Antibacterial activities of Averrhoa bilimbi L. (Oxalidaceae) fruit extract inhibiting Salmonella typhi

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Antibacterial activities of *Averrhoa bilimbi* L. (Oxalidaceae) fruit extract inhibiting *Salmonella typhi*

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ABSTRACT IN ENGLISH

Traditional medicine (*Usada*) can treat typhoid fever, and one such plant is *Averrhoa bilimbi* L. The fruit's flavonoids and triterpenoids are considered to have antibacterial properties. This study investigates the antibacterial activities of *Averrhoa bilimbi* L. (Oxalidaceae) fruit extract inhibiting *Salmonella typhi*. Experiments in the laboratory employed a Posttest Only Control Group Design. 6 treatment groups received *Averrhoa bilimbi* L. extract at 25%, 50%, 75%, 100%, and positive control (Ciprofloxacin) and negative control (sterile distilled water). The extract's inhibitory activity was determined using *Salmonella typhi* bacteria cultured on Mueller Hinton medium. Kruskal Wallis and U-Mann Whitney tests with a 95 % level of evidence (p0.05) were employed to analyze the data. The results indicated that *Averrhoa bilimbi* L. (Oxalidaceae) fruit extract inhibited *Salmonella typhi* by 25% (0 0 mm), 50% (13,000 1,414 mm) strong criterion, 75% (18,750 1,500) strong criterion, and 100% (20.250 1.707 mm) extremely strong criterion. Statistical analysis revealed a statistically significant difference in concentrations of *Averrhoa bilimbi* L. fruit extract and *Salmonella typhi* in all groups, with a p-value of 0.000 0.05. As a result, the fruit extract possesses antibacterial properties and has the potential to be turned into a herbal medication (*Usada*).

ABSTRACT IN BAHASA

Pengobatan penyakit demam tifoid dapat memanfaatkan bagian tumbuhan sebagai obat tradisional (*usada*) salah satunya buah *Averrhoa bilimbi* L. Kandungan flavonoid dan triterpenoid pada buah diyakini bermanfaat sebagai antibakteri. Penelitian ini berupaya untuk menguji aktivitas antibakteri ekstrak buah *Averrhoa bilimbi* L. (Oxalidaceae) dalam menghambat *Salmonella typhi*. Penelitian eksperimental laboratorium dengan rancangan *Posttest Only Control Group Design*. Terdapat 6 kelompok perlakuan ekstrak *Averrhoa bilimbi* L. dengan konsentrasi 25%, 50%, 75%, 100%, kontrol positif (*Ciprofloxacin*) dan kontrol negatif (aquadest steril). Daya hambat ekstrak diujikan pada bakteri *Salmonella*

typhi yang ditumbuhkan pada media *Mueller Hinton*. Analisis data menggunakan uji *Kruskal Wallis* dan *U-Mann Whitney* dengan level of evidence 95% (p<0,05). Hasil penelitian diperoleh ekstrak buah *Averrhoa bilimbi* L. (Oxalidaceae) dalam menghambat *Salmonella typhi* sebesar 25% (0 ± 0 mm), 50% (13,000 ± 1,414 mm) kriteria kuat, 75% (18,750 ± 1,500) kriteria kuat dan 100% (20,250 ± 1,707 mm) kriteria sangat kuat. Hasil uji statistik menunjukkan terdapat perbedaan signifikan antara konsentrasi pemberian ekstrak buah *Averrhoa bilimbi* L. terhadap *Salmonella typhi* di semua kelompok dengan nilai *p-value* 0,000 < 0,05. Dengan demikian, ekstrak buah memiliki aktivitas antibakteri dan berpotensi dikembangkan sebagai obat herbal (*Usada*).

***Keywords*:** antibacterial, *Averrhoa bilimbi* L., typhoid fever, *usada*.

INTRODUCTION

Typhoid fever is one of Indonesia's endemic diseases. This disease is caused by feces and urine contamination of water and food, which introduce *Salmonella typhi* bacteria into the human body [1]. The *S. typhi* bacteria assault the digestive and excretory systems, resulting in systemic infections shown by recurrent fever, bacteremia, and inflammation of the intestines and liver [2]. *Typhoid* fever frequency and incidence have grown in Indonesia, particularly in places with inadequate sanitation, with 21 million cases documented and over 700 deaths [3].

Efforts to manage and control typhoid fever in Indonesia have been made. However, the disease continues to spread in several places. *Typhoid* fever is treated with first-generation antibiotics (*chloramphenicol*, *co-trimoxazole*, *Ciprofloxacin*, and *amoxicillin*) that inhibit *S. thypi* growth.

However, long-term use results in antibiotic resistance in the form of *Multi-Drug Resistance Salmonella typhi* (MDR-ST) [4,5]. Thus, deliberate measures are required to prevent the emergence of antibiotic resistance by using plant components with antibacterial properties that are safe, affordable, and capable of inhibiting the development of typhoid fever pathogens [6].

Averrhoa bilimbi L. (Oxalidaceae) is a plant species widely employed in Indonesian traditional medicine, particularly in the province of Bali. In Bali, traditional medicine (*Usada*) is derived from *Lontar Usada Taru Pramana* plant. According to the *papyrus*, *Averrhoa bilimbi* L. is an effective medication, particularly for fever relief. So it is critical to perform empirical investigations on the discovery and development of traditional medicine in Bali. According to Kimbal [7], Sunti acid content produced from Averrhoa bilimbi L. extract fermentation can inhibit *Escherichia coli Multidrug-Resistant* (ECMR). Furthermore, the *ethanolic* extract of *A. bilimbi* L. has antibacterial activity on all MDR (*Multidrug-Resistant*) bacteria with an inhibitory diameter of 10-14.5 mm on agar diffusion wells. It has potential as an antibacterial agent, especially for MDR strains [6].

The leaves of *A. bilimbi* L. contain an ethanol compound with an ethanol concentration of 82.82 mg GAE/g DW in the DPPH test and are capable of inhibiting the growth of *Salmonella sp.*, *Escherichia coli*, and *Staphylococcus aureus* bacteria, suggesting that it may be used to prevent diseases caused by oxidative stress and bacterial infection [8]. Additionally, the fruit of *A. bilimbi* L. can be used as an antipyretic, lowering fever effectively [9]. After reviewing the advantages of *Averrhoa bilimbi* L. as traditional medicine and its potential as an antibacterial agent, This study investigates the antibacterial activities of *Averrhoa bilimbi* L. (Oxalidaceae) fruit extract inhibiting *Salmonella typhi*. The results of this study are intended to serve as a scientific reference for the advancement of traditional medicine (*Usada*), particularly for the investigation of the content and inhibitory potential of *A. bilimbi* L. fruit extract against the pathogen that causes *typhoid* fever.

MATERIALS AND METHODS

Study Design

This research used an experimental laboratory design with a *Posttest Only Control Group*. This study included six treatment groups: negative control (sterile distilled water), positive control (*Ciprofloxacin*),

and *Averrhoa bilimbi* L. concentrations of 25%, 50%, 75%, and 100%, with four replications determined using the *Federer* equation [10]. For six months, the research was carried out at the Biopesticide Laboratory and Clinical Microbiology Laboratory, Faculty of Medicine, Udayana University.

Approval Study

The research series has been approved by the Department/KSM Clinical Microbiology, Faculty of Medicine, Udayana University or Central General Hospital (RSUP) Sanglah Denpasar with Number 155/UN.14.2.2.VIII.6/2018.

Instruments and materials

The instruments and materials in this study were: 96% ethanol, sterile distilled water, *Ciprofloxacin* antibiotic disk, *Mueller Hinton Agar* (MHA), SS *Agar* media, blank disk, 0.9% NaCl, sterile cotton sticks, water bath, blender, rotary evaporator, glass beaker, petri dish, spirit lamp, incubator, caliper, autoclave, filter paper.

Study procedure

***Mueller Hinton Agar* (MHA) media creation**

Suspension of 38 grams of MHA powder in 1 liter of distilled water in an Erlenmeyer. Stir until the MHA powder is dissolved. After

dissolving, the pH of the media was measured using a pH stick (optimal pH 7.3 ± 0.1). Sterilized in an autoclave at 121oC for 15 minutes. The cold liquid MHA was poured into a petri dish aseptically and then stored.

Averrhoa bilimbi L. extract creation

The *ethanolic* extract of *A. bilimbi* L. fruit was obtained by maceration of *simplicia* powder with 96% ethanol. One hundred grams of *simplicia* powder of *A. bilimbi* L. fruit was soaked (maceration) in 60 mL of 96% ethanol solution for three days while stirring once a day. The results obtained were filtered and re-maceration with 40 mL of 96% ethanol solution and allowed to stand for two days. All the macerated were then concentrated using a rotary evaporator until a thick extract was obtained.

Dilution of a thick extract and preparation of a concentration

The viscous extract of the macerated *A. bilimbi* L. fruit was an antibacterial extract with a concentration of 100%. Each dilution was made as 5 mL and prepared in four glass beakers. Each coded A, B, C. D. Each glass beaker was given a letter code to accommodate: Code A was used to accommodate extracts with a concentration of 100%, and code B was used to accommodate extracts with a concentration of 100%. With a concentration of 75%, code C is used to accommodate extracts with a

concentration of 50%, and code D is used to accommodate extracts with 25%. Then each glass beaker was inserted: Code A: 100% extract. Code B: 3.75 mL of *A. bilimbi* L. fruit extract + 1.25 mL of sterile distilled water. Code C: 2.5 mL of *A. bilimbi* L. fruit extract + 2.5 mL of sterile distilled water. Code D: 1.25 mL of *A. bilimbi* L. fruit extract + 3.75 mL of sterile distilled water. Each dilution was homogenized, and the extract was ready for use.

Salmonella typhi bacterial suspension preparation

One to three oses of Salmonella typhi colonies from pure cultures were taken and suspended into a tube containing 3 mL of 0.9% physiological NaCl solution. This suspension is compared to standard *McFarland* turbidity.

Assay of antibacterial activity

Salmonella typhi bacterial suspension was inoculated using a dispersion technique using a sterile cotton swab on *Mueller Hinton Agar* (MHA) media. Discs impregnated with fruit extract of *A. bilimbi* L. with different concentrations were placed on each test medium. Ciprofloxacin antibiotic disks were also placed on each test medium as a positive control and sterile distilled water as a negative control. The media was incubated

at 37°C for 1x24 hours. The zone of inhibition formed around the disc, measured in mm (millimeter).

Data Analysis

The zone of inhibition is determined by the strength with which the inhibition is carried out, which is expressed in millimeters (mm). Among the criteria for an inhibition zone are the following: 5 mm (weak); 5-10 mm (medium); 10-20 mm (strong); and > 20 mm (very strong). The data from the inhibition zone for *S. typhi* growth were evaluated statistically using the Statistical Package for the Social Sciences (SPSS) Inc. version 21.0 software. The Kruskal Wallis test was used to examine the data, followed by the U-Mann Whitney test at a 95% confidence level ($p<0.05$). Tables, figures, and narratives are used to convey all data.

RESULT

Testing the antibacterial activity of *A. bilimbi* L. (Oxalidaceae) fruit extracts at various concentrations in inhibiting *S. typhi* revealed that a concentration of 100% (P4) has a potent inhibitory activity. In comparison, concentrations of 75% (P3) and 50% (P2) have a moderate inhibitory activity, and a concentration of 25% (P1) has no inhibitory activity and is equivalent to negative control (sterile distilled water). The

test results indicate that there are data that do not pass the basic assumption test, necessitating a non-parametric test to determine comparability. The Chi-Square score is 22.201, corresponding to a probability of 0.05 (p=0.000). Thus, there is a substantial variation in the ability of *A. bilimbi* L. (Oxalidaceae) fruit extract to inhibit *S. typhi* inhibition across all treatment groups. The U-Mann Whitney test revealed significant differences in the majority of treatment groups except for the negative control group with a concentration of 25% (P1) and a concentration of 75% (P3) with a probability value of 100% (P4) (p>0,05). The antibacterial activity of *A. bilimbi* L. fruit extract against *S. typhi* is demonstrated in Table 1 and Figure 1.

TABLE 1. Antibacterial activity of *A. bilimbi* L. fruit extract in inhibiting *S. typhi*

Group	Replication				Inhibition Average Zone	Inhibition Criteria	Shapiro Wilk test	Levene Test	Chi-Square	P
	I	II	III	IV						
K(+)	33	37	34	33	34,250 ± 1,892a	Very strong	0,086			
K(-)	0	0	0	0	0 ± 0b	-	0			
P1	0	0	0	0	0 ± 0b	-	0			
P2	15	12	12	13	13,000 ± 1,414c	Strong	0,161	0,019	22,201	0,000*
P3	20	17	18	20	18,750 ± 1,500d	Strong	0,224			
P4	21	18	20	22	20,250 ± 1,707d	Very strong	0,850			

Information: * = there is a significant difference ($p<0.05$); Different letter = there is a significant difference ($p < 0.05$); Same letter = no significant difference ($p > 0.05$).

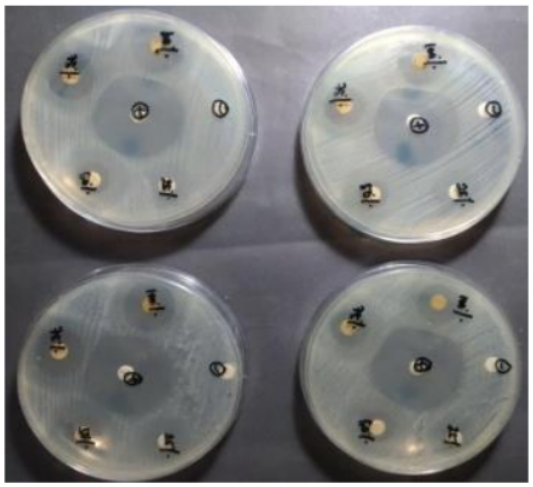

FIGURE 1. *S. typhi* bacteria have been inoculated on *Mueller Hinton Agar* (MHA) media with the addition of discs according to the concentration of the treatment.

DISCUSSION

Averrhoa bilimbi L. (Oxalidaceae) is a commonly cultivated and planted plant species in Indonesia. The fruit of *A. bilimbi* L. has a highly sour flavor due to the high concentration of oxalic acid, which exceeds

70% [11]. Additionally, the fruit of *A. bilimbi* L. includes bioactive substances that may be utilized to make herbal medication (*Usada*), including saponins, flavonoids, triterpenoids, tannins, and peroxides, formic acid, glucose, calcium oxalate, and sulfur [12]. The fruit's bioactive chemicals were shown to inhibit the growth of disease-causing bacteria, including *Salmonella typhi*, which causes typhoid fever.

According to the results of testing the antibacterial activity of *A. bilimbi* L. fruit extract at a 25% concentration, it failed to inhibit the growth or destruction of *S. typhi* bacteria. This occurred because the concentration of bioactive compounds in the extract was insufficient to inhibit the growth or destruction of the bacterial cell wall, rendering it ineffective at this concentration. This is consistent with previous studies, which demonstrated that bioactive chemicals could suppress infections if they are present in appropriate amounts and proportions and are capable of causing harm to target organs through specific pathways [13].

Furthermore, at a concentration of 50%, an average inhibition of 13,000 ± 1,414 mm was obtained, and a 75% concentration of an average inhibition of 18,750 ± 1,500 with decisive criteria. Meanwhile, at 100% concentration, it inhibited 20.250 ± 1.707 mm with decisive criteria. The high level of inhibition carried out by the extract of *A. bilimbi* L. was due

232 to the presence of bioactive compounds that worked and formed an
233 inhibition zone which became an indicator that the extract with this
234 concentration had been able to inhibit the bacteria available on MHA
235 media [11,14,15]. The results showed that the inhibitory capacity of the extract
236 of *A. bilimbi* L. depended on the concentration used. The greater the extract
237 concentration given, starting from a concentration of 50%, 75%, and
238 100%, the greater the inhibition zone formed by *S.typhi*. It indicates that
239 the bioactive compounds in the extract's concentration work well against
240 pathogens [16–18].

241 *A. bilimbi* L. fruit extract is documented to include a variety of
242 bioactive chemicals with antibacterial, antioxidant, antimicrobial,
243 antiaging, and antioxidative effects. [19,20]. Furthermore, the antibacterial
244 benefits of *A. bilimbi* L. fruit were obtained because it contains alkaloids,
245 saponins, flavonoids, and tannins [14]. Flavonoid compounds have a toxic
246 effect on bacteria by damaging the bacterial cell wall so that it is
247 hydrolyzed and unable to sustain life [21]. Alcohol groups in flavonoids will
248 react with lipid compounds and amino acids in bacteria so that the bacterial
249 cell wall will be damaged [22,23]. Tannin compounds in the fruit extract of *A.*
250 *bilimbi* L. work by lysing cell walls, inactivating enzymes, and genetic
251 functions of *S. thypi* bacteria [9]. This is in line with previous research that

stated that the ethyl acetate compound in *A. bilimbi* L. fruit which was formulated in the form of gel and extract, was able to provide an excellent inhibitory effect on *Propioni*bacterium acnes and *S. aureus* so that it was effectively used as an antibacterial and antimicrobial agent [17,24]. The essential oil contained and produced from the fruit extract of *A. bilimbi* L. is helpful as an antibacterial, antioxidant, and *antibiofilm* [16].

Identifying flavonoid and *triterpenoid* compounds in plant parts is generally done by dissolving certain substances [25]. The orange color is formed to detect flavonoid compounds and reddish-purple to detect *triterpenoid* compounds [18,26]. Flavonoids in sufficient concentrations can inhibit the growth of *S. thypi* by forming complex compounds with extracellular and dissolved proteins, causing damage to the bacterial cell membrane, followed by the release of effective intracellular compounds that are effective and have antibacterial potential [19]. Meanwhile, the *triterpenoid* content reacts with the *porin* (transmembrane protein) on the outer membrane of the bacterial cell wall, forming a polymeric solid bond that results in bacteria lysis and growth arrest; this is why the triterpenoid content in the fruit of *A. bilimbi* L. is used as an antiradical or antioxidant [27,28].

In vitro and molecular docking studies have been conducted to determine the effectiveness of chemicals found in *A. bilimbi* L. fruit, particularly polyphenol compounds often employed as antibacterial and antioxidant agents in daily life [29]. Triterpene saponin chemicals found in *A. bilimbi* L. fruit operate as antimicrobials by affecting the integrity of bacterial cell membranes, which results in cell wall lysis. [23]. Saponins are semipolar compounds that are soluble in lipids and water, which can lead to the concentration of substances in microbial cells [11,30]. Concentrating the extract to a sufficiently high concentration enabled the fruit extract of *A. bilimbi* L. to be beneficial as an antibacterial, particularly for its inhibitory efficacy against *S. typhi*. A concentration of 100% can block the bacterium *S. typhi*, which meets the rigorous requirements for *Ciprofloxacin*.

CONCLUSION

At doses of 50%, 75%, and 100%, the fruit extract of *Averrhoa bilimbi* L. (Oxalidaceae) effectively inhibits the bacteria *Salmonella typhi*, which causes *typhoid* fever. The highest extract concentration has the most substantial inhibitory effect and is equal to Ciprofloxacin (decisive criteria). Further study is required to determine the composition, chemical

profile, and bioactive chemicals employed as antibacterial and antimicrobial agents, particularly in reducing the development of Salmonella typhi bacteria. Experimental animal testing is strongly recommended to establish a more thorough effect.

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