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ORIGINAL ARTICLE

In Vitro Antibacterial Activity of *Averrhoa bilimbi* Leaves Ethanol Extract against *Salmonella typhi*

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A B S T R A C T

Introduction: Some native plants in Indonesia have potential effects on health and medication. This study aimed to determine the potential antibacterial effect of *Averrhoa bilimbi* (*A. bilimbi*) leaves ethanol extract against *Salmonella typhi* (*S. typhi*) bacteria.

Methods: This was an experimental study. The antibacterial potency of *A. bilimbi* leaves was measured against *S. typhi* in vitro. Different concentrations of the leaves' ethanol extract were prepared. The minimum inhibitory concentration (MIC) was evaluated by a macro-dilution method using Mueller Hinton broth. The minimum bactericidal concentration (MBC) value was observed by subculturing the specimen from the previous dilution tube to the nutrient agar.

Results: The MIC of *A. bilimbi* leaves ethanol extract against *S. typhi* bacteria could not be determined because all the treatment tubes' colors were dark and turbid. Meanwhile, the MBC value was at 500 mg/ml.

Conclusion: *A. bilimbi* leaves extract had potential bactericidal effects against *S. typhi* with a MIC value that could not be determined.

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Introduction

Typhoid fever is an acute systemic illness caused by *Salmonella typhi* (*S. typhi*) bacteria. It is still a major health problem in Asia and Africa, including Indonesia. The global estimation of typhoid fever cases is more than 21 million. The deaths were predicted to be approximately 200 thousand each year.[1] The disease is mostly derived from ingesting food or drink contaminated by feces and is rarely caused by urine or vomitus of patients and carriers.[2,3] According to some studies, washing hands with soap at critical moments reduces the incidence of the disease.[4] Approximately 10^5 *S. typhi* is needed to reach an infective dose, resulting in clinical symptoms.[5] The incubation period is usually 3-6 days. Common symptoms are fever, weakness, abdominal pain, constipation, and headaches. After manifestation or subclinical infection, some people become carriers for an uncertain time.[5]

Some strains of *S. typhi* have undergone antibiotics against multidrug resistance: ampicillin, chloramphenicol, and cotrimoxazole.[6] The bacteria are also already resistant to fluoroquinolone.[7] Therefore, new strategies are needed to reduce further resistance. An alternative way is by using herbs. *Averrhoa bilimbi (A. bilimbi)*, locally known as '*Belimbing wuluh*' in Indonesia, has been used for traditional medicine for a long time.[8] The effectiveness of *A. bilimbi* products for infectious and non-infectious diseases has been proved by several studies. Commonly, people process the leaves into a paste to be used as a remedy for some skin problems like itches, swollen skin due to insect bites, and some skin infections. It is also believed to be beneficial for treating rheumatism and mumps.[9] Leaves infusions are used as a tonic after childbirth and to treat cough.[8] The bioactive compounds in *A. bilimbi* leaves, such as saponins, tannins, alkaloids, phenols, and flavonoids, have some antimicrobial activity.[10] The leaf extract had been proven effective against some gram-positive and negative bacteria.[11] Previous studies have proven that *A. bilimbi* leaves extract has antidiabetic, antimicrobial, antihyperlipidaemic,[11] anti-inflammatory, cytotoxic,[12] and antioxidant effects.[13] This study aimed to measure the antibacterial potency of *A. bilimbi* leaves against *S. typhi.*

Methods

This was an experimental study. This study had received ethical clearance from the Ethics Committee in Health Research, Faculty of Medicine, Universitas Airlangga, Surabaya.

Plant materials: *A. Bilimbi* leaves fine powders were collected and determined from the technical implementation unit (UPT) of Balai Medika, Batu.

Plant extracts: Ethanol 96% was used as a solvent. The process of extraction was performed by the maceration method. It was performed at the Laboratory of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga.

Bacteria cultures: *S. typhi* colonies were gained from the Laboratory of Microbiology, Faculty of Medicine, Universitas Airlangga.

Preparation of *A. bilimbi* extract: The crude extract was prepared in concentrations of 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, and 15.625 mg/ml by suspension with sterile H_2O.

Preparation of *S. typhi* bacteria: The turbidity standard of *S. typhi* suspension corresponded to 0.5 McFarland.

Antimicrobial assay: The macro-dilution method was used in determining antibacterial activity. In minimum inhibitory concentration (MIC) susceptibility testing, Mueller-Hinton broth medium was prepared on eight tubes, consisting of six treatment tubes (O1-O6) and two control tubes (ON & OP). Each treatment tube contained an extract of *A. bilimbi* leaves suspension with various concentrations from 500mg/ml (O1), 250 mg/ml (O2), 125mg/ml (O3), 62.5 mg/ml (O4), 31.25 mg/ml (O5), and 15.625 mg/ml (O6) respectively, *S. typhi* bacteria (0.5 McFarland), and Muller-Hinton broth media. ON tube, as the negative control, was filled with 500mg/ml *A. bilimbi* leaves extract. As the positive control, the OP tube was filled with a suspension of *S. typhi* bacteria. All tubes were then incubated for a day at 37°C. Finally, with unaided eyes, MIC was visually observed as the lowest concentration in the clear tube due to the absence of *S. typhi* bacterial growth.

A subculturing specimen determined the minimum bactericidal concentration (MBC) from the previous dilution tube to the nutrient agar. Each diluted tube was then streaked to a nutrient agar plate. Next, all tubes were incubated for another 24 hours. The MBC was determined visually by the unaided eye as the lowest concentration on the clear plate without the growth of *S. typhi* bacterial colonies. According to Federer's formula, this experiment requires replication to be performed at least four times.

Results

Minimum Inhibitory Concentration (MIC)

With unaided eyes, MIC was observed as the lowest level of the diluted treatment tube, which appeared clear due to the absence of microbial growth. Figure 1 and Table 1 show the colors and turbidity of each tube. The color of *A. bilimbi* extract was dark and turbid. The OP tube color which contains only *S.thyphi* bacteria was white and turbid. This turbidity was due to the growth of *S. typhi* bacteria. On the other hand, ON and O1-O6 tubes color were brown and turbid. Bacterial growth could not be observed in all these treatment tubes. Therefore, the MIC value could not be determined. The results were the same for 4x replications.

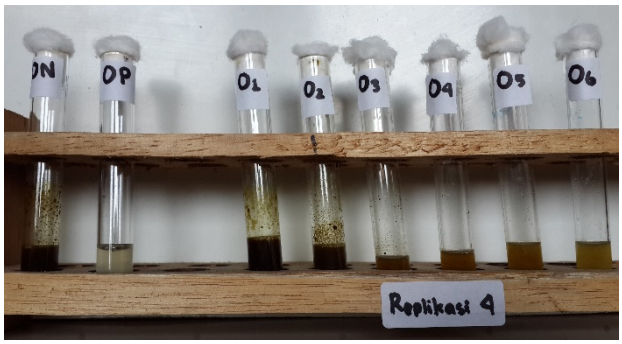

Figure 1. Dilution test to determine the MIC

Table 1. MIC dilution test result

Tube	Observation of bacterial growth			
	1st Replication	2nd Replication	3rd Replication	4th Replication
OP	X	X	X	X
ON	X	X	X	X
O1	X	X	X	X
O2	X	X	X	X
O3	X	X	X	X
O4	X	X	X	X
O5	X	X	X	X
O6	X	X	X	X

X = turbid
O = clear
OP = positive control
ON = negative control
O1-O6 = treatment tube with various concentration
O1 = 500 mg/ml
O2 = 250 mg/ml
O3 = 125 mg/ml
O4 = 62.5 mg/ml
O5 = 31.25 mg/ml
O6 = 15.625 mg/ml

Minimum Bactericidal Concentration (MBC)

MBC was visually determined as the lowest concentration of the leaf extract without the growth of the bacterial colonies in the plate. Figure 2 and Table 2 show the MBC susceptibility dilution testing. The positive control plate (O'P) was fully covered with bacterial colonies. The negative control (O'N) plate showed the absence of a bacteria colony. The largest concentration of *A. bilimbi* extract treatment tube 500 mg/ml (O'1) also showed no bacteria colony. Other treatment tubes (O'2-O'6) were covered with bacterial colonies. The results were the same for 4x replications. From this analysis, the value of MBC was 500 mg/ml.

Figure 2. Subculturing specimen from the previous dilution tube to the nutrient agar to determine the MBC

Table 2. MBC dilution test result

Plate	Observation of Bacterial Growth			
	1st Replication	2nd Replication	3rd Replication	4th Replication
O'P	+	+	+	+
O'N	-	-	-	-
O'1	-	-	-	-
O'2	+	+	+	+
O'3	+	+	+	+
O'4	+	+	+	+
O'5	+	+	+	+
O'6	+	+	+	+

- : There were no growth of *S. typhi* colonies
+ : There were growth of *S. typhi* colonies
O'P = positive control
O'N = negative control
O'1-O'6 = treatment plate with various concentration
O'1 = 500 mg/ml
O'2 = 250 mg/ml
O'3 = 125 mg/ml
O'4 = 62.5 mg/ml
O'5 = 31.25 mg/ml
O'6= 15.625 mg/ml

Discussion

The antibacterial ability of *A. bilimbi* leaves extract is due to bioactive compounds, such as alkaloids, saponins, tannins, flavonoids, and phenols.[10] Natural alkaloids act as antibacterial in many ways. They inhibit nucleic acid and protein synthesis,[14] disrupt the integrity of the peptidoglycan components of bacterial cells,[15] and inhibit energy and efflux pump metabolisms in bacteria.[14] This mechanism blocks bacterial toxins and inhibits bacterial growth.[14] Saponins work as an antibacterial property by increasing the permeability of bacterial cell membranes.[16] Saponin is hydrophilic and lipophilic in characteristic. This property has similarities with surfactants, which can lower the surface tension of water. Surface tension drop is caused by the presence of soap-like compounds, which can damage the bacterial cell membrane due to the formation of lipid bonds from the cell membrane. Therefore, the membrane tension of the bacteria decreases.[16,17] Tannins can pass bacterial cell walls and interfere with cell metabolism, thus depriving substrates required for bacterial

growth. They inhibit extracellular microbial enzymes and make a complex with metal ions to be used as an antibacterial agent. However, since *S. typhi* is a gram-negative bacterium, they have a thin layer of peptidoglycan and a bilayered membrane.[18] Thus, the ability of tannin to pass through the bacterial cell wall is slower than gram-positive bacteria.[18] Flavonoids have several antimicrobial actions, such as inhibiting synthase, which is involved in nucleic acid synthesis, respiratory chain and energy metabolism, cytoplasmic membrane function, and cell envelope synthesis.[19] The bioactive compounds of *A. bilimbi* seem to cooperate in producing an adequate antibacterial mechanism effect.

The previous study has proven the antibacterial activity of *A. bilimbi* leaf extract against several bacteria using the disc diffusion method to determine the inhibition zone.[13] The effectiveness of *A. bilimbi* leaves extract as an antimicrobial agent against gram-positive and gram-negative bacteria has been reported in several studies.[17] The inhibition zone was higher on *S. aureus* than *E. coli* and *Salmonella sp.*[13] Another study concluded that *A. bilimbi* leaf extract provides growth inhibition zones in disc diffusion test against *S. typhi* at a concentration of 300 mg/ml-500 mg/ml.[20] *A. bilimbi* leaf extract also has antibacterial action with the MIC at 30% and the MBC at 35% against *Enterococcus faecalis* bacteria.[19]

Strength and Limitations

This study is useful for discovering new antibacterial agents from plants. By utilizing native plants of Indonesia, hopefully it can be useful to be used as a drug that can reduce antibiotic resistance. The method used was easy to be used in many research areas. The conclusion was obvious to determine cause and effect.

However, differences in the extraction methods and the type of extract solvent can also affect the antibacterial effectiveness of *A. bilimbi* leaves extract. Further research is important to determine the activity of *A. bilimbi* in vivo. In the future, it is expected that the use of *A. bilimbi* for treating several diseases will be successful.

Conclusion

Based on this study, *A. bilimbi* could become an alternative treatment for people infected with *S. typhi*.

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Conflict of Interest

The authors stated there is no conflict of interest.

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Ethical Clearance

This study had received ethical clearance from the Ethics Committee in Health Research, Faculty of Medicine, Universitas Airlangga, Surabaya (no. 455/EC/KEPK/FKUA/2016) on 1 March 2015.

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