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The Effect of Concentration Eco-Enzyme *Averrhoa Bilimbi* L. Fruit On Antimicrobial Activity

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Abstract: This study determined the antimicrobial activity of the eco-enzyme of *A. bilimbi* L. fruit with various concentrations against *Staphylococcus aureus, Escherichia coli*, *Salmonella typii*, and *Candida albicans*. The antimicrobial test was carried out using the disc diffusion method and analyzed by measuring the inhibition zone produced. The eco-enzyme of *A. bilimbi* L. fruit effectively inhibits the growth of *S. typii* and *E. coli* at a concentration of 75%. It generates an inhibitory zone measuring 7.12 mm in *E. coli* and 4.92 mm in *S. typii*. Eco-enzyme concentrations of 50% with an inhibitory power of 5.42 mm are good antibacterial concentrations for *S. aureus.* In the antifungal test, *C. albicans* growth may be inhibited by 3.54 mm at a 100% concentration of eco-enzyme. The eco-enzyme of *A. bilimbi* L. fruit can inhibit the growth of gram-positive bacteria (*S. aureus*), gram-negative bacteria (*E. coli* and *S. typii*) and fungi (*C. albicans*).

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Introduction

A. bilimbi L. With the local name 'belimbing wuluh' is a fruit that commonly found in Indonesia. *A. bilimbi* L. is rarely in demand by the public because it has a sour taste, so that rotten *A. bilimbi* L. fruits are often found scattered. *A. bilimbi* L. is a medicinal plant that is often used in tropical and subtropical countries in the world. *A. bilimbi* L. is used as an antimicrobial agent (Alhassan & Ahmed, 2016).

Antimicrobial agents are among the most frequently used drugs in human medicine and veterinary practice. Extracts from *A. bilimbi* L. were reported to show considerable antimicrobial activity against several pathogenic microorganisms i.e. *Bacillus cereus*, *B. megaterium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermis*, *Aspergillus ochraceous*, *Cryptococcus neoformans*, and many more (Alhassan & Ahmed, 2016).

The use of eco-enzyme *A. bilimbi* L. has been developed to prove its ability to inhibit microbial activity. Eco-enzyme is a fermented liquid from organic waste such as fruit peels and vegetable scraps which in the process is added with sugar and water. Eco-enzymes were introduced to utilize enzymes found in organic waste in cleaning environmentally friendly products (Astuti et al., 2016). Eco-enzymes have a good impact from a global perspective because eco-enzymes will produce O_3 (ozone) and CH_3COOH (acetic acid). Acetic acid can inhibit and even kill bacteria and viruses (Rochyani et al., 2020; Safitri et al., 2021). Eco-enzymes also have a good impact on the economy because they can save expenses on buying cleaning fluids, fertilizers, and pesticides for plants (Dewi et al., 2021).

Antimicrobials are active compounds used to inhibit or kill microbes such as bacteria, viruses, and fungi (Dwinta, 2021). There are two kinds of antimicrobials based on the ingredients, synthetic antimicrobials, and natural antimicrobials. Synthetic antimicrobials have been proven to prevent and kill pathogenic microbes. However, if synthetic antimicrobials are used excessively, it will cause pathogenic microbes to become resistant (Pratiwi, 2017). In addition, the excessive use of synthetic antibiotics will harm

health (Wang et al., 2018). Therefore, it is necessary to switch to natural antimicrobials, which are safer in the long term use, minimize resistance, environmentally friendly and economical (Cahyono, 2017).

Eco-enzymes as antimicrobial agents will be more beneficial than synthetic antimicrobials. Eco-enzyme contains lipase, protease, and amylase enzymes. This enzyme acts as an antimicrobial that can control gram-positive and gram-negative bacteria. (Benny et al., 2023) reported that lipase, protease and amylase were found in eco-enzymes from citrus peels. In the antimicrobial test, the Eco-enzyme could inhibit the growth of *E.coli*, *Pseudomona*s ssp., *Bacillus* spp. The clear zone formed, indicates the activity of lipase, protease, and amylase in the eco-enzyme. Eco-enzymes contain some organic acids, such as citric acid, acetic acid, malic acid, lactic acid, and oxalic acid. Those organic acids are useful for killing microbes (Ginting & Prayitno, 2022).

Several studies reported that eco-enzymes from pineapple peels, orange peels, onion peels and leaves of *A. bilimbi* L. have antibacterial activity against acne bacteria, *Enterococcus faecalis* (Astuti et al., 2016). (Ginting & Prayitno, 2022) also reported that eco-enzymes from waste fruits (pineapple, papaya and banana) can inhibit the growth of *S. aureus*. Based on the report, it can be seen that there has been no antimicrobial activity test that utilizes eco-enzymes from *A. bilimbi* L. fruit. The urgency of this research is to utilize eco-enzymes as eco-environmentally friendly antimicrobials. Researchers were inspired to test the antimicrobial activity using eco-enzymes from *A. bilimbi* L. fruit with several concentrations on several microbes. The objective of this research was to determine the effect of eco-enzyme concentrations from *A. bilimbi* L. fruit on antimicrobial activity.

Method

Preparation and Characterization of Eco-enzymes

A. bilimbi L. fruit eco-enzyme is made using three main ingredients, namely *A. bilimbi* L. fruit, brown sugar and water. The eco-enzyme manufacturing process begins by pouring clean water into a 20-litre bucket. The ratio of water: *A. bilimbi* L. fruit: brown sugar is 10 : 3: 1. A total of 15 litres of water was put into a container containing 8 kg of A. bilimbi L. fruit pieces and 6 kg of brown sugar. Water, *A. bilimbi* L. fruit and brown sugar are homogenized and fermented for three months. The variables observed in this study include scent, colour, pH and MPN of the eco-enzymes produced (Septiani et al., 2021).

The scent and colour of the eco-enzyme were observed through organoleptic tests (Maryanti & Wulandari, 2023). Eco-enzyme pH of *A. bilimbi* L. fruit was measured using a pH Meter-Mettler Teledo. MPN analysis of Eco-enzymes *A. bilimbi* L. fruit used a series of 9 tubes (3-3-3) of the Thomas formula consisted of two tests, the presumptive test (introduction) and the confirmative test (affirmation). The presumptive test was carried out using Lactose Broth (LB) media. The presumptive test was started by preparing 9 test tubes containing 10 mL of sterile LB media. The first three test tubes were inoculated with 10 mL eco-enzyme, the second 3 test tubes were inoculated with 1 mL eco-enzyme and the last 3 test tubes were inoculated with 0.1 mL eco-enzyme. Inoculation was carried out by aseptic method. Then slowly homogenize the LB media that has been inoculated with eco-enzyme. Incubation at 37°C for 48 hours. A positive test is indicated by the presence of gas bubbles formed in the Durham tube. Positive results in the presumptive test were further confirmed with a confirmative test using Brilliant Green Lactose Broth (BGLB) media. The number of coliform and colifecal bacteria was calculated based on SNI 06-4158-1996 (Sipriyadi et al., 2021).

Preparing Bacterial and Fungal Cultures

50 ml of Nutrient Broth (fungal growth medium) and Potato Dextrose Broth (bacterial growth medium) was made. The media is homogenized on the hotplate. Put 10 ml of Nutrient Broth in each test tube to grow *S. aureus, E. coli, S. thypii* and 10 ml of Dextrose Broth to inoculate *C. albicans.* Bacterial and fungal cultures were incubated for 24 hours at 37ºC (Ramadani et al., 2022).

Antibacterial Assays

An antimicrobial test was carried out using the disc diffusion method (Razmavar et al., 2014). Nutrient Agar (150 ml) and paper discs were sterilized using an autoclave. Eco-enzyme was prepared in several concentrations, 100% eco-enzyme, 75% eco-enzyme, 50% eco-enzyme and 25% eco-enzyme. Chloramphenicol solution was used as a positive control (25%). A sterile paper disc is soaked in each solution. Bacterial cultures (*S. aureus*, *E. coli*, *S. thypii*) were inoculated using the spread plate method (0.1 ml). The soaked paper disc is placed on the media that has been inoculated with bacteria. Antibacterial activity was determined by measuring the inhibition zone formed. Inhibition zone is expressed in millimeters (mm) (Ramadani et al., 2022).

Antifungal Assay

In the antifungal assay, the method was the same as in the antibacterial test. Potato Dextrose Agar is used in this assay. Nystatin solution was used as a positive control (25%). Eco-enzyme was prepared in several concentrations, 100%, 75%, 50% and 25%. A sterile paper disc is soaked in each solution. *C. albicans* culture (0.1 ml) was inoculated using the spread plate method. The soaked paper disc is placed on the media that has been inoculated with bacteria. Antibacterial activity was determined by measuring the inhibition zone formed. Inhibition zone is expressed in millimeters (mm) (Razmavar et al., 2014).

Result

Characterization of Eco-enzymes

The eco-enzyme produced from *A. bilimbi* L. fruit has a brownish colour with a fresh sour scent (Figure 1.). Eco-enzyme from *A. bilimbi* L. fruit has a high level of acidity with a pH value of 1.84 (Table 1.). The presence of coliform and colifecal bacteria in eco-enzymes from *A. bilimbi* L. fruit was calculated using the MPN (Most Probable Number) method. The MPN value is an important indicator to determine the

quality of the eco-enzyme produced. Based on SNI 06-4158-1996 calculations, the eco-enzyme from *A. bilimbi* L. fruit does not contain coliform and colifecal bacteria (Table 1.).

Figure 1. Eco-enzyme of *A. bilimbi* L. Fruit

Table 1. Eco-enzyme characteristics of *A. bilimbi* L. fruit

Indicator	Unit	Result
Colour	-	Brownish
Scent	-	Fresh sour
pH	-	1,84
MPN	MPN index per 100 ml	<3

Antibacterial Assay

In the antibacterial test, the inhibition zone is obtained and presented in Table 2. Chloramphenicol has strong rate of inhibition against *E. coli* compared to eco-enzymes (Table 2.). However, the use of eco-enzymes as natural antimicrobials is also quite a large influence in inhibiting the growth of *E. coli*. The best eco-enzyme concentration to inhibit the growth of *E. coli* is 75%, with an inhibition of 7.12 mm.

The *S. thypii* bacteria are the same as *E. coli*. It turns out that chloramphenicol has a stronger inhibitory as a control than the eco-enzyme *A. bilimbi* L. and the eco-enzyme *A. bilimbi* L., which is great to use as a natural antimicrobial for *S. thypii* is an eco-enzyme 75% with an inhibition of 4.92 mm (Table 2.). The *S. aureus* is the same as *E. coli* and *S. thypii*. Chloramphenicol has a stronger inhibtion zone than the eco-enzyme *of A. bilimbi* L. However, for the concentration of the use of eco-enzyme *A. bilimbi* L., which is good to use as a natural antimicrobial *S. aureus* is 50% with a resistance of 5.42 mm (Table 2.).

Table 2. Antibacterial assay

Concentration	Inhibition Zone (mm)		
	E. coli	*S. thypii*	*S. aureus*
Control positive (25%Chloramphenicol)	23.70 ± 2.01	18.59 ± 5.39	27.01 ± 3.97
100%	2.87 ± 0.14	4.00 ± 0.39	2.65 ± 0.45
75%	7.12 ± 3.15	4.92 ± 0.77	2.96 ± 0.63
50%	3.39 ± 0.74	3.59 ± 0.75	5.42 ± 0.77
25%	2.52 ± 0.87	3.07 ± 0.89	3.16 ± 0.30

Antifungal Assay

Based on the antifungal test, data on the area of the inhibition zone are presented in the Table 3. Eco-enzyme *A. bilimbi* L. has a very strong inhibition of *C. albicans* compared to nystatin. It is proven that eco-enzyme is very good to be used as a substitute for nystatin as an antimicrobial against fungi. The best eco-enzyme concentration to inhibit the growth of *C. albicans* is 100% eco-enzyme with the rate of inhibition of 3.54 mm (Table 3.).

Table 3. Antifungal assay

Concentration	Inhibition Zone (mm)
Control positive (25% Nystatin)	3.17 ± 0.21
100%	3.54 ± 0.61
75%	2.95 ± 0.88
50%	1.35 ± 0.74
25%	0.73 ± 0.56

Discussion

Three indicators consisting of colour, scent and pH (Figure 1. and Table 1.) show that the fermentation process is successful. Larasati et al. (2020) stated that the success indicator of making eco-enzymes can be seen from colour, scent and pH. The success of eco-enzyme fermentation is marked by a brownish colour in the eco-enzyme liquid, a fresh sour scent (like a fruity scent as a basic ingredient) and a very acidic pH value (< 4) (Larasati et al., 2020).The acidic pH of eco-enzymes is caused by organic acids such as citric acid and acetic acid which are products of the fermentation process. The acidity level of the eco-enzyme produced is determined by how high the accumulated organic acids are. The higher the organic acid content, the more acidic the eco-enzyme.

The MPN test is an indicator that determines the quality of eco-enzymes. This test aims to detect and quantify the number of coliform and colifecal bacteria present in the eco-enzyme of *A. bilimbi* L fruit. The test results showed that no gas bubbles were formed in each dilution series. The MPN value obtained is <3 MPN index per 100ml (Table 1.). Referring to SNI 06-4158-1996, these values indicate that the eco-enzyme of A. bilimbi L. fruit does not contain coliform and colifecal bacteria. The eco-enzyme of *A. bilimbi* L. fruit is protected from polluting bacteria and has good quality.

There are bioactive compounds in *A. bilimbi* L. that function as antibacterial substances, such as flavonoids, alkaloids, tannin, phenol and saponins. These bioactive compounds can produce complex

antibacterial effects (Nathania et al., 2023). Flavonoids are a group of secondary metabolite compounds often found in plants and belong to the class of phenolic compounds with a chemical structure of C6-C3-C6 (Redha, 1985). One of the functions of flavonoids is as an antibacterial which can release transduction energy in the bacterial cytoplasmic membrane and inhibit nucleic acid synthesis, inhibit the function of the cytoplasmic membrane, and inhibit the energy metabolism of bacteria (Manik et al., 2014). According to (Eskundari et al., 2022), flavonoids can inhibit protein kinases, which are used for cell proliferation in bacteria. Inhibition of protein kinase will make the cell's physiological processes disturbed, and the cell will carry out apoptosis.

Yan et al. (2021) reported that alkaloids can inhibit bacterial growth by interfering with protein synthesis and metabolism and damaging the peptidoglycan structure. Meanwhile, tannins work by inhibiting the activity of bacterial extracellular enzymes so that the substrate for metabolism is not fulfilled (Kaczmarek, 2020). *A. bilimbi* L. also contains saponins which can be used as antibacterials (Aristyantari et al., 2022). Saponins can damage the permeability of the bacterial cell wall. It can result in cell death(Nurzaman et al., 2018). Terpenoids are also secondary metabolites contained in *A. bilimbi* L. Terpenoids as antibacterial by reacting with transmembrane proteins (porins) found in the bacterial cell membrane and forming strong polymer bonds resulting in damage to the porins (Safitri & Leliqia, 2021).

In addition to bioactive compounds, enzymes contained in eco-enzymes from *A. bilimbi* L. fruit also inhibit bacterial growth. (Ginting & Prayitno, 2022) reported that the clear zone in the antibacterial activity test was caused by amylase, protease and lipase activity. The presence of enzymes on the substrate can suppress bacterial growth through the destruction of membranes and cell walls.

E. coli is a gram-negative rod-shaped bacterium (bacillus). It is classified in the Enterobacteriaceae family, the Gammaproteobacteria class. *E. coli* is commonly found in the digestive tract of humans and animals (Jang et al., 2017). *E. coli* is one of the microorganisms contained in faeces and is a bacterium often found in waters which is used as a quality bioindicator to indicate the high or low level of contamination of water contaminated with human and animal faeces (Anggara et al., 2021). *E. coli* is a bacterium that causes diseases, watery diarrhea, bloody diarrhea, urinary tract infections, meningitis, and sepsis, which can cause death (Cho et al., 2018). *S. thypii* is a gram-negative bacterium that causes septicemia, typhoid fever, and gastroenteritis. *S. typii* is transmitted via faecal-oral, which is experienced by people who have poor sanitation and consume contaminated drinks and food (Yogita et al., 2018). *S. aureus* is a gram-positive pathogenic bacterium. *S. aureus* is found on human skin where its presence does not cause disease, but if it exceeds the limit, it will become a toxin for the skin (Novitasari & Inayati, 2019). *S. aureus* is one of the bacteria that cause infectious diseases such as dermatitis, mastitis, toxic shock syndrome, and infections of the respiratory tract (Wikananda et al., 2019).

Differences in the structure bacterial cells can affect antimicrobial activity. According to (Hamidah et al., 2019), differences in the structure of the cell walls of gram-negative and gram-positive bacteria can affect the rate of antimicrobials. The cell wall of gram-negative bacteria is more complex than the cell wall of gram-positive bacteria. The cell wall of gram-positive bacteria has a thicker peptidoglycan and does not have a lipopolysaccharide layer. The absence of lipopolysaccharide causes gram-positive bacteria to be more sensitive to antibacterials because they can absorb more of the given antibacterials. Gram-negative bacteria have a thick lipopolysaccharide which is a defense for gram-negative bacteria from foreign substances such as antimicrobials. The lipopolysaccharide layer makes gram-negative bacteria more resistant to antimicrobials than gram-positive bacteria (Eskundari et al., 2022).

C. albicans is a type of fungi that exists in humans that causes candidiasis. *C. albicans* are often found in the oral cavity, digestive tract, vaginal mucosa, upper respiratory tract, and under the nails. *C. albicans* pathogen will attack when the body's immunity decreases and causes candidiasis disease in the mouth, skin, vagina, lungs, bronchi or nails. This fungi can attack all sexes and not look at the age level (Talapko et al., 2021).

Based on the results, it is known that the eco-enzyme *A. bilimbi* L. can work effectively as an antifungal against *C. albicans* due to the presence of secondary metabolites in *A. bilimbi* L. These compounds include alkaloids, flavonoids, saponins, tannins, terpenoids, eugenol and polyphenols. (Alhassan & Ahmed, 2016) confirmed that *A. bilimbi* L. has alkaloids, flavonoids, saponins, tannins, triterpenoids, and polyphenols in its fruits and leaves. Antifungal compounds will neutralize enzymes or toxins associated with fungal invasion, damage cell membranes, inhibit cells so that they can inhibit the formation of haustorium and appressorium, and affect the biosynthesis of nucleic acids and proteins in fungi (Wahyuni et al., 2014).

Eugenol is a phenol group compound that plays an active role in destroying *C. albicans* cells. When cell division occurs, the DNA will undergo a G2 phase and an M DNA phase. The fungi whose growth is inhibited will decrease in the proportion of the S-G2-M stage. This process will affect the proliferation index of the fungus. Fungal cells that are given antifungal effects will experience shrinkage of the cellular membrane and loss of cell organelles, nucleus, and cytoplasm, which are covered by electron areas. This results in damage to death in cells (Setiari et al., 2019).

Besides eugenol, alkaloid compounds are compounds that can be used as antifungals. The mechanism of alkaloids as antifungals is by entering the cell wall. DNA replication is disrupted and inhibits the formation of DNA and RNA. In addition, alkaloids will prevent the biosynthesis of nucleic acids in fungi so that fungi cannot grow(Yan et al., 2021). Saponin compounds can also be antifungal agents because saponin compounds can bind to sterols in fungal cell membranes and prevent the development of fungal spores (Wahyuni et al., 2014).

Apart from being antibacterial, flavonoids can be used as antifungals. Flavonoids can damage the permeability of cellular walls, which are related to functional cell proteins and DNA. It prevented the growth of fungi (Aboody & Mickymaray, 2020). Tannins are secondary metabolites that can be used as antifungals with a mechanism of action that prevents the biosynthesis of ergosterol, which is the main constituent of sterols in fungal cell membranes. If the main sterols are inhibited, it will damage the permeability of the fungal cell membrane (Yan et al., 2021). Triterpenoids are secondary metabolites found in *A. bilimbi* L.

Triterpenoids are active compounds that are toxic to fungi. If triterpenoid compounds are absorbed by fungi, it will cause damage to the cellular organelles. It eventually prevents the growth of fungi (Ismaini, 2011).

Conclusion

The eco-enzyme of *A. bilimbi* L. fruit can inhibit the growth of gram-positive bacteria (*S. aureus*), gram-negative bacteria (*E. coli* and *S. thypii*) and fungi (*C. albicans*). The fruit eco-enzyme *A. bilimbi* L. inhibits the growth of *C. albicans* at a concentration of 100%. It inhibits the growth of bacteria (*S. aureus*, *E. coli* and *S. thypii*) at a concentration of 50-75%. Eco-enzyme *A. bilimbi* L. can be used as an alternative antimicrobial to substitute for chloramphenicol and nystatin. Further studies can be carried out to utilize the potential of eco-enzyme *A. bilimbi* L. as an antimicrobial in various related fields.

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