Effectiveness of tannins extract from leaf guava (Psidium guajava L) on the growth and damage of cell morphology Escherichia coli

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RESEARCH ARTICLE

Effectiveness of tannins extract from leaf guava (*Psidium guajava* L) on the growth and damage of cell morphology *Escherichia coli*

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Abstract

This study aims to determine effectiveness of tannins extracted from guava leaf (*Psidium guajava* L) on the growth and damage cell morphology of *Escherichia coli*. The method used for qualitative analysis with the tannins are formed by the intensity of the color is blackish green $FeCl_3$ compounds. In the quantitative analysis of tannins used variations of organic solvent (ethanol with a concentration of 30%, 50%, 70%). Levels of tannins in the sample solution was calculated with Tannates Acid Equivalent (EAT). Test of antimicrobial activity using agar diffusion method, while bacterial cell damage test using SEM (scaning electron microscopy). The best solvent to extract the highest levels of tannins with Ethanol 30% by value of tannin levels 2,351mg /g. The results showed that the extract tannins can inhibit the growth and destroy bacteria by reacting with the cell membrane of *E. coli*. Damage to the cell membrane of *E. coli* can prevent the entry of food ingredients or nutrients that are necessary for bacteria to generate energy as a result of bacteria will experience growth retardation and even death. Tannin compounds including polyphenols, these compounds can inhibit bacteria by destroying the bacterial plasma membrane is composed of 60% protein and 40% lipid which is generally in the form of phospholipids. In the cell membrane of tannin will react with proteins to form hydrogen bonds so that the protein will be denatured, but it can also react with tannins contained phospholipids in cell membranes, resulting in tannin will damage the cell membrane of *E. coli*.

Introduction

The bacteria are very diverse in nature and much, have specific requirements both nutritional and physical needs. Some bacteria have a simple life requirements, while others have a complex life requirements. *Escherichia coli* is a commensal and pathogenic microbes that live in important human and animal digestive tract. These bacteria have been known to show resistance to several antibiotics. This can be an important source of spread of resistance to other pathogens of humans or animals. Resistance genes from bacteria resistant to antibiotics can be spread by human or animal feces to other organisms in the environment. In response to the problem of resistance, which means it can increase mortality and morbidity, required an alternative treatment without the use of chemical antibiotics, namely by using active ingredients found in plants (herbs). Guava leaves (*Psidium guajava* L) is part of the guava tree commonly used as a traditional medicine to cure diarrhea and thrush. Guava leaves (*Psidium guajava* L) containing the active chemical compound saponins, flavonoids, tannins, eugenol and triterpenoids. Polyphenolic compounds dominate guava leaves are flavonoids (> 1,4%) and tannins (BPOM, 2004). Polyphenolic compounds are antibacterial compounds that can inhibit the growth of bacteria. Tannin compounds are polyphenolic compounds that are in plants , food and beverage (Makkar and Becker,1998) and water soluble organic solvent (Haslam,1996). Tannins can be obtained from almost any kind of green plants , plants low level and high level it with the content

and quality varies. Tannins are polyphenolic compounds that are very complex. The effectiveness of antibacterial compounds found in plant tannins guava leaves is influenced by the concentration of tannins. The higher levels of tannin antibacterial activity will increase. According Fardiaz (1992), that may be antimicrobial compounds inhibit the growth of bacteria or mold (bacteristatic or fungistatic) or be kill bacteria (bactericidal) or fungus (fungicidal). Selection of appropriate organic solvents for extracting bioactive components is a decisive factor for the achievement of the goals and objectives of obtaining the extract components. To obtain a good extract needs to be done in stages starting with the extraction of non-polar solvent, then the semi-polar and polar solvent to obtain an extract containing a row of nonpolar compounds, semi-polar and polar (Zainuddin,2006). Based on this assessment should be conducted to determine the effectiveness of the ethanol extract of guava leaves (*Psidium guajava* L) on the growth of *Escherichia coli* in vitro.

Material and Methods

Equipment and Materials

The tools used in this study include analytical scales, blenders, sieves, desiccator, petridish, stir bar, beaker, beakers, pipettes, Erlenmeyer flask, evaporator, filter paper, aluminum foil, vortex and UV-Vis spectrophotometer. Materials used in this study is the guava leaves, distilled water, technical ethanol 30%,50% and 70%. *Folin-Ciocalteu* reagent, Na_2CO_3 7%, $FeCl_3$ 1%,nutrien agar.

Research procedures

1. Sample Preparation of Guava Leaf Wind dried guava leaves for 1 week. Once dried guava leaves seed blend to a powder and sieved using a sieve.
2. Guava Leaf Extraction. The sample extraction by maceration. Weighed as much as 50 g of guava leaves, soaked in 150 ml of ethanol with a concentration of 30%,50% and 70% for 24 hours and then filtered to obtain a filtrate. Treatment was for 3 days. Filtrate obtained together then evaporated to obtain ethanol extracts. The evaporated extract was cooled in a desiccator before further analysis.
3. Phytochemical test Tannins Using $FeCl_3$ 1% phytochemical test is a qualitative test for the suspected presence of tannin in the extract of guava leaves. Phytochemical test conducted in this study that adding extracts with $FeCl_3$ 1% reagent indicated by the color change of green or blue-black ink. Phytochemical test using $FeCl_3$ 1% is used to determine whether a sample contains a phenol group is indicated by a green color blackish or dark blue after being added with $FeCl_3$ 1%, so if phytochemical with $FeCl_3$ 1% test gives a positive result it made possible the samples contained phenolic compounds and possible one of them is tannin. Because tannins are polyphenolic compounds. This was confirmed by Harborne (1996) classic way to detect simple phenol extract is added to a solution of $FeCl_3$ 1% in water, which cause the color green, red, purple,blue and black strong. Formation of green or blue-black ink on the extract after added with $FeCl_3$ 1% as tannins will form complexes with Fe^{3+} ions.
4. Determination of levels of total tannins manufacture of standard solution. Carefully weighed 10 mg gallic acid, then dissolved in distilled water and paid back the volume up to 10 ml to obtain a level of 1 mg / ml as a stock solution. And the stock solution pipetted their respective 10, 15, 20, 25 and 30 ml, was added to 0.2 ml of *Folin-Ciocalteu* (after diluted with distilled water 1:1), homogeneously mixed for 10 seconds and then allowed to stand for 5 minutes. Then add 2 ml of Na_2CO_3 7% w/v (in distilled water), homogeneously mixed for 30 seconds, then paid back the volume to 5 ml with distilled water in a pint flask in order to obtain a final concentration of 2, 3, 4, 5, and 6 mg/ml. Allowed to stand for 95 minutes. Measured on a UV-Vis spektrofometer the maximum wavelength.

Preparation of samples

Carefully weighed 0.1 mg of guava leaf extract was then added to 0.2 ml of *Folin-Ciocalteu* (after diluted with distilled water 1:1), and then allowed to stand for 5 minutes. Then add 2 ml of Na_2CO_3 7% w/v (in distilled water), homogeneously mixed for 30 seconds, then paid back the volume with distilled water to 10 ml in a pint flask. From this stock solution pipetted 1 ml and diluted with water to 10 ml of distilled water. Allowed to stand for 95 minutes. Measured on a UV-Vis spektrofometer at maximum wavelength. Replication is done 3 times.

Antimicrobial Activity Test Methods Diffusion

Solid media that has been heated until melted, cooled to a temperature of ± 40°C and poured in a sterile petri dish which was added 0.1 ml of a solution of active bacterial cultures, homogenized and allowed to solidify. Paper discs (diameter 6 mm) impregnated with a way to shed 20 mL extracts from several concentrations (0,1 % ,1 % , 10 % , 20 % and 30 %) and negative control (DMSO) (Zakaria *et al*., 2007). Subsequently incubated at 37° C for

18-24 hours for bacteria and fungi 35°C for 48 hours. The diameter of the inhibition zone formed was measured using calipers (Volk and Wheeler,1993).

Result and Discussion

Extraction tannin

Guava Leaf Extraction According Harborne (1996) to extract the tannins in a total network of plants required a solvent capable of dissolving polar compounds especially tannins. Water is a good solvent for most of the tannins, but the best solvent is a mixture of organic solvents and water. Extraction is the process of separating a substance based on differences in solubility of the two immiscible liquids are different. Extraction method used in this study is extracted by maceration method. Maceration is a simple extraction method. Maceration is done by immersing the sample in an organic solvent. Organic solvents will penetrate the cell wall and into the cavity of the cell that contains the active substance so that the active substance will dissolve. Due to the difference between the solution concentration of active substance in the cell, then the solution is concentrated pushed out. The advantage this extraction method, is the method and the equipment used is simple and easily cultivated (Cheong *et.al*, 2005). Material to be macerated 50 g soaked in a mixture of organic solvents (ethanol): water (1:3) with a concentration of 30%, 50% and 70% for 24 hours and treatment was repeated up to 3 times (Harborne 1996). Maceration is used because this process has a fairly high absorption effectiveness of the active substances contained in the leaves of guava include tannins. Fluid results maceration then evaporated with rotary evaporator (Rotavapor) to obtain a crude extract of guava leaves thick and brown, then to dry it using Frezdryer. Concentration aims to determine the % yield as well as ease in terms of storage when compared in an extract that is still strong (there are still solvent). The yield difference on one of the six samples because the content of bioactive extracted by solvents, so that the results obtained yield was varied. Yield obtained can be seen in Table 1. Percentage yield calculated from the weight of dry extract extracted by looking at the weight of the initial sample.

Table 1. Sample Results Ekstrasktif Guava Leaf With Variations Solvent Concentration

Solvent	Dry Sample Weight (g)	Extract Weight (g)	Yield (%)
Ethanol 30%	50	4.593	9.186
Ethanol 50%	50	4.944	9.888
Ethanol 70%	50	5.686	11.371

Yield results obtained as shown in Table 1 indicate that the solvent composition of ethanol 70% has a higher yield is 11.37% rather than solvent composition.

Qualitative and quantitative test tannins

Phytochemical test conducted in this study that adding crude extract of guava leaves with reagent $FeCl_3$ 1%. Results indicate a change in the color of blackish green, as shown in Figure 1.

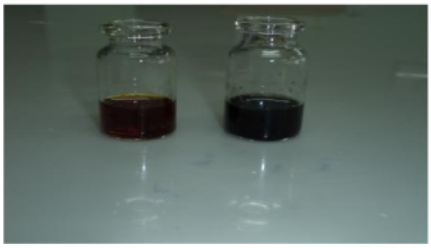

Figure 1. Qualitative test Tannins Using $FeCl_3$ Determination of Content of Total Tannins

Tannins are generally defined as polyphenolic compounds have high molecular weight (over 1000) and can form a complex with the protein. Determination of total content of tannins in leaves of guava using total phenol method using *Folin-Ciocalteu* reagent and standard tannat acid. Determination of total phenol is used to determine the content of tannin contained in each sample. This method has advantages including better color rendition, can minimize the differences at the time of testing and more specific (Rita, 2006). Folin method does not distinguish between types of phenolic components. The more the number of phenolic hydroxyl group, the greater the concentration of phenolic components were detected (Khadambi, 2007). Tabel 2 shows that the highest content of total tannins contained in the 30% ethanol extract tanin higher levels compared with the other extracts.

Table 2. Total Tannin Content Testing Data In Guava Leaf Mean Number of Deuteronomy solvent (mg/g)

Solvent	Replay			Number	Average (mg/g)
	1	2	3		
Ethanol 30%	2.118	2.484	2.452	7.054	*2.351*
Ethanol 50%	1.688	1.710	1.785	5.183	1.728
Ethanol 70%	1.871	1.796	1.839	5.506	1.835

Based on table 2 it can be seen that by using different solvents and concentrations, the amount of extractable tannins are also different, although the solvent used is the same.

Antibacterial Activity Test Extract Tannins

The selection of the best solvent in this study are based on the total tannin assay with Folin Ciocalteu method . The best solvent to extract the highest levels of tannins with Ethanol 30 % by value of tannin levels 2.351mg /g . The selection was based on the best solvent extracts tannins that have the highest levels of value, so it can provide antimicrobial inhibition greater. This statement is confirmed by Zulaekah (2005) based on research results show that the higher the concentration or level of tannins in the tea leaf extract that is used in the manufacture of pickled eggs produce salted boiled eggs with the least number of total bacteria. This suggests that the higher levels of tannins in a sample it is possible to be more effective in killing or inhibiting the growth of bacteria. Antimicrobial activity assay in this study using a crude extract of tannin extract of guava leaves have not yet done but the process of separation or fractionation based phytochemical test and determination of tannins using the *Folin-Ciocalteu* method was enough to show that in the extract contains tannin.

Testing antimicrobial activity of tannin extract of guava leaves using *E. coli* as test bacteria. This is done to determine whether the tannin extract of guava leaves can inhibit the growth of *E. coli*, because there is the possibility of tannin which is a chemical that most of the spread in the plant is capable of inhibiting bacterial cell wall synthesis and bacterial cell plasma membrane damage, so it needs to be investigated tannin activity against these microbes.

Extract concentration of solvent ethanol 30 % used is 0,1 % , 1 % 10 % , 20 % and 30 % to determine the antimicrobial activity of tannin from guava leaves. Test the activity of tannin extract as antimicrobial done by measuring the inhibition zone formed around the discs. The test results demonstrated antimicrobial activity through inhibition diameter tannin extracts against microbes can be seen in Figure 2 below.

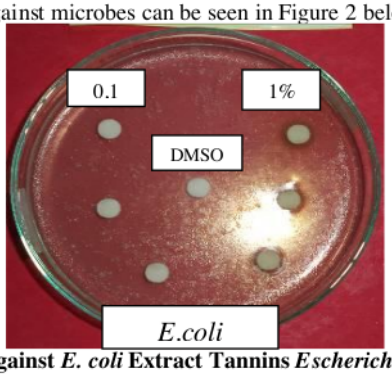

Figure 2. Diameter of Inhibition Against *E. coli* Extract Tannins *Escherichia coli*

ANOVA results showed that the treatment concentrations of tannin extract of guava leaves provide a significant influence (P< 0.01) against *E. coli* diameter inhibitors. Honestly significant difference test results (indicating that the extract with a concentration of 0,1 % was not significantly different from the concentration of 1 % and 10 %, but significantly different from the concentration of 20% and 30%. Figure 2 shows that with increasing concentration of the extract, the greater the diameter of inhibition which means that the greater the concentration of active ingredient that acts as an antibacterial, so its ability to inhibit the growth of *E. coli* bacteria. The results of this study demonstrate using tannin extract concentration at low concentrations has been able to give effect to the inhibition bacteria *E.coli*. This can happen because the cell wall of Gram- negative bacteria are thinner so the tannins it easier to attack the protein found in the cell wall of the bacteria *E.coli*. Proteins in bacteria is one of the constituent components of the cell wall and plasma membrane, where the protein on the cell wall of the damaged or denatured bacterial cell wall will be easily penetrated by a chemical substance which causes impaired metabolism of bacteria. Cell wall of Gram-positive bacteria contain peptidoglycan mukopeptida or 90 % and the cell wall has a thickness of 25-30 nm, whereas the Gram-negative cell wall is thinner that has a thickness of 10-15 nm and consists of three layers. The inner layer is Mukopeptida, the outer layer is composed of two layers, namely lipopolysaccharide and lipoproteins.

Tannins can form hydrogen bonds with the protein contained in bacterial cells, if the hydrogen bonds formed between tannins with proteins will be denatured proteins possibility that bacterial metabolism becomes impaired. The reaction of tannins with proteins to form a bond taninprotein. Section reactive protein and has the ability to bind with tannin is a peptide bond, hydroxyl and amide groups. Bonding is the dominant hydrogen bonding between the carboxyl group of the peptide bond with the hydroxy groups of the tannins. The formation of hydrogen bonds between tannin with protein causes a conformational change in the protein molecule that biochemical activity is reduced. This conformational change is called denaturation of proteins, clotting proteins normally preceded by denaturation that work well on isoelectric point, so that the protein will undergo coagulation. Allegedly based on the reaction of tannins can inhibit the growth of bacteria *E. coli*.

Tannin can also inhibit growth and kill bacteria by reacting with the cell membrane. Tannin compounds including polyphenols, these compounds can inhibit bacteria by destroying the bacterial membrane plasma is composed of 60 % protein and 40 % lipids are generally in the form of phospholipids, in the cell membrane of tannin will react with proteins to form hydrogen bonds so that the protein will be denatured, besides the tannins also can react with phospholipids found in cell membranes, resulting in tannin will damage the cell membrane, causing leakage of essential metabolites that inactivate the bacterial enzyme system. Damage to the cell membrane to prevent the entry of food ingredients or nutrients that are necessary for bacteria to generate energy will experience as a result of bacterial growth inhibition and even death (Volk and Wheller,1988). Inhibition of growth of *E. coli* extracts tannins from leaves of guava is also suspected to be caused by this mechanism .

Analysis of Damage Cell *E.coli* With SEM (scaning electron microscopy)

Based on the test results and the effectiveness of antimicrobial activity, we used a concentration of 1% tannin-rich extract of guava leaves to see the effect caused by tannin extracts against microbes through Scaning Electron Microscope (SEM). Normal cells of *E. coli* short rod-shaped, Gram-negative, size 0,4-0,7 µm x 1,4 µm, as shown in (Figure 4a). Effect of the addition of tannin extract with a concentration of 1% of the bacteria *E. coli* can be seen in (Figure 4b). The observation of morphology cell *E. coli* with SEM as shown in Figure 4.

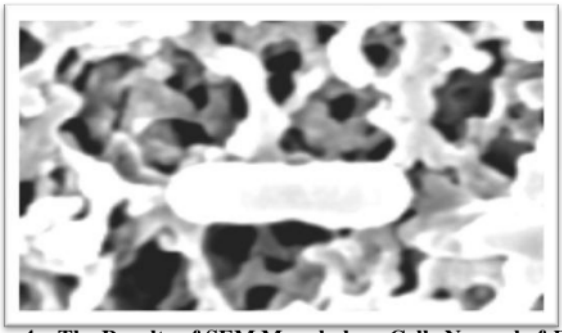

Figure 4a. The Results of SEM Morphology Cells Normal of *E. coli*
Source: Suliantari 2009

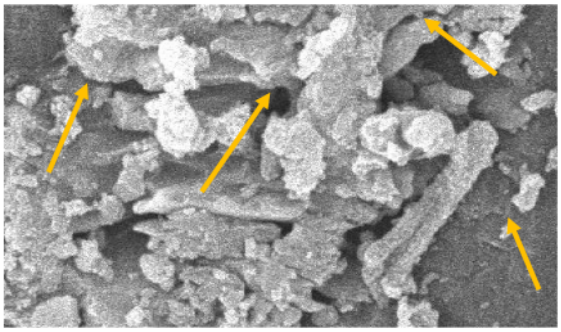

Figure 4b . The Results of SEM The morphology cell of *E. coli* Given The Tannins Extract of Guava Leaf

Based on the SEM observations (Fig.4b) the effect of tannin extract with a concentration of 1 % of the bacteria *E. coli* can cause cell walls *E. coli* become wavy, there are indentations, bends and bulges are indicated via arrows in Figure 4b . The presence of this bulge in the cell wall may be due to increased cell membrane porusitas due to weakening of the cell wall by an extra rich guava leaf tannins. Increased permeability membrane porusitas cause cell changes that can lead to cell leakage. Changes in cell wall permeability resulting in fluid seeping out cytoplasm to form intercellular space membrane cytoplasm. This space will be even greater with the weakening of the cell wall. In membram state can not withstand the pressure from the cytoplasmic membrane to leak, the flow out of the cell cytoplasm where the cytoplasm when the amount of fluid that came out in large numbers to cause the cells become shriveled and dead .

Phenolic compounds can react with the phospholipid component of the outer membrane cells of *E. coli*, causing cell walls into the lysis (Branen and Davidson 1993). Cell lysis can be caused by disruption of the enzymes that synthesize the cell wall, resulting in weakened cell walls and increased porusitas (Gilbert 1984). Lysis of the cell wall can cause partial or quasi- separated. In Gram-negative bacteria by treatment with tannin extract of guava leaves causing a portion of the outer membrane a protein-lipopolysaccharide- peptidoglycan discharged while still remaining detached, the cell is called spheroplas. According to Conte and Barriere (1992) spheroplas form as is also found in the cells *E. coli* were treated with penicillin for 90 minutes .

Conclusion

1. The best solvent to obtain tannin extract of guava leaf is ethanol 30 %. This is supported by the calculation of tannin levels in each extract, tannin extract levels in the ethanol 30 % of 2.351 mg/g.
2.Tannin extract of guava leaves have antibacterial activity against *E.coli*.
3.Mechanism of action of the tannin extracts on cell bacterial damage depends on the type of bacterial. Extracts tannins are given in *E. coli* led to cell wall damage suffered.

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