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In vitro Inhibitory Activity of Ethanolic Fruit Extract from *Averrhoa bilimbi* L. against *Streptococcus pyogenes* Bacteria

Puspita Dewi¹, Gusti Ayu Ratih^{2(CA)}, Burhannuddin³, I Gede Sudarmanto⁴¹Department of Medical Laboratory Technology, Health Polytechnic of Denpasar, Indonesia; boerhan28@gmail.com²Department of Medical Laboratory Technology, Health Polytechnic of Denpasar / Postgraduate Program of Pharmacy, Airlangga University, Indonesia; iga_ratihkurada@yahoo.com (Corresponding Author)³Department of Medical Laboratory Technology, Health Polytechnic of Denpasar, Indonesia⁴Department of Medical Laboratory Technology, Health Polytechnic of Denpasar, Indonesia

ABSTRACT

Averrhoa bilimbi L., popularly known as belimbing wuluh, is a common plant used as traditional medicine and cooking ingredients in Indonesia. *Averrhoa bilimbi* fruit contains many active substances as antibacterial such as tannin, formic acid, flavonoids, saponins, peroxides, and triterpenoids. *Streptococcus pyogenes* is a pathogenic bacteria that can cause respiratory infections. This study was carried out to investigate the inhibitory activity of *Averrhoa bilimbi* L. fruit extract at various concentrations on the growth of *Streptococcus pyogenes*. This study was a true experimental with post test only control design. The inhibitory activity was assessed by the Kirby-Bauer's disc diffusion method with four different extract concentrations (20%, 40%, 60%, 80%), 96% EtOH as negative control, and Amoxicillin antibiotic as positif control. The EtOH extract showed the mean diameter of inhibition zone of 20% extract was 11.43 mm (strong), 40% extract was 19.13 mm (strong), 60% extract was 24.58 mm (very strong), 80% extract was 30.99 mm (very strong), and the negative control (96% EtOH) was 0.00 mm. Least Significant Difference test showed there were significant different of inhibition zone in each extract concentrations of *Averrhoa bilimbi* L. against *Streptococcus pyogenes* with the strongest inhibitory activity is 80% extract concentration.

Keywords: *Averrhoa bilimbi*, Inhibitory activity, *Streptococcus pyogenes*

INTRODUCTION**Background**

Averrhoa bilimbi (Family Oxalidaceae) is popularly known as belimbing wuluh, is a common plant used as traditional medicine and cooking ingredients in Indonesia. This plant is widespread across Indonesia, Malaysia and as exotic in Argentina, Australia, Brazil, Colombia, Cuba, Ecuador, Guyana, India, Jamaica, Myanmar, Philippines, Puerto Rico, Singapore, Sri Lanka, Surinam, Tanzania, Thailand, Trinidad & Tobago, US, and Venezuela. Fruit ellipsoid, obovoid or nearly cylindrical, faintly 5-sided, 4-10 cm long, capped by a thin, starshaped calyx at the stem-end and tipped with 5 hair like floral remnants at the apex. Crispy when unripe, the fruit turns from bright green to yellowish-green, ivory or nearly white when ripe and falls to the ground. The outer skin is glossy, very thin, soft, juicy and extremely acid⁽¹⁾.

A. bilimbi has been used in the traditional medicine for the treatment of a variety of ailments. Infusions and decoctions of the leaves are used as an antibacterial, antiscorbutic, astringent, postpartum protective medicine, in the treatment of fever, inflammation of the rectum, and diabetes⁽²⁾. Fruit juice is used in the treatment of scurvy, bilious colic, whooping cough, hypertension, obesity, and diabetes^(3,4,5). Several studies have found that *A. bilimbi* contains high amount of bioactive compounds. Preliminary phytochemical studies of the fruit extracts using chemical methods and thin layer chromatography revealed the presence of carbohydrates, proteins, amino acids, flavonoids, tannins, bitter principles, essential oil, valepotriates, coumarin, and terpenes^(6,7). The bark extracts of *A. bilimbi* revealed moderate antibacterial activity against some microorganisms and the phytochemical investigation suggested the presence of flavonoids, saponins and alkaloids⁽⁸⁾. The leaves and fruit extract of *A.*

bilimbi also contains a number of flavonoids with the type of luteoin. This compound, together with apigenin is known to be effective enough to inhibit the growth of several types of bacteria such as *Bacillus cereus* and *Corney bacterium diphtheria*⁽⁹⁾. The fruits are also rich in Vitamin C and oxalic acid^(6,7). The previously study also reported that the content of oxalic acid in *Averrhoa* species can become a potent source of antioxidant and antimicrobial against *S. aureus*⁽¹⁰⁾. The aqueous and chloroform extracts of *A. bilimbi*'s leaves and fruits (100 mg/ml) showed a positive antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermis*, *Bacillus cereus*, *Salmonella typhi*, *Citrobacter freundii*, *Aeromonas hydrophila*, *Proteus vulgaris*, and *Kocuria rhizophila*⁽¹¹⁾. In another study, fruits and roots extracts of *A. bilimbi* were also found to exhibit the positive activity against *Mycobacterium tuberculosis* with MIC of 1600 µg/ml⁽¹²⁾. The previously study also reported that EtOH extract of *A. bilimbi* fruit inhibits the growth of *Streptococcus mutans* with the mean diameter of inhibition zone formed at concentration of 25% was 10.1 mm, 50% was 15.1 mm, 75% was 18.1 mm, and 100% was 20.1 mm⁽¹³⁾.

Purpose

The aim of current study was to analysis the inhibitory activity of *Averrhoa bilimbi* L. fruit extract at various concentrations on the growth of *Streptococcus pyogenes* by using *in vitro* techniques. The concentration variations used in this study were 20%, 40%, 60%, and 80%. This concentration variation is based on the preliminary test that has been carried out. Concentration of 20% extract produces a zone of inhibition of 10 mm, so the concentrations of 20%, 40%, 60%, and 80% are chosen in this study.

METHODS

The inhibitory activity was assessed by the Kirby-Bauer's disc diffusion method with four different extract concentrations (20%, 40%, 60%, 80%), 96% EtOH as negative control, and Amoxicillin antibiotic as positif control. The method of this research used True Experimental. The research design used Posttest Only Control Group Design⁽¹⁴⁾. Unripe *Averrhoa bilimbi* L. fruit (green to yellowish green) with a size of 4-5.6 cm were collected from local area of Denpasar district-Bali, and authenticated by an expert botanist from Udayana University, Bali. Inhibitory activity was evaluated by measuring inhibition zone diameters. Data analysis began with the Kolmogorov Smirnov test, then One Way ANOVA test was used to determine the inhibition zone and LSD (Least Significant Deference) was used to determine the inhibitory zone variation between each concentration of *A. bilimbi* fruit EtOH extract. The time of the study began on June 1st to August 31st, 2018.

RESULTS

Inhibition zone of *Averrhoa bilimbi* L. fruit extract against *Streptococcus pyogenes* bacteria growth

In this study the inhibitory test of *A. bilimbi* fruit extracts concentrations of 20%, 40%, 60% and 80% was carried out on the growth of *Streptococcus pyogenes* bacteria using 96% EtOH as a negative control and Amoxicillin 25 µg as a positive control.

Table 1. Zone of inhibition of *A. bilimbi* fruit extract at various concentrations on the growth of *Streptococcus pyogenes* using the Kirby-Bauer's disc diffusion.

Extract/Control	<i>In vitro</i> assays		Mean of Inhibition Zone ± Standard Deviation (mm)	Category
	Inhibition Zone ± Standard Deviation			
	Replication I	Replication II		
20%	11.7 ± 0.46	11.15 ± 0.10	11.42 ± 0.26	Strong
40%	19.95 ± 0.06	18.30 ± 0.23	19.12 ± 0.14	Strong
60%	25.05 ± 0.06	24.10 ± 0.84	24.57 ± 0.42	Very strong
80%	29.10 ± 0.49	32.87 ± 0.25	30.99 ± 0.30	Very strong
Positive control	35.00 ± 0.0	36.40 ± 0.0	35.70 ± 0.0	Sensitive

Values represent mean ± standard deviation, n = 4.

The mean diameter of negative controls showed there was none inhibitory activity of *Streptococcus pyogenes* bacteria growth. The result of the largest inhibition zone was produced by 80% extract concentration which was equal to 30.99 mm, while the smallest diameter was obtained from 20% extract concentration which was 11.43 mm. The results of minimum inhibitory zone diameter and its inhibitory category are presented in Table 1. The inhibitory activity of *A. bilimbi* fruit extracts at concentration of 20% and 40% was categorized as strong inhibition because it has a diameter of inhibition zone 10-20 mm. While the inhibition of *A. bilimbi* fruit extracts at concentration of 60% and 80% was categorized as very strong with diameter of the inhibition zone >20 mm.

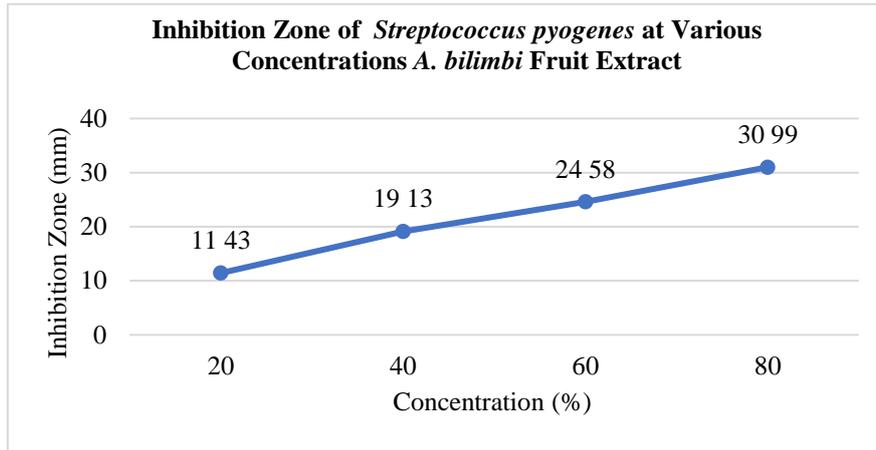


Figure 1. Inhibition Zone Differences of *Averrhoa bilimbi* L. fruit extract at various concentrations

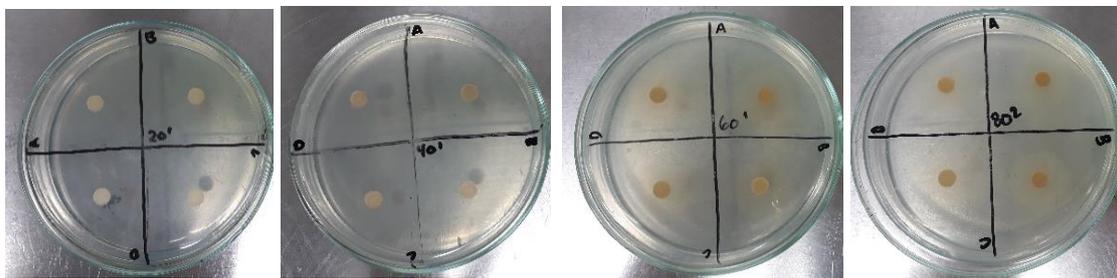


Figure 2. Diameter of inhibition zone at concentration of 20%, 40%, 60%, and 80% of *A. bilimbi* fruit extracts

Data analysis of differences inhibitory activity of *Averrhoa bilimbi* L. fruit extract

The Kolmogrov Smirnov test results obtained p-value was 0.476 (>0.05), than the p-value of One Way Anova test was 0.000 (<0.05). The results shown there was a differences inhibition zone of *Streptococcus pyogenes* bacteria growth at various concentrations of *A. bilimbi* fruit extract. The differences of inhibitory activity from various concentrations was analyzed by LSD test. The LSD test showed that p-value was 0.000 ($p < \alpha$), there were significant differences between each of the extracts concentration.

DISCUSSION

The inhibitory test was conducted to determine the antibacterial potential of *Averrhoa bilimbi* L. against *Streptococcus pyogenes* bacteria. *A. bilimbi* fruit extract was obtained through maceration method using 96% EtOH, the extract obtained then tested for its inhibitory activity at various concentrations with the Kirby-Bauer diffusion method. Based on the results of the observation, all the concentrations of the ethanol extract of *A. bilimbi* fruit have inhibition on the growth of *Streptococcus pyogenes* bacteria. Inhibition can be seen from the clear zones that formed around the disk containing *A. bilimbi* fruit extract. In this method, the extract on the disk will diffuse and the active substances contained in it will inhibit bacterial growth. The wider the clear zone formed shows the greater inhibitory activity. In this study, 96% ethanol was used as a negative control to determine whether there is an effect of ethanol on the growth of *Streptococcus pyogenes* bacteria which is also used as a solvent in the extraction process. The mean diameter of negative controls showed there is no inhibitory activity of *Streptococcus pyogenes* bacteria growth with a measured value of 0.00 mm. The same thing was also obtained from the study ethanol extract and decoction of *Myrmecodia sp.* on *Escherichia coli* Bacteria⁽¹⁵⁾.

In this study, the positive controls used Amoxicillin antibiotic discs as work controls. Amoxicillin is a broad-spectrum antibiotic that is widely used to treat human and animal diseases, which is included in the class of penicillin (β -lactam antibiotics). This antibiotic has been proven effective against various types of infections caused by gram-positive and gram-negative bacteria and used to treat and prevent respiratory, gastrointestinal, urinary and skin bacterial infections due to its pharmacological and pharmacokinetic properties⁽¹⁶⁾. The mean diameter of the inhibition zone from Amoxicillin antibiotic discs was 35.70 mm. These results are according to

CLSI (*Clinical and Laboratory Standards Institute*), where Amoxicillin belongs to the sensitive category. It is indicated the bacterial isolates used and the procedures carried out in this study are confirmed with testing standards for inhibiting antibacterial substances⁽¹⁷⁾. This study also shows that Amoxicillin is still effective against *Streptococcus pyogenes* bacteria, with a minimum inhibition zone diameter of > 24 mm⁽¹⁷⁾.

The results of this study shows that the fruit extracts of *A. bilimbi* at various concentrations were able to inhibit the growth of *Streptococcus pyogenes* which was characterized by the formation of clear zones around the discs. Inhibition activity is divided into four categories: inhibiting weak (<5 mm), medium (5-10 mm), strong (10-20 mm), and very strong (> 20 mm)⁽¹⁸⁾. The concentration of 20% (11.43 mm) and 40% (19.13 mm) have a strong inhibitory activity; 60% (24.58 mm) and 80% (30.99 mm) have very strong inhibitory activity. This study also shows that different diameter inhibition zones are formed for each concentrations. It might be caused *A. bilimbi* fruit contains various active compounds as antimicrobial properties such as flavonoids, alkaloids, tannins, and saponins; and antioxidant agent such as oxalic acid^(10,19). Oxalic acid also reported effective in reducing initial populations and controlling growth of spoilage bacteria on raw chicken breasts⁽²⁰⁾. The previously study also reported that the content of oxalic acid in *Averrhoa* species can become a potent source of antioxidant agent and antimicrobial against *S. aureus*⁽¹⁰⁾. The presence of the inhibition zone in this study indicates that extracts from unripe fruits contains of bioactive compounds that might be contribute to the antimicrobial properties of the fruits. The previously study reported the extracts from *A. bilimbi* unripe fruits are more effective against against *Salmonella spp*⁽²¹⁾. This is similar to the result of the present study where the microbiological stability of some fruits and vegetables can be attributed in part to the presence of oxalic acid. The antibacterial effect of oxalic acid is attributed to a reduction in the extracellular pH and cellular uptake of the undissociated form of the acid. This uptake results in intracellular pH imbalance and subsequent death of the bacteria cell⁽²⁰⁾. Figure 1 shows that the higher the concentration, the inhibitory zone formed becomes wider. The similar result was reported in the study of the inhibitory activity of Binahong Leaf (*Anredera cordifolia* (Ten.) Steenis extract against *Streptococcus pyogenes*⁽²²⁾. Factors that can influence the differences of inhibition zone are media thickness and concentration of active substance in the extract⁽²³⁾.

CONCLUSION

Based on the results of the research, it can be concluded that Unripe *A. bilimbi* fruit extract at various concentrations were able to inhibit the growth of *Streptococcus pyogenes*. The mean diameter of the largest inhibition zone was produced by 80% extract concentration with very strong inhibition category, while the smallest diameter was produced by 20% extract concentration with strong inhibition category. That might be caused *A. bilimbi* fruit contains various active compounds as antimicrobial properties and antioxidant agent, especially the presence of oxalic acid. There was a significant differences between the inhibition zones of the growth of *Streptococcus pyogenes* bacteria at the various concentrations of 20%, 40%, 60%, and 80%.

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