shown in Figure 3 and the wave number for each vibration is shown in Table 4. The IR spectrum shows the presence of CH absorption at a wave number of 2950.83 cm^{-1} and C = O ester absorption at a wave number of 1712.88 cm^{-1}, aromatic C=C bond at wave number 1635.86 cm^{-1}, C=O ester at wave number 1714 cm^{-1}, CH = CH bond at wave number 979.81 cm^{-1} and CH bond at wave number 1450.46 cm^{-1}. Based on the data from the interpretation of infrared spectra, it can be seen that the biotransformation product is a methyl cinnamate compound. This shows that there are no new derivative products from laja gowah oil.

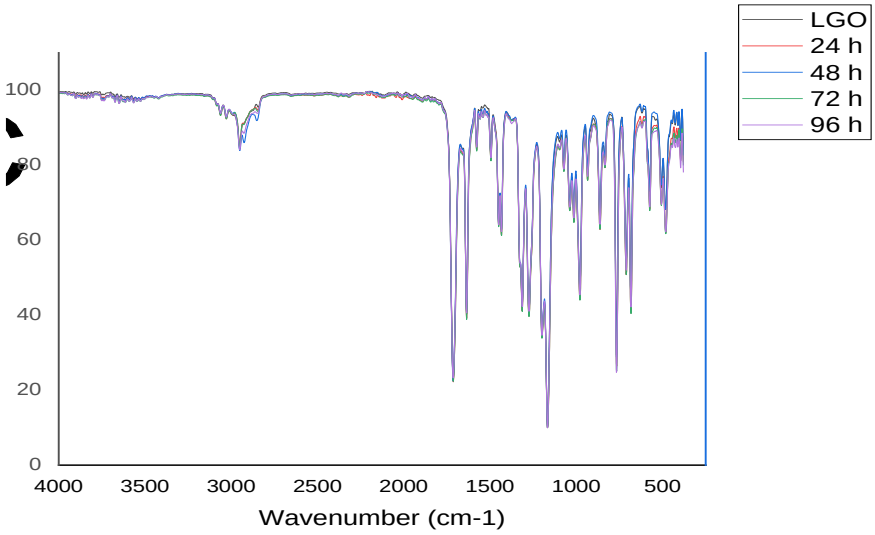

FIGURE 3. Infrared spectrum of laja gowah oil and biotransformation products

The biotransformation product of laja gowah oil obtained was extracted with a non-polar solvent, then analyzed for its compound content using GC-MS. Chromatogram and mass spectrum data of each peak of the biotransformation product compound at each incubation time are tabulated and analyzed. Table 3 shows the results of biotransformation based on variations in incubation time. Biotransformation of laja gowah oil with *A. niger* produces four compounds, namely eucalyptol, camphor, methyl cinnamate, and decane. Figure 4 shows a graph of the relationship between methyl cinnamate levels at each incubation time, there is an increase in methyl cinnamate levels in the exponential phase. Biotransformation products showed an increase in methyl cinnamate levels from 0 hours to 72 hours of incubation time. At the 96-hour incubation time, there was a decrease in methyl cinnamate levels.

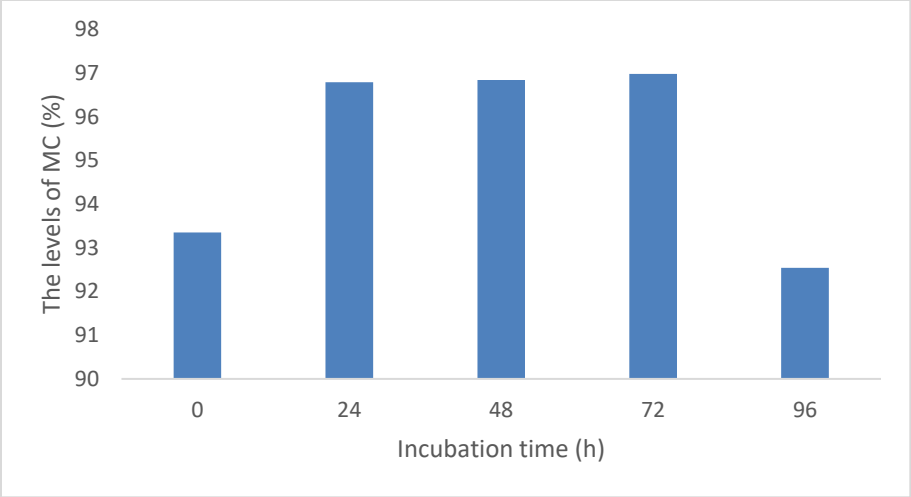

FIGURE 4. The level of methyl cinnamate in various incubation time

TABLE 3. The composition of the constituent chemical compounds of biotransformation products

Compound Name	Incubation time (h)				
	0	**24**	**48**	**72**	**96**
Trans-ocimene	0.55	-	-	-	-
Beta-pinene	1.34	0.69	0.51	-	0.25
Para cymene	0.94	-	-	-	-
Eucalyptol	1.5	0.98	1.25	0.95	3.24
Camphor	0.88	0.87	0.92	1.00	2.97
Methyl cinnamate	93.35	96.79	96.84	96.98	92.54
Decane	1.44	0.67	0.49	0.79	0.88

Biotransformation of laja gowah oil with incubation time of 24 hours, 48 hours, and 72 hours occurred in the exponential phase of *A. niger*. In the exponential phase, *A. niger* cells divide optimally because of the availability of many nutrients so that cell activity increases [11]. This is evidenced by the high levels of methyl cinnamate (96.98%) at the 72 hour incubation time. At the 96-hour incubation period, the methyl cinnamate content decreased from 96.98% to 92.54%. The 96-hour incubation time has entered the stationary phase of the *A. niger* growth curve. In the stationary phase, the number of living A. niger cells is the same as the number of dead cells [15]. At the 96-hour incubation time there was an increase in eucalyptol, beta pinene, camphor, and decane, this is presumably because in the stationary phase A. niger produces more diverse secondary metabolites.

Extracellular enzymes of *A. niger* caused the transformation of minor compounds in laja gowah oil including trans-ocimene and para-cymene into methyl cinnamate compounds (Figure 5). This assumption is supported by research that the carbon skeleton of minor compounds in laja gowah oil is a substrate for *A. niger* [16]. The reaction mechanism that is thought to occur is a condensation reaction that causes changes in the carbon skeleton by adding carbonyl and alkoxy groups [17]. The mechanism of the biotransformation reaction needs to be explained by molecular analysis with other instruments.

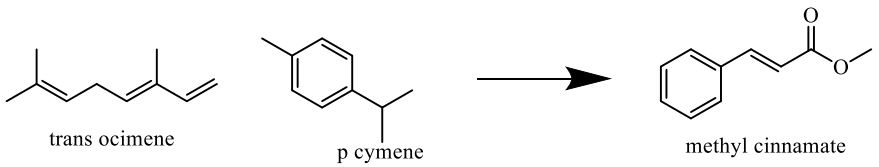

FIGURE 5. Biotransformation of minor compounds in laja gowah oil into methyl cinnamate

Antibacterial activity of the biotransformation product of laja gowah oil

Antibacterial test aims to determine the antibacterial activity of laja gowah oil and its biotransformation products associated with the compound content. Antibacterial tests were carried out on *Escherichia coli* (Gram negative) and *Staphylococcus aureus* (Gram positive) bacteria. The main difference between Gram positive and Gram negative bacteria is the structure and composition of their cell walls. Table 4 shows the antibacterial activity data of laja gowah oil and its biotransformation products. In the antibacterial test against *E. coli* bacteria, laja gowah oil had a larger inhibition zone than the biotransformation product. The higher the methyl cinnamate level, the lower the antibacterial activity against E. coli. In the antibacterial test against *S. aureus* bacteria, the higher the methyl cinnamate level, the higher the antibacterial activity against *S. aureus*.

TABLE 4. Inhibition zone of laja gowah oil and its biotransformation products

Sample	Inhibition zone (cm)	
	E. coli	***S. aureus***
Laja gowah oil	0.9	0.5
LG-24	0.4	0.4
LG-48	0.3	0.6
LG-72	-	0.7
LG-96	-	0.9
DMSO 15%	-	-
Ciproflaxacine	1.84	2.73

The difference in bacterial sensitivity to laja gowah oil and its biotransformation products may be due to differences in cell wall structure between Gram-positive and Gram-negative bacteria [18]. The sensitivity of bacteria to antibiotics depends on differences in the composition of their cell walls, such as the amount of peptidoglycan, the presence of receptors and lipids, the nature of cross-linking, the activity of autolytic enzymes that determine penetration, binding, and drug activity [19]. The content of chemical compounds from the test sample affects the resulting inhibition zone. Table 4 shows that laja gowah oil containing methyl cinnamate, trans-ocimene, para cymene, eucalyptol, camphor, and beta pinene compounds inhibited the growth of *E. coli* bacteria more than *S. aureus*. The absence of trans-ocimene and para-cymene compounds caused a decrease in antibacterial activity against *E. coli*. In Gram negative bacteria, the peptidoglycan layer on the cell membrane is thinner than in Gram positive bacteria. The outer membrane of Gram-negative bacteria is composed of phospholipids and lipopolysaccharides so that antibacterial substances that interfere with the integrity of the cell membrane will more easily attack Gram-negative bacteria by dissolving phospholipids. Phospholipids will break down into glycerol, carboxylic acid, and phosphoric acid so that the membrane cannot maintain its shape, as a result the membrane leaks, substances can enter and leave the cell uncontrollably so that metabolism is disrupted and bacteria are lysed [20].

Gram positive bacteria have a thicker layer of peptidoglycan than Gram negative bacteria. This thicker layer of peptidoglycan causes the cell wall permeability of Gram-positive bacteria to be lower than that of Gram-negative bacteria. The incubated biotransformation product contained higher methyl cinnamate than laja gowah oil. High levels of methyl cinnamate affect the zone of inhibition against S. aureus bacteria. This is in contrast to research conducted by Martiana et al. (2016) which showed that methyl cinnamate compounds and their derivatives were unable to inhibit the growth of *S. aureus* [21]. Methyl cinnamate has a better inhibitory activity value against S. aureus than E. coli because of the influence of the structure of the methyl cinnamate compound and its polarity [22].

CONCLUSION

The biotransformation process of A. niger is influenced by the growth phase of *A. niger*. The optimum incubation time for biotransformation of laja gowah oil was 72 hours with a methyl cinnamic content reaching 96.98%. Biotransformation of laja gowah oil by *A. niger* did not produce new derivatives but increased levels of methyl cinnamic compounds. Biotransformation of laja gowah oil can be used as an alternative method of improving the quality of laja gowah oil which is environmentally friendly and increases its selling price. Methyl cinnamate levels affect the antibacterial activity of the biotransformation product, which is more effective at inhibiting *S. aureus* than *E. coli*.

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