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Antibacterial Effectiveness of *Syzygium cumini* (L.) Skeels Leaves to *Escherichia coli* pBR322

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Abstract

Bacterial resistance is a serious problem that until now still has become a global problem. The search for new antibacterial compounds is needed to overcome this problem. *Syzygium cumini* (L.) Skeels is a plant known to contain bioactive compounds that can be used as an antibacterial agent. This study aims to explore the leaves of this plant as an antibacterial against *Escherichia coli* pBR322 resistant bacteria. Based on the antibacterial test results, extracts and leaf fractions of this plant can inhibit bacterial growth. Ethyl acetate fraction at a concentration of 800 ppm showed strong antibacterial activity with an inhibition zone diameter of 10.36±0.02 mm followed by ethanol extract and other fractions, namely 8.43±0.01 mm (ethanol extract), 7.24±0.01 mm (water fraction), and 1.45±0.01 mm (n-hexane fraction). The results of spectrophotometric MIC determination also showed inhibition against bacterial growth, which was marked by a decrease in the absorbance value at the lowest antibacterial concentration of 600 ppm from 0.454 to 0.070 at wavenumber 600 nm after 24 hours of incubation. The decrease in the absorbance value indicated that the antibacterial properties of the plant leaves at this concentration were effective in inhibiting bacterial growth.

Keywords: Antibacterial, Escherichia coli pBR322, resistant, Syzygium cumini (L.), skeels.

INTRODUCTION

Bacterial resistance is a serious problem in the medical world which until now has become a global problem. The use of antibiotics that are not following the service rules is one-factor causing bacterial resistance. It's because bacteria can quickly build a defense system that can inactivate the working mechanism of the antibiotic by overhauling the target receptors that bind to the antibiotic or expressing a flux pump gene that can pump the antibiotic out of the cell so that treatment becomes ineffective (Cesur & Demiröz, 2013). In developing countries, especially Indonesia, cases of bacterial resistance are not new. Data on cases of bacterial resistance in 2009, Indonesia ranks 8th out of 27 countries as the Country with the title of the highest multidrug-resistant issues in the world (Estiningsih, Puspitasari, & Nuryastuti, 2016).

The increase in cases of bacterial resistance, which is expecting to continue to increase every year, is the main reason for researchers to keep trying to find new antibacterial substances to overcome this problem (Rendowaty, Djamaan, & Handayani, 2017). Various types of research related to the search for antibacterial compounds have generally been carried out either by synthetic means or using herbal ingredients derived from plants. The use of antibacterial agents derived from plants currently tends to be the leading choice to continue to be developed because, empirically, it has been proven safe and almost does not cause harmful side effects to the body (Zakaria et al., 2017). According to (Costa, Lins, Le Hyaric, Barros, & Velozo, 2017) medicinal plants are natural resources that can be used as natural medicines because of the content of bioactive compounds in it. One type of plant that has been widely used as natural medicines empirically in curing various types diseases is Jamblang (*Syzygium cumini* (L.) Skeels).

Jamblang (*Syzygium cumini* (L.) Skeels) is a typical Indonesian flora that currently has a rare population (Dewi & Wahyuni, 2016). However, in the area of Central Malaka-Timor-Indonesia, the existence of jamblang plants is still relatively abundant. Since long ago, some local people have traditionally used the leaves of this plant to treat various types of diseases, including diarrhea, one of the factors caused by infection with the bacteria

of *Escherichia coli*. Although classified as normal intestinal flora, other strains of this bacteria are also known to be dangerous and pathogenic because they can cause infection, both spores and endemic (Idrus & Mustapa, 2021).

Research on the use of *Syzygium cumini* (L.) Skeels leaves have been done widely in other countries, one of which is Brazil. The results showed that the leaf extract of this plant had antibacterial activity against several *Streptococcus* bacteria *Streptococcus uberis*, *Streptococcus agalacteae*, and *Streptococcus dysgalacteae* with minimum inhibitory concentrations of 3.1, 1.5, and 1.9%, respectively (Voigt Mota et al., 2013). Although it has been used as an antibacterial agent, the use of this plant leaves as an antibacterial is still limited to bacteria that are sensitive to antibiotics, while its application against diarrhea-causing pathogens such as *Escherichia coli* that contains resistant plasmids like pBR322 has never been done. Therefore, further research is needed to obtain more detailed information about the potential of this plant in terms of its antibacterial properties. This study aims to test the antibacterial effectiveness of jamblang leaves against the bacteria *Escherichia coli* pBR322, which has been resistant to ampicillin and tetracycline antibiotics. In addition, the minimum concentration test for bacteria will also be determined spectrophotometrically so that information on the smallest antibacterial concentration of these plant leaves can be obtained in inhibiting the growth of the Escherichia coli pBR322 bacteria.

METHODOLOGY

Instruments and Materials

The research materials used were old jamblang leaves (*Syzygium cumini* (L.) Skeels) from Central Malaka, Timor Island, East Nusa Tenggara Province, and the *Escherichia coli* pBR322 bacterial isolate. Other ingredients, namely; distilled water, 70% ethanol (E-merk), n-hexane (E merk), ethyl acetate(E merk), 1.175% $BaCl_2$ (E merk), 1% H_2SO_4 (Pa), physiological NaCl (E merk), Luria Bertani Broth, Luria Bertani Agar, filter paper (no brand), paper discs (Oxoid Antibacterial susceptibility test discs), Kanamycin (Kanamycin Meiji sulfate) 30 ppm (and 20% DMSO (E merk). The equipment used in the study were glassware (Pyrex), 60 mesh sieve (Test sieve analys), sonicator water bath (Ultrasonic cleaner GRANBO Stainless Steel 304), funnel glass (Pyrex), evaporator (IKA Rotary Evaporator RV 10 Digital), oven (Memmert UNB 400), ose needle (no brand), Petri dish (Pyrex), incubator (Memmert IN 30 Incubator), vortex (Nueation Vortex mixer), analytical balance (Fujitshu FSR-A), Bunsen burner (no brand), autoclave (GEA Medical LS-50 LJ), micropipette (Scilogex) and spectrophotometer UV-Vis (Genesys 10S UV-Vis).

Experimental

Preparation and Determination of Water Content

Old jamblang leaves were taken from a tree with a tree age of about 20 years from Fahiluka Village, Malaka Regency, East Nusa Tenggara, washed clean and dried for 7 days. The dried leaves were mashed into 60 mesh, and then determined the water content.

Extraction and Fractionation

The sample was extracted using 70% ethanol solvent with a ratio of 1: 10 (w/v). The extraction was carried out by sonication at an ultrasonic frequency of 40 kHz for 25 minutes at a temperature of 25 ºC. The sonication results are then filtered using a Buchner funnel. The filtrate is then evaporated at 60 ºC. The extracts obtained were tested for phytochemical content and continued with fractionation (1: 1 v/v) using 3 solvents with different polarity levels: water, ethyl acetate, and n-hexane.

Preparation of Bacterial Suspension

The culture of *Eschericha coli* pBR322 bacteria that has been prepared is taken 1 to 2 colonies and then put in 10 mL of 0.9% physiological NaCl. The bacterial suspension was then vortexed and turbidity adjusted with a standard solution of Mc. Farland 0.5. Each mL of the suspension is equal to a bacterial concentration of 1.5×10^8 CFU/mL.

Antibacterial Activity Test

As much as 15 mL of Luria Bertani Agar which already contains ampicillin-tetracycline antibiotics (50 µg/mL; 15 µg/mL) is put into a sterile Petri dish then allowed to stand until solidified. The bacterial suspension has been adjusted according to Standard Mc. Farland 0.5 pipette 100 µL aseptically then poured on the surface of the media and leveled using a spreader glass. Furthermore, each disc paper was given an extract and fraction with a 200, 400, 600, and 800 ppm concentration. The positive control was kanamycin 30 ppm, while the negative control was 20% DMSO. The amount of material dropped on each disc paper was 20 µL. Disc paper was then placed on the surface of the media and then incubated at 37 ºC for 24 hours. Antibacterial activity is measured by looking at the clear zone that forms around the disc after 24 hours.

Minimum Inhibitory Concentration (MIC) Determination of Antibacterial.

In this test, bacterial suspension as much as 1 mL according to Mc. Farland 0.5 was put into 7 tubes containing 2 mL of Luria Bertani broth and antibiotics (ampicillin, 50 µg/mL; tetracycline 15 µg/mL). Into the 4 tubes, The extract or the best fraction of the antibacterial activity test results was inserted into Luria Bertani Broth media with concentrations 200, 400, 600, and 800 ppm, respectively. The following 2 tubes were inserted with 30 ppm antibiotic kanamycin (control +) and DMSO 20% (control -). Then the last tube was used as a control medium without bacterial suspension. The minimum inhibitory concentration (MIC) of antibacterial can be determined by comparing the absorbance value from the spectrophotometer of each tube after the incubation process with the pre-incubation process. The lowest absorbance resulting from the antibacterial concentration will considering as MIC of antibacterial.

Data Analysis

Antibacterial activity was analyzed using one-way analysis of variance (ANOVA) at a 95% confidence level ($p<0.05$) with the application of Minitab 18.0 to determine the difference in the effect of the jamblang leaves extract and fraction on *Escherichia coli* pBR322 bacteria.

RESULTS AND DISCUSSION

Determination Of Water Content

The sample's water content was determined gravimetrically by heating the sample at 105 ºC for 3 hours. Water content determination was carried out to ensure that the amount of water content in the sample exceeded 10%. The high water content in the sample can trigger microbial growth and cause enzymatic reactions resulting in chemical composition changes. So that sample did not last a long enough storage. According to (BPOM, 2019), the water content of a good sample is less than 10%. Based on the results of measuring the water content, it was found that the water content of the sample in this study was 7.85%. It shows that the sample used has met the standard of a good sample water content.

Extraction and Fractionation

Extraction of jamblang leaves was carried out by sonication using an ultrasonic bath with an ultrasonic frequency of 40 kHz at a temperature of 25 for 25 minutes. The choice of the sonication method is based on its ability to extract natural compounds faster than other extraction methods. It's because the effect of ultrasonic vibrations generated from the sonicator can quickly penetrate cell tissues so that the cells become lysis. The compounds in plant cells will be extracted into the solvent (Jos, Pramudono, & Aprianto, 2012).

The ethanol extract obtained from the extraction then separated the metabolite content through a liquid-liquid fractionation. Fractionation was carried out using three types of solvents: water, ethyl acetate, and n-hexane. The purpose of fractionation is to separate the content of secondary metabolites in the extract according to differences in polarity based on the principle of "Like dissolves like" (Uthia, Arifin, & Erfianti, 2017). Based on the fractionation results, the yield percentage data of each fraction of 20 g of ethanol extract as shown in Table 1.

Table 1. Rendament of ethanol extract fraction

Etanol extract (20 g)	Fraction weight (g)	Rendament (%)
Water fraction	8.26	41.32
Ethyl acetate	2.58	12.92
N-hexane fraction	0.13	0.69

Based on the data in Table 1, it can be seen that the yield percentage produced from each fraction is different. The yield of the n-hexane fraction was smaller than the water fraction and ethyl acetate fraction. The high yield of water fraction and ethyl acetate fraction was probably due to differences in the polarity of the compounds in the sample into the solvent.

Phytochemical Screening

Phytochemical screening aimed to identify the secondary metabolites in the ethanol extract of jamblang leaves from Timor Island. It is also intended to prove that the differences in the region's location and the rainfall from the origin of this plant will show the difference in the composition of the secondary metabolites contained in it.

Based on previous research conducted by (Ramos & Bandiola, 2017), the results of phytochemical screening of the ethanol extract of jamblang leaves from the Philippines as a whole did not show positive results for all tests. In contrast in this study, the whole phytochemical test showed positive results for all tests as shown in Table 2.

Based on the data in Table 2. It is consistent with the statement of (Salim, Sitorus, & Ni, 2016) that the secondary metabolite content of a plant is strongly influenced by geographic location, altitude, and rainfall where the plant originates. According to (Dulay & De Castro, 2016), the content of secondary metabolite compounds in plants is an essential component in its biological activity related to treatment. Several secondary metabolite compounds such as flavonoids, terpenoids, tannins, and saponins are also said to have biological activities that can act as antimicrobials, antioxidants, anti-cancer, anti-allergic and anti-inflammatory. It is supported by the research results of (Fartyal & Kumar, 2016) which prove that several metabolite compounds such as alkaloids, flavonoids, and steroids also have an important role, which can act as natural antibacterial agents.

Antibacterial Activity Test

The antibacterial activity of jamblang leaves was carried out using the disc diffusion method. The effect of giving ethanol extract and fraction on its antibacterial ability was determined by measuring the inhibition zone diameter formed around the disc. Based on the results of the antibacterial activity test at various antibacterial concentrations, it can be seen in Table 3 that the increase in the concentration of each antibacterial is directly proportional to the inhibition zone formed from each test.

The data in Table 3 of four types of antibacterial at various antibacterial concentrations of 200 ppm, 400 ppm, 600 ppm, and 800 ppm shows that the antibacterial types differed significantly ($p<0.05$) on the diameter of the formed inhibition zone. The antibacterial test results show that ethyl acetate gave a better antibacterial activity value than the other extracts and fractions with an inhibition zone diameter of 10.36 ± 0.02 mm at a concentration of 800 ppm. Meanwhile, the ethanol extract was 8.43 ± 0.01 mm,

Table 2. Phytochemical Screening of Ethanol Extract of Jamblang Leaves.

Phytochemical group	Test result according to literature		Test result
Flavonoids	Test material will turn to brownish-yellow		+
Terpenoids and Steroids	Terpenoid:The color becomes red, purple, or brown. Steroid:The color of the test material will change to green		+ +
Alkaloids	Wagner's reagent test: brown precipitate. Meyer reagent test : White precipitate. Dragendroff test:red-orange precipitate		+
Tannins	The test material changes color to blackish green or blackish blue		+
Saponins	Test material produces stable foam		+

the water fraction was 7.24 ± 0.01 mm, and the n-hexane fraction was 1.45 ± 0.01 mm. The difference in the inhibition zone diameter resulting from each antibacterial is strongly influenced by the type of bioactive components contained therein.

Table 3. Antibacterial Activity of Jamblang Leaves to *Escherichia coli* pBR322.

Antibacterial Concentration	Inhibition zone diameter of antibacterial (mm); Mean ± SD			
	Ethanol Extract	Water fraction	Ethyl acetate fraction	N-hexan fraction
200	3.13±0.01^{c}	2.13±0.01^{d}	3.20±0.02^{b}	1.20±0.02^{e}
400	5.17±0.25^{b}	2.52±0.02^{c}	5.43±0.01^{b}	1.27±0.01^{d}
600	6.03±0.20^{c}	5.28±0.01^{d}	8.25±0.01^{b}	1.43±0.01^{e}
800	8.43±0.01^{c}	7.24±0.01^{d}	10.36±0.02^{b}	1.45±0.01^{e}
Control (+)	16.67±0.02^{a}	16.67±0.02^{a}	16.67±0.02^{a}	16.67±0.02^{a}

Note: Antibacterial activity data were interpreted by mean ± standard deviation of 3 repetitions; Different superscripts on the same line stated significantly different ($p< 0.05$) on Tukey's test.

The higher the antibacterial activity value produced, it can be said that the more bioactive components that act as antibacterials (Suciari, Luh Kadek., Mastra, Nyoman., HS, 2018). Therefore, the high antibacterial activity value of the ethyl acetate fraction is thought to have high bioactive compounds in its activity as an antibacterial. Its ability to inhibit bacterial growth is better than other extracts and fractions. This is supported by (Nugraha, Suryadi Achmad, & Erly Sitompul, 2019) research data that shows ethyl acetate can dissolve bioactive components that can act as antibacterials, including flavonoids, tanin and saponins. Other research data also indicates that ethyl acetate can dissolve several bioactive compounds from different plants with bioactive components in the form of flavonoids, saponins and alkaloids (Sri Widyawati, Budianta, Kusuma, & Wijaya, 2014).

The antibacterial mechanism of each of these compounds is different from one another. According to (Górniak, Bartoszewski, & Króliczewski, 2019), the inhibition of flavonoids groups as bioactive compounds against antibacterials has a complicated mechanism and cannot be fully understood. Several references state that flavonoid compounds can inhibit bacterial growth by remodelling the structure of the cell membrane. Flavonoid compounds can be bound to the surface of the bacterial cell membrane, disruption of the function of the bacterial cell membrane, they are causing death in bacterial cells. The mechanism of alkaloids as antibacterials is known to act as DNA intercalators in inhibiting the topoisomerase enzyme in bacterial cells. The inhibition of this enzyme causes the DNA replication process in bacterial cells to be disrupted, resulting in inhibition of bacterial cell growth (Ernawati & Sari, 2015). Apart from being an intercellator agent, another alkaloids mechanism is to disturb the peptidoglycan constituent components of bacterial cells so that the cell walls are not formed intact due to the bacterial cells' death (Supari, Leman, & Zuliari, 2016). The mechanism of tannins and saponins as antibacterial compounds is to work by damaging the function of the cytoplasmic membrane in bacteria, causing cell leakage in bacteria. Saponins act as antibacterials by reducing the surface tension of bacterial cells, which causes the leakage and discharge of the bacterial intracellular fluid that caused death cells bacteria (Nugraha et al., 2019).

Minimum Inhibitory Concentration (MIC) of Antibacterial

Antibacterial MIC is determined spectrophotometrically using the best fraction of the antibacterial activity test results at "λ" 600 nm. The measurement at 600 nm is because bacterial cells can absorb light at that wavelength (Astutiningsih, Setyani, & Hindratna, 2014). Antibacterial MIC needs to be determined to see how much influence the concentration of bioactive compounds in antibacterials inhibits bacterial growth. The antibacterial ability at the lowest concentrations in inhibiting bacterial growth can be known from the value of antibacterial absorbance from the smallest to the highest concentrations. The determination of MIC spectrophotometry obtained absorbance value information from antibacterial fractions of ethyl acetate, as shown in Table 4.

Table 4. Minimum Inhibitory Concentration (MIC) of Ethyl Acetate Fraction

Antibacterial concentrations	Absorbance at "λ" 600 nm	
	Before incubation	After incubation
200 ppm	0.211	0.248
400 ppm	0.316	0.335
600 ppm	0.454	0.070
800 ppm	0.648	0.114
DMSO 20% (-)	0.062	1.205
Kanamycin 30 ppm (+)	0.068	0.054
LB Media control	0.050	0.051

The increase in the absorbance value at the antibacterial concentration of 200 ppm and 400 ppm, respectively, from 0.211 to 0.248 and 0.316 to 0.355, indicates that antibacterials at these concentrations have not worked optimally in inhibiting bacterial growth so that bacteria can still thrive and absorb a lot of light when measured on the spectrophotometer. It can also be seen from the increasing absorbance value shown by the 20% DMSO (-) control. The high absorbance value produced exceeds 1.0 in DMSO control because there is the very high amount of light absorbed by bacteria due to bacterial growth in the sample after incubation 24 hours.

Therefore increases the concentration of bacteria, blocking the light transmitted to the detector based on Lambert Beer's Law that absorbance is directly proportional to substance (bacteria) absorbing the light (Akinduti et al., 2019). While the data for measuring the absorbance value at antibacterial concentrations of 600 ppm and 800 ppm, it can be seen that there is a decrease in the absorbance value which is also shown by the control (+) kanamycin 30 ppm. The decrease in the absorbance value is estimated at a concentration of 600 ppm, 800 ppm, and control (+) kanamycin 30 ppm, antibacterials can inhibit bacterial growth. A low absorbance value indicates that little light is absorbed by the bacteria because bacterial growth has been inhibited by antibacterial substances in the sample.

CONCLUSION

Extract and fraction of jamblang leaves are effective as antibacterial agents against *Escherichia coli* pBR322 (resistant to ampicillin and tetracycline). The best antibacterial activity from antibacterial testing was ethyl acetate fraction at a concentration of 800 ppm with an inhibition zone diameter of 10.36 ± 0.02 mm and a minimum inhibitory concentration of 600 ppm against bacteria.

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