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In vitro evaluation of antibacterial activity of *Annona squamosa* Linn leaf extract against *Escherichia coli*

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Abstract

Background. *Escherichia coli is normal bacterial flora that located in the lower gastrointestinal tract. Most of Escherchia coli strains are harmless, however some of them can cause mild infection until severe life-threatening complication. Recently, it has been reported that rapidly rising antibiotics resistance on this bacteria because the relatively high use of antibiotics. Therefore, new innovations are needed to reduce the increasing number of antibiotic resistant bacteria. One of them is by using medicinal plants that has antimicrobial activity. Flavonoids, alkaloids, tannins, and terpenoids contents on Annona squamosa L. leaf extract have been proved potentially as antibacterial.*
Objectives. *This study aimed to evaluate the potential activity of Annona squamosa L. ethanolic leaf extract against the growth of Escherichia coli bacteria.*
. ***Methods.*** *This is an experimental laboratory study using the agar well diffusion method in the Microbiology Laboratory, Faculty of Medicine, University of North Sumatra. The fresh leaves of Annona squamosa L. were extracted by maceration method using the 96% ethanol. The various concentration of the extract (6.25%, 12.5%, 25%, and 50%) were added to Muller Hinton agar that already applied with 24-hours old culture of Escherichia coli ATCC 25922, with gentamicin as positive control and DMSO as negative control.*
Results. *The diameters of the inhibition zone with concentrations of 6.25%, 12.5%, 25%, 50% Annona squamosa L. leaf extract consecutively 5,525± 0,450 mm, 6,275 ± 0,350 mm, 6,525± 0,471 mm, 7,925± 1,072 mm.*
Conclusion. *In conclusion, the ethanolic leaf extract of Annona squamosa L. has antibacterial activity against Escherichia coli.*
Keywords *: Annona squamosa L.,antibacterial, Escherichia coli, ethanolic extract.*

Introduction

Escherichia coli is a gram-negative bacteria that naturally presents in the human lower gastrointestinal tract. Most of the *E. coli* strains are non-pathogenic, however some of them can cause several infections. The most common is urinary tract infection, moreover diarrheal disease, sepsis/meningitis also caused by *Escherichia coli*.[1] This bacteria can be transmitted through contaminated food (65%) and water (15.4%), person-to-person contact (10%), direct or indirect contact with animals or their environment (10%).[2]

Antibiotics are the best choice for tackling bacterial infections. But due to the indiscriminate, incessant and misuse of antibiotics, the number of antibiotic-resistant bacteria increased.[3] The existence of multidrug-resistant bacteria is a serious threat and particular concern for global health. It has been reported that *E. Coli* isolated from the food handler's hands at a primary school in Malaysia is resistant to Penicillin and Chloramphenicol (85.71%), Sulfamethoxazole-Trimethoprim, Ampicillin and Trimethoprim (57.14%), Kanamycin and Tetracycline (28.57%) and Ciprofloxacin (14.29%).[4] Therefore, new innovations are needed to reduce the increasing number of antibiotic resistant bacteria. One of them is by using medicinal plants that has antimicrobial activity.

In recent years, the use of herbal medicine has significantly increased in the developing countries. World Health Organization estimated that 80% of the world's population in the developing countries rely mainly on herbal medicine for their primary health care needs.[5] Antimicrobial substances of various extracts from many plants have recently been of great interest in researches, because of their possibility in replacing synthetic antimicrobials with natural ones. Thus medicinal plants are a potential source for the development of newer drugs because of the effectiveness, less side effects and relatively low cost.[6]

Annona is the second largest genus of flowering plants in the family Annonaceae family.[5] *Annona squamosa* Linn, commonly known as sugar apple, is the most developed tropical fruit in the family.[7] During this time, custard apple only known for its edible fruit, though various parts of *Annona squamosa* L. are traditionally used for treatment of various diseases. *Annona squamosa* seeds extracts are reported to have anti-tumor activities against human hepatoma cells, the petroleum ether extract from the bark of *Annona squamosa* is studied for its analgesic and anti-inflammatory activity, *the ethanolic extract of Annona squamosa leaves has been reported to have antidiabetic effect*, the aqueous extracts of *A. squamosa* leaves show antifungal and antioxidant activities.[5,8,9,10] Therefore, this study is conducted to evaluate the antibacterial activity of various concentration ethanolic leaf extract of *Annona squamosa* L. against the growth of *Escherichia coli*.

Methods

This research is an experimental research with posttest only control group design. The *Annona squamosa* L. leaf extract was made in the Biology Laboratory, Faculty of

Pharmacy, University of North Sumatra. The research on the antibacterial activity of *Annona squamosa* L. leaf extract against the growth of *Escherichia coli* bacteria was conducted in the Microbiology Laboratory, Faculty of Medicine, University of North Sumatra. This study was conducted from July to October 2019.

The sample of this study is *Escherichia coli* bacteria that has been given various concentrations of *Annona squamosa* L. leaf extract. Escherichia coli ATCC 25922 is obtained from the Laboratory of Microbiology, Faculty of Medicine, Universiy of North Sumatra, while the *Annona squamosa* L. leaf is obtained from a garden located in Lubuk Pakam area, Medan.

In this study, the sample is given six treatments using *Annona squamosa* L. leaf extract with concentrations of 6.25%, 12.5%, 25%, 50%, gentamicin as the positive control, and Dimethyl Sulfoxide (DMSO) as the negative control. These six treatments are repeated four times so that the total number of samples is 24.

- **Collection of plant material**

The fresh leaves of *Annona squamosa* L. were collected from a garden located in Lubuk Pakam area, Medan, Indonesia in July 2019. The plant specimen was identified and authenticated at the Herbarium Medanese Laboratory, University of North Sumatra. After identification, the plant material was processed for extraction procedure.

- **Preparation of the plant extract**

The leaves were washed thoroughly with running tap water and shade dried for 24 hours. Then, the leaves were drained in a drying cabinet with a 40-watt lamp for 5 days. Leaves were then crushed into small pieces and finally powdered using an electric blender. The leaves powder was then stored in plastic bags for further utilization. The 300 grams of dried powdered leaves were macerated by mixing 3 L ethanol 96% in the container and then sealed tightly and soaked for 24 hours while occasionally stirred. After 24 hours, the solution of the extract was filtered using Whatman paper No. 1, the process was repeated twice. After that, all filtrate were collected and evaporated using the rotary vacuum evaporator, then the remaining filtrate was concentrated using a steam cup in the waterbath so that a viscous extract was obtained. The extract was stored in the refrigerator at 4 °C for future use.

- **Assessment of antibacterial activity**

Escherichia coli ATCC 25922, which were collected from Microbiology Laboratory, Faculty of Medicine, University of North Sumatra were used in this study. The antibacterial activity of the leaf extracts was evaluated by the agar well diffusion method. Petri dish that contains Muller Hinton agar were applied each with 24-hours old culture of *Escherichia coli*. Wells was bored using a sterile cork's pit with approximately 6 mm diameter and the various concentrations of extract were added. The extract was dissolved in Dimethyl Sulfoxide (DMSO) with concentration levels of 6,25%, 12,5%, 25%, and 50%. Gentamicin was used as a positive control and DMSO as a negative control. The plates were incubated at 37°C for 24 hours in an upright position. The antibacterial

activity was assayed by measuring the diameter of the inhibition zone formed around the well using caliper.

- **Statistical analysis**

Statistical analysis were performed using SPSS Statistics version 25. Data were analyzed using one-way analysis of variance (ANOVA).

Results

The diameters of the inhibition zone of *Escherichia coli* that has been given *Annona squamosa* L. leaf extract with concentrations of 6.25%, 12.5%, 25%, 50%, the positive control and negative control are shown in the table 1.

Table 1 : The diameter of inhibition zone of ethanolic leaf extract of *Annona squamosa* L.

Diameter of inhibition zone (mm)						
Repetition	DMSO	**Concentrations of extract**				Gentamicin
		6,25 %	12,5%	25 %	50 %	
I	0	5,5	6,7	7,2	8,9	19,6
II	0	5	6,4	6,5	7,1	17,8
III	0	5,5	5,9	6,2	8,8	16,4
IV	0	6,1	6,1	6,2	6,9	18
Mean ±SD	0	5,525 ± 0,450	6,275 ± 0,350	6,525 ± 0,471	7,925 ± 1,072	17,950± 1,310

Table 1 showed that *Annona squamosa* L. leaf extract with a concentration of 6.25%, 12.5%, 25%, and 50% had a smaller diameter of inhibition zone than positive control gentamicin. This suggests that *Annona squamosa* L. leaf extract has antibacterial activity against *Escherichia coli* bacteria, but the antibacterial effects of *Annona squamosa* L. leaf extract are not as strong as the positive control. According to the table 1, it can be concluded that the higher the concentration of *Annona squamosa* L. leaf extract, the greater the diameter of the inhibition zone was formed.

Based on the results of One Way ANOVA variant test, obtained significance value 0.000 ($P < 0.05$). This indicates that there is significant difference in the various concentrations of *Annona squamosa* L. leaf extract in inhibiting the growth of the *Escherichia coli* bacteria.

Discussion

In this study, *Annona squamosa* L. leaf extract was obtained by maceration method with 96% ethanol as solvent. The maceration method has an advantage in phytochemical constituents isolation because it avoids the phytochemical modification. The selection of ethanol as solvent plays an important role in the results because it affects the amount of phytochemicals contained in the extract. The active compounds in *Annona squamosa* L. leaf extract are polar, so it will be easier to dissolve in polar solvent. Based on the research, among the four (Petroleum ether, Ethanol, Water, Chloroform) leaf extracts, the ethanolic leaf extract of *Annona squamosa* L. was found to contain the major phytochemicals.[11]

The average diameter of the inhibition zone formed in this study is 5.525 mm in 6.25% concentration,

6.275 mm in 12.5% concentration, 6.525 mm in 25% concentration, and 7.925 mm in 50% concentration. It shows that the diameter formed on each concentration of *Annona squamosa* L. leaf extract increases with increasing concentration. The results of this study is in accordance with a previous research which stated that the higher the concentration of *Annona squamosa* L. leaf extract, the higher the content of phytochemical constituents resulting in a more maximal inhibition of baceria growth[6]. Phytochemical analysis revealed that the presence of flavonoids, alkaloids, tannins, and terpenoids in the ethanolic leaf extract of *Annona squamosa* L. are responsible for antibacterial activity.[11]

The mechanism of flavonoids as antibacterial is the protein denaturation of bacteria cell wall by binding proteins through hydrogen bonds, damaging the cytoplasmic membrane, causing leakage of important metabolites and activates enzymes on bacteria. This breakdown causes the leakage of nucleotides and amino acids and prevents the inclusion of active ingredients into cells, this condition could lead to the death of the bacteria.[12] Flavonoids have H^+ ions that are capable of attacking polar groups (phosphate clusters) so that the phospholipids molecule will decompose into glycerol, carboxylic acid and phosphoric acid resulting in phosphorpides which are incapable of maintaining the form of cytoplasmic membrane. Hydrogen bonds in the flavonoids also play a role in disrupting DNA synthesis in bacteria and disrupting bacterial energy metabolism.[13]

In the alkaloids structure, there is a base group containing nitrogen which could react with the amino acid compounds that compose the bacteria cell walls and bacteria DNA. The reaction will result in changes in the structure and arrangement of amino acids, causing a change in the genetic balance of the DNA chain in which it will suffer damage and trigger the occurrence of bacterial lysis that will cause bacteria cell death.[14] In addition, the OH group in the structure of alkaloids can also increase the activity of inhibiting the growth of bacteria with protein denaturation, resulting in the increase of cell membranes permeability. The increased permeability of bacteria cell membranes causes leakage of bacterial intracell components to the outside, consequently the bacteria will gradually die.[15]

Tannins are the most commonly secondary metabolites found in plants. Tannin can precipitate the proteins of the bacteria so that the enzymes produced by bacteria and the transport proteins of the bacteria cell wall become inactivated, damaging the bacteria cell wall.[16]

Terpenoids have a toxic effect on the cell wall of bacteria. It can interact with proteins present in cell membranes and intracellinal components, resulting in the disruption of the structure of bacterial membrane of *Escherichia coli*. This causes the cell membrane to undergo degradation structurally and

functionally. The damage of the cell membranes causes the cytoplasm coagulate and the cell membrane permeability increase, resulting in the leakage of intracellular components and reduced synthesis of ATP.[17]

Conclusions

In conclusion,the ethanolic extract of *Annona squamosa* L. leaves has antibacterial activity against the growth of *Escherichia coli* bacteria. The higher the concentration of the extract, the greater the inhibition zone was formed.

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