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Antibacterial activity test of (+)- katekin and gambir (uncaria gambier roxb.) against some types of grambled bacteries negative bacteria and their mechanism

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ABSTRACT

There is evidence that catechins and aqueous extracts of the gambir plant (Uncaria gambier Roxb.) has antibacterial action. The purpose of this research was to further evaluate the antibacterial activity of (+)- catechins and an aqueous extract of gambir against Shigella flexneri, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris, and Proteus mirabilis. As part of the procedures that were carried out, sensitivity testing to gram-negative bacteria was performed to validate the activity. antimicrobial (+)- catechins and gambier water extract, calculation of the Minimum Inhibitory Concentration (MIC), and analysis of the mechanism of inhibition against gram-negative bacteria were all part of this study. The findings of this research show that the minimum inhibitory concentration (MIC) level for (+)- catechins against Shigella flexneri bacteria is 7.5 mg/ml. The mechanism of action of (+)- catechins is to disrupt cell membranes, which then leads to the leakage of cell components. Ca2+ ions and K+ ions, together with proteins and nucleic acids, were found to be present in the bacterial cell medium, which provided evidence that this was the case. In the meantime, the macrodilution technique revealed that the gambier water extract did not exhibit any inhibitory effect at any concentration up to 25 mg/ml.

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1. INTRODUCTION

Plants are a storehouse of various types of chemical compounds, ranging from simple structures and properties to complex and unique. Various types and chemical compounds contained in plants will have a positive correlation with the properties and benefits they have. Efforts to search for medicinal plants have long been carried out, both to find new compounds or to add to the diversity of existing compounds (Djauhariya and Hernani, 2004).

In recent years, many studies have been carried out to find natural antioxidants and antibacterials derived from plants, especially native Indonesian plants. Based on research that has been conducted on a number of plant extracts commonly used as spices and traditional medicine, some of them have the potential as a source of antioxidants. One of the plants studied is the gambier plant (Uncaria gambier Roxb.) which has been used by the traditional community for a long time as an antiseptic and medicine for stomach aches. As well as a mixture of betel nut. Until now, not many studies have explored the antibacterial activity of gambier leaves (Kresnawaty et al., 2009).

In Indonesia, traditional medicines from plants are simplicia and herbs. Currently, Indonesia is one of the potential medicinal plant producing countries with its biodiversity. Indonesia's biodiversity ranks third in the world after Brazil and Zaire. Around 30,000 species of flowering plants grow in Indonesia's tropical forests and it is estimated that around 3,689 species of them are medicinal plants. Of these medicinal plants, only 283 species of medicinal plants have been used in the traditional medicine industry (Djauhariya and Hernani, 2004).

The need for traditional medicinal raw materials, especially those derived from plants, is mostly still taken from nature, so that some types are becoming scarce. Most of the plants are useful for the treatment of various types of diseases, including allergic diseases, metabolic diseases, and degenerative diseases related to aging (Djauhariya and Hernani, 2004). Species Uncaria gambier Roxb. is one of the important sap-producing annual plants that is widely used for industrial and pharmaceutical purposes. The role of this species is felt to be increasingly important from time to time, but efforts to improve the genetic potential of these plants have so far not received serious attention (Jamsari et al., 2007). Gambir is efficacious as an astringent. And Gambir is also useful for treating dysentery, burns (external medicine), wounds (external medicine),

Traditionally, gambier is used as a complement to betel nut and as medicine, such as in Malaysia, gambier is used to treat burns, in addition to a decoction of young leaves and shoots, it is used as a medicine for diarrhea and dysentery and as a mouthwash for sore throats. Modernly, gambier is widely used as a raw material for the pharmaceutical and food industries, including raw materials for liver disease medicines with the patent "catergen", raw materials for candy which soothes the throat for smokers in Japan because gambier is able to neutralize nicotine. Meanwhile, in Singapore, gambier is used as a raw material for stomachaches and toothaches (Dhalimi, 2006).

Leaves and twigs are part of the gambier plant which has economic value. The compounds contained in the extract or sap of the leaves and twigs of the gambier plant have various potential uses. The dried extract or sap of leaves and twigs is a product known as gambier, while in world trade it is known as gambier, cutch, catechu or pale catechu. The main compounds contained in gambier are pseudotannin catechins and phlobatanin catechutanic acid with the percentage of each compound being 7-30% and 22-55%. The difference in catechin levels in gambier is influenced by the condition of the extracted leaves (Utami et al., 2008).

Gambir is the main commodity of West Sumatra province. Gambir has long been used as a complement to betel nut which is chewed and is believed to strengthen teeth. Gambir extract contains (+)- catechins as the main component, a polyphenolic compound, which has potential as an antioxidant and antibacterial reported that gambier extract has inhibitory power against Streptococcus mutans bacteria which causes dental plaque. The occurrence of dental plaque can cause caries on the teeth and continue with gingivitis. (+)- catechins are weak acids (pKa1 = 7.72 and pKa2 = 10.22), poorly soluble in water, and unstable in open air. It is easily oxidized at a pH close to neutral (pH 6.9) and is more stable at a lower pH (2.8 and 4.9). Its phytochemical properties are a challenge in itself in the formulation of catechins into medicinal preparations. Utilization of catechins to prevent the occurrence of dental plaque by administering mouthwash that can be taken to obtain the antioxidant activity of catechins (Lucida et al., 2007).

Gambir is a product of the gambier plant (Uncaria gambier Roxb.) which contains functional compounds belonging to the group of polyphenolic compounds. Gambir commercially obtained by processing gambier leaves with the method of boiling, pressing and drying solids. In trade, one of the components of gambier quality is determined based on its catechin content. The extraction of a material is principally affected by temperature. The higher the temperature used, the higher the extract obtained. However, the extracted materials at various temperature levels do not necessarily have a different effect on their antibacterial properties (Pembayun et al., 2007).

2. RESEARCH METHOD

Time and Place of Research

This research was conducted from May to August 2010 at the Phytochemical Laboratory, Botany Field, Biology Research Center, Indonesian Institute of Sciences (LIPI) Cibinong.

Research Principles

The sample used in this study was aqueous extract of dried gambier, extracted from the leaves and plants of gambier (Uncaria gambier Roxb). The gambier water extract to be studied comes from Payakumbuh, West Sumatra. (+)- Catechins were isolated from Gambir and then tested for its antibacterial activity. Likewise with the water extract of Gambir which will be evaluated for its antibacterial activity against gram-negative bacteria.

Preparation of Materials, Media and Tools

a. Preparation of test material

Gambier samples. In this study, the samples used were extracts of the sap of the gambier plant (Uncaria gambier Roxb.) which had been printed. Obtained from the Payakumbuh plantation, West Sumatra. Preparation of Powder, Gambir chunks finely ground to a powder. 500 grams of gambier powder dissolved in 1 liter of hot water. Once dissolved, the soaking results obtained are separated (filtration) with filter paper to produce a filtrate. 250 ml of the water fraction obtained in freeze drying which will then be tested for its antibacterial activity and the other 250 ml will be fractionated with ethyl acetate. Fractionation with ethyl acetate was repeated until a clear filtrate was obtained. After obtaining the ethyl acetate fraction, the filtrate was concentrated with a rotary vacuum evaporator,

b. Sterilization of Tools and Materials

All tools to be used are washed, dried and sterilized beforehand. The test tube, measuring cup and Erlenmeyer were closed with cotton. The petri dish is wrapped in paper. Then everything is put in a heat-resistant plastic and sterilized by autoclaving at 121ºC, for 30 minutes. The loop needle is sterilized by flambir on a Bunsen flame. Laminar Air Flow is sterilized with a UV lamp and sprayed with 70% alcohol. Laminar sterilization is carried out before and after working in it.

c. Preparation of Bacterial Suspensions

An inoculum culture of gram-negative bacteria that has been rejuvenated for 24 hours is taken in one ose that has been ignited and then put in a test tube containing 5 ml of MHB medium and then vortexed and shaken in an incubator shaker at 37°C for 18-24 hours.

3. RESULTS AND DISCUSSIONS

Results

The material used in this research is dried gambier extract.

Figure 1. Dry gambier extract

Isolation of (+)- catechins from the fractionation of ethyl acetate with chloroform : methanol (4:1) resulted in 70.44 grams of dry extract. And the identification of (+)- catechin compounds with TLC produced 1 spot with the same Rf value as (+)- standard catechins.

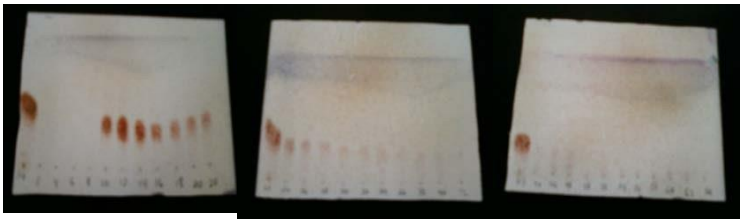


Figure 2. Photo of TLC of the Column Chromatographic Fraction

The results of the combined column chromatography containers are: 9-10, 11-28, 29-40, 41-75. After each fraction was combined and then the solvent was removed, TLC was carried out again to confirm, the results obtained were that fractions 9-40 were (+)- catechin compounds. And produce 1.2079 grams of 1.5 grams of footage. And the percentage of (+)- catechin content is 22.54%.

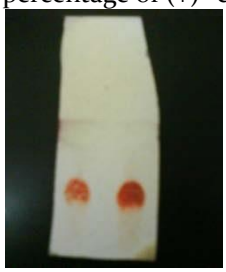


Figure 3. Photo of TLC of Column Chromatography Fractions

From the results of the research that has been done, it shows that the determination of the activity test of gambier and (+)- catechins at a concentration of 7 mg/ml produces a diameter of inhibition of the test bacteria, as shown in the table below:

Table 1. Inhibition diameter of gambier aqueous extract and (+)- catechins against S. flexneri, P. aeruginosa LIPI MC 0103, E. coli LIPI MC 0136, P. vulgaris, P. mirabilis at a concentration of 7 mg/ml.

Bacteria	Diameter of Inhibition Area (mm)			
	Gambir	Control – (DMSO 10%)	(+)- catechins	Control – (Acetone)
Shigella flexneri	7	-	5	-
Pseudomonas aeruginosa	4	-	2	-
Escherichia coli	4	-	3	-
Proteus vulgaris	7	-	6	-
Proteus mirabilis	1	-	3	-

Determination of the MIC value of (+)- catechins and gambier was carried out at concentrations of 5 mg/ml – 25 mg/ml (w/v) with concentration intervals of 2.5 mg/ml.

Table 2. MIC values of gambir water extract and (+)- gambir catechins against Shigella flexneri, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris, Proteus mirabilis after using macro dilution method

ConcentrationExtract (b/v)	*S. flexneri*		*P. aeruginosa*		*E. coli*		*P. vulgaris*		*P. mirabilis*	
	K	G	K	G	K	G	K	G	K	G
5mg/ml	√	√	√	√	√	√	√	√	√	√
7.5mg/ml	-	√	√	√	√	√	√	√	√	√
10mg/ml	-	√	√	√	√	√	√	√	√	√
12.5mg/ml	-	√	√	√	√	√	√	√	√	√
15mg/ml	-	√	√	√	√	√	√	√	√	√
17.5mg/ml	-	√	√	√	√	√	√	√	√	√

20mg/ml	-	√	√	√	√	√	√	√	√	√
22.5mg/ml	-	√	√	√	-	√	√	√	√	√
25mg/ml	-	√	√	√	-	√	-	√	-	√

Information :
√ : There is bacterial growth
- : No bacterial growth
K : (+)- catechins
G : Gambir water extract

Determination of leakage of the bacterial cell wall/membrane was analyzed by measuring the absorbance at a wavelength of 260 nm (nucleic acid) and 280 nm (protein) and measuring the levels of metal ions (Ca2+ and K+) in the test solution after being contacted with the concentration of the test solution 1 KHM and 2 KHM. We can see the measurement results in Figure 4 below:

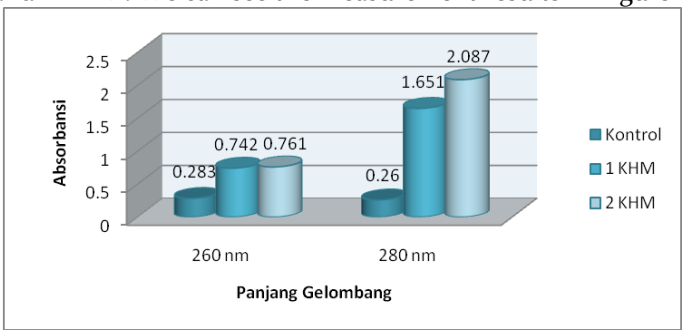


Figure 4. Graph of measurements of cellular metabolite compounds against bacteria Shigella flexneri

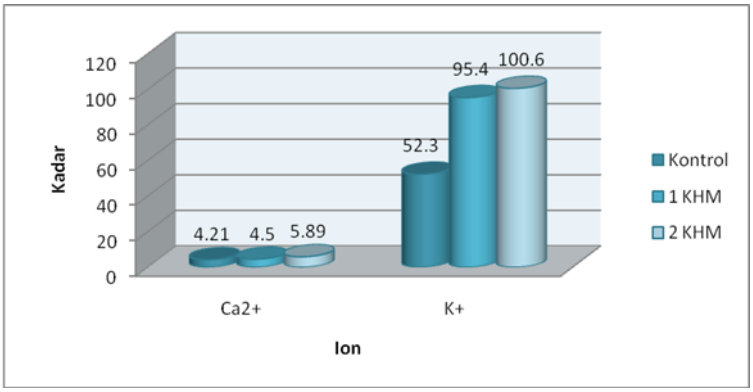
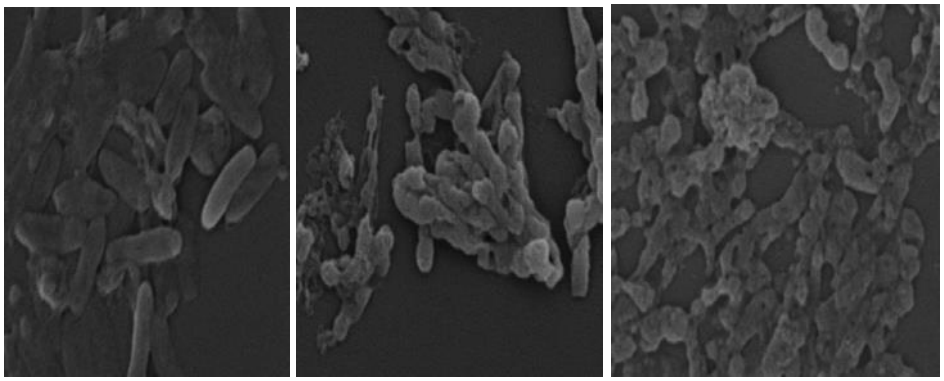


Figure 5. Graph of metal ion content in S. flexneri bacterial culture media treated with 1 MIC and 2 MIC (+)- catechins

Observation of the morphology of the bacterial cell wall/membrane was carried out with the help of a scanning electron microscope (SEM). There was a change in the bacterial wall/membrane after treatment at concentrations of 1 MIC and 2 MIC. The result is as follows:



Control 1 KHM 2 KHM

Figure 7. SEM photographs of S. flexneri cells treated with (+)- catechins.
Magnification 15,000 x.

Discussion

Gambir (Uncaria gambier Roxb.) has long been used as a mixture of betel nut which is believed to strengthen teeth. Gambir extract contains (+)- catechins as the main component, which has the potential as an antibacterial (Lucida et al., 2007). The potency and antibacterial activity of gambier and (+)- catechins can be identified by looking at the diameter of the inhibition and MIC values produced at various concentrations.

In gambier product extract, total phenolic compounds are the most important components related to their antibacterial properties, so far (+)- catechins have been reported as one of the main phenolic compounds in gambier extract (Pembayun et al., 2007). Isolation of (+)- catechins was carried out by column chromatography using chloroform : methanol (4:1) as the eluent. The fractions resulting from column chromatography were then returned to TLC to ensure that the fractions obtained were (+)- catechins by comparison with (+)- standard catechins. Next, the fractions with the same spot are combined and the solvent is evaporated. The yield of catechins obtained was 22.54%. The results of the catechins obtained were then tested against gram-negative bacteria.

Antibacterial sensitivity test used Shigella flexneri, Pseudomonas aeruginosa, Eschericia coli, Proteus vulgaris and Proteus mirabilis with a concentration of 7 mg/ml (Taguri et al., 2006), while for the antibacterial activity test used a concentration of 5 mg/ml – 25 mg/ml. In table 1, it can be seen that the bacteria S. flexneri and P. vulgaris are bacteria that are sensitive to gambier and catechins. Based on the sensitivity test for the five types of test bacteria using the disc diffusion method, it is known that the diameter of the inhibition produced by S. flexneri and P. vulgaris in gambier is 7 mm, while for catechins are 5 mm and 6 mm. The diameter of this inhibition area is larger than that of P. aeruginosa, E. coli, P. mirabilis. It was proven in a study conducted by Voravunthikunchai et al (2004) that the extract from Uncaria gambier produced an inhibition zone against all strains of Escherichia coli O157:H7. And the difference in the size of this inhibition area shows that each bacterium has a different sensitivity, the more sensitive the test bacteria, the greater the diameter of the inhibition produced. The size of the inhibition zone is influenced by the sensitivity of the organism, the culture media, the incubation conditions, the concentration of antimicrobial substances on the disc paper (Lorian, 1980). Substances that produce larger inhibition zones are not necessarily more active than substances that produce smaller zones (Brock, 1973). the more sensitive the test bacteria, the greater the diameter of the resulting inhibition. The size of the inhibition zone is influenced by the sensitivity of the organism, the culture media, the incubation conditions, the concentration of antimicrobial substances on the disc paper (Lorian, 1980). Substances that produce larger inhibition zones are not necessarily more active than substances that produce smaller zones (Brock, 1973). the more sensitive the test bacteria, the greater the diameter of the resulting inhibition. The size of the inhibition zone is influenced by the sensitivity of the organism, the culture media, the incubation conditions, the

concentration of antimicrobial substances on the disc paper (Lorian, 1980). Substances that produce larger inhibition zones are not necessarily more active than substances that produce smaller zones (Brock, 1973).

Administration of (+)- catechins and aqueous extract of gambir with a concentration of 5 mg/ml; 7.5 mg/ml; 10mg/ml; 12.5 mg/ml; 15mg/ml; 17.5 mg/ml; 20mg/ml; 22.5 mg/ml and 25 mg/ml greatly influenced the growth activity of the test bacteria which can be seen in table 2. The results in table 2 show that gambier at a concentration of 5 mg/ml – 25 mg/ml could not inhibit bacterial growth, whereas (+)- catechins at a concentration of 7.5 mg/ml can inhibit the growth of Shigella flexneri. For Eschericia coli, its growth was inhibited at a concentration of 22.5 mg/ml, Proteus vulgaris and Proteus mirabilis at a concentration of 25 mg/ml, while Pseudomonas aeruginosa growth still occurred. The phenol group can inhibit antibacterial activity due to the presence of OH groups (Cowan, 1999). The phenol group found in gambier is catechins and tannins.

At a concentration of 5 mg/ml all the bacteria, both gambier and catechins, were still able to grow, so the micro-dilution method was replaced with the macro-dilution method, besides the resulting extract was red, so it could not be identified by adding INT (Iodonitro Tetrazolium). In the macro-dilution method, antibacterial activity can be seen from the type of bacteria that grows or does not grow. The bacteria that have the lowest inhibitory concentration value in this extract are used for the next stage of research. Leakage in the bacterial cell wall/membrane can be identified by analyzing the presence of proteins and nucleic acids as well as metal ions such as Ca2+ and K+. Leakage of cellular metabolites from bacteria due to the addition of (+)- catechins was measured with a UV-Vis spectrophotometer and was characterized by an increase in the absorbance value at a wavelength of 260 nm for nucleic acids and an increase in absorbance values at a wavelength of 280 nm for proteins (Miksusanti et al. , 2008). Meanwhile, to determine the occurrence of metal ion leakage can be measured with AAS. In Figure 5, it can be seen that the bacterial cell wall/membrane is leaking in the presence of nucleic acids and proteins in the test bacterial culture media solution detected by UV-Vis at a wavelength of 260 nm and 280 nm. From Figure 5, it can be seen that administration of (+)-catechins at a concentration of 1 MIC causes cell leakage which causes an increase in absorbance for nucleic acids (260 nm).

The increase in absorbance at a wavelength of 260 nm is in line with the increase in absorbance for proteins, namely at a wavelength of 280 nm (figure 5). When compared with the increase in absorbance for nucleic acids, the increase for protein (280 nm) is higher. At a wavelength of 280 nm, the absorbance of 1 MIC concentration increased from 0.260 to 1.651 and at 2 MIC concentrations there was an 8-fold increase to 2.087 compared to the control.

According to Davidson and Branen (1999), phenol compounds will react with the cytoplasmic membrane and can increase membrane permeability. Inhibition of bacterial growth is thought to be related to the bacterial cell structure (Ultee et al., 2002). And the presence of membrane damage will result in the release of intracellular components such as amino acids and other materials absorbed at a wavelength of 260 nm, such as nucleic acids and proteins (Maillard., 2002). Nucleic acids can absorb UV light at a wavelength of 260 nm due to the presence of aromatic nitrogenous bases, whereas phosphates and sugars do not contribute to UV absorption (Stansfield et al., 2006).

Not much different from the measurement of cellular metabolites, namely nucleic acids and proteins, the measurement of metal ions (Ca2+ and K+) shown in (figure 6) also shows an increase with increasing concentration of MIC in the test/extract solution. In Figure 6, there was an increase in Ca2+ ion levels from 4.21 ppm – 5.89 ppm and K+ ion levels from 52.3 ppm – 100.6 ppm. The increased Ca2+ and K+ ions released by the test bacterial cells indicated that there had been damage to the cell wall and cytoplasmic membrane. To defend themselves, in general, the cell membrane has a layer of lipids. From the results of research conducted by Seok et al (1999), Lactobacillus sp bacteria in very acidic environmental conditions will cause the main components of the bacterial cell membrane to be damaged and consequently intracellular components such as Ca2+, Mg2+, K+ and lipids will be released. An indication of damage to the cytoplasmic membrane is leakage of cytoplasmic K+ content and an increase in the released K+ content is a sign of damage to membrane permeability (Cox et al.,

2001). Ca2+ and Mg2+ function to maintain the stability of the bacterial membrane and with the leakage of these ions, the stability of the membrane will be disrupted which in turn will result in the death of the bacteria. As happened in cell leakage, the higher the concentration of MIC extract used, the morphology of the test bacterial cells also underwent changes compared to normal cells. Damage to the morphology of bacterial cells was observed by SEM with a magnification of 15,000 times. With 1 MIC extract treatment, the surface of the bacterial cells became rough and uneven and somewhat elongated, and with 2 MIC treatment, the cell surface became rougher and the edges of the cell walls became jagged (figure 7). S. flexneri is normally rod-shaped. Colonies of these bacteria are smooth, small, smooth and flat surface.

4. CONCLUSION

From the results of the research that has been done, several conclusions can be drawn as follows. The content of (+)- catechins in Gambir is 22.54%. (+)- Catechins have antibacterial activity against Shigella flexneri bacteria, where the minimum inhibitory concentration (MIC) value is 7.5 mg/ml (w/v). (+)-Catechins have an antibacterial inhibition mechanism by damaging the cell wall/membrane of the Shigella flexneri bacteria. Gambir water extract has no antibacterial activity and inhibition mechanism against Shigella flexneri, Pseudomonas aeruginosa, Eschericia coli, Proteus vulgaris and Proteus mirabilis.

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