

Antibacterial Activity of Various Extracts of *Averrhoa bilimbi* against Multidrug Resistant Bacteria

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Abstract. The multi-drug resistance (MDR) bacteria is a global health problem that causes high mortality every year. Therefore, novel antibacterial agents are needed from natural biological sources. This research aimed to investigate the antibacterial activities of various crude extracts of *Averrhoa bilimbi* against MDR bacteria. The antibacterial activity was calculated based on the use agar well diffusion assay and the minimum bactericidal concentration (MBC) using Mueller–Hinton broth in a microdilution method. Bacteria from wells were subcultured using inoculating loop onto a 5% sheep BAP. The best antibacterial activity, calculated as the most widely inhibitory zone and the smallest MBC values. The ethanolic extract showed antibacterial activity against the all MDR bacterial test in the agar well diffusion assay (10-14.5 mm inhibition diameter). The MBC of water extract against ES β L + CR *Pseudomonas aeruginosa* showed the best antibacterial activity (12.5 mg/mL). The fruit of bilimbi was shown to be potentially developed as antibacterial agents, especially for MDR strains. Further in vivo research and discovery of action mode are needed to shed light on their antibacterial effects. This study can provide new information about the benefits of bilimbi as a source of natural antibacterial agents against MDR-bacteria.

Key words: *Averrhoa bilimbi*; Antibacterial activity; Minimum Bactericidal Concentration; Resistance bacteria; Secondary metabolites

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INTRODUCTION

The antibiotic resistant bacterial strains have been a menace to public health globally. Particularly, multi-drug resistant (MDR) bacterial strains remain a serious cause of concern since they cause treatment failures and impose huge economic burdens especially in developing countries (Ballani and Babby, 2016). MDR bacteria are the main cause of severe complications in the therapies of contagious diseases and has become a serious problem that causes high mortality every year (Mekes et al., 2019). The MDR bacteria is a global health problem caused by inappropriate use of antibiotics. Patients with infections caused by MDR bacteria have a worse risk due to their difficult treatment and the likelihood of high mortality. Infections caused by MDR bacteria also consume more health-care resources than patients infected with non-resistant strains of the same bacteria (World Health Organization, 2018). So, novel antibacterial agents are needed from natural biological sources (Valle Jr et al., 2015). Biological antibacterial agents can be obtained from the mushrooms (Prastiyanto et al., 2016), bacteria (Ryandini et al., 2018), bacteriocins (Lestari et al., 2019), and plant (Tillah et al., 2017; Wahyuni et al., 2019). There have been many reports found in the medical literature concerning the significance of traditional medicinal plants as alternatives to antibacterial agents (Akhtar, 2015; Aumeeruddy-elalfi et al., 2015).

Plants is a good source of antibacterial compounds, because of the variety and diversity of the chemical structures of the compounds contained therein (Ngameni et al., 2013). *Averrhoa bilimbi* Linn. (bilimbi) also known as belimbing wuluh in Indonesia is one of the medicinal plants that is found in tropical and subtropical countries and useful as a medicine, such as antidiabetic (Ahmad et al., 2019), hepatoprotective (Dnyaneshwar et al., 2010), anti-cancer, and antithrombotic agent (Ali et al., 2014).

Research related to antibacterial activity of bilimbi has been reported. The extracts of bilimbi fruit can inhibit bacteria *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923, *Vibrio parahaemolyticus* ATCC 17802 (Seebaluck-sandoram et al., 2019). The fruits and roots extracts of bilimbi were also inhibit *Mycobacterium tuberculosis* (Mohamad et al., 2011). Research on antibacterial activity of extracts of bilimbi fruits against MDR bacteria have not been reported, so it is necessary to investigate the antibacterial potential of bilimbi fruit extract against MDR bacteria. In this study, we used five variations of bilimbi fruit extracts.

The study on antibacterial activity of bilimbi with various solvent (Methanol, Ethanol, Chloroform, N-Hexane and water) are expected to provide new information about the benefits of bilimbi. In addition, it also can support the bilimbi as a source of natural antibacterial agent againsts MDR-bacteria. The re-

search aim was to investigate the antibacterial activities of fruit extracts of bilimbi with various solvents against MDR bacteria from clinical specimen.

METHODS

Plant materials

Fruits of bilimbi Figure 1 (green, not overly ripe) were freshly picked from a home garden in Kedungmundu, Semarang, Indonesia. The fruits were washed with water to remove all unwanted materials and then rinsed with sterilized distilled water, then dried under sunlight for two days. The dried bilimbi were then milled into fine powder using a milling machine and stored in a sterile airtight container until further use.



Figure 1. Bilimbi fruits (*Averrhoa bilimbi*)

Plant extract preparation

Bilimbi extracts were prepared using the maceration method with various solvents i.e.: methanol, eth-

anol, chloroform, N-Hexane and water. One hundred grams of powdered bilimbi was soaked in 300 mL of each solvent for 24 h at room temperature and protected from light with shaking. Solvent replacement was done every day. Replacement of solvent was done until the solution became clear with the assumption that there was no active compound contained in the dry powder. The supernatant was filtered through Whatman filter paper No.1 (Whatman). Maceration solutions were concentrated under reduced pressure using a rotary evaporator at 50°C. The crude extracts were collected and allowed to dry at room temperature.

Bacterial preparation

The organisms used for in vitro antibacterial screening in this study is summarized in Table 1. MDR bacteria were isolated from patients of the dr. Kariadi Hospital, Semarang City, Indonesia. All isolates were identified and susceptibility patterns were obtained using Vitek®MS (bioMérieux, Marcy l'Etoile, France). The bacteria were subcultured overnight (24 h) at 35±2 °C on 5% sheep blood agar (BAP). The bacterial colonies were homogenized and adjusted to 0.5 McFarland standards (5×10⁸ CFU/mL) using spectrophotometry.

Table 1. The organisms for in vitro antibacterial screening in this study

Species	Source	Antibiotic resistance pattern
ESβL, <i>K. pneumoniae</i> ssp <i>pneumoniae</i>	52 / male, swab wound	<i>Ampicillin, Sulbactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Gentamicin, Sulfamethoxazole</i>
ESβL, <i>E. coli</i> ,	29 / male, swab wound	<i>Ampicillin, Sulbactam, Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Gentamicin, Ciprofloxacin, Sulfamethoxazole</i>
ESβL + CRE, <i>E. coli</i> ,	2 / male, pus	<i>Ampicillin, Sulbactam, Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Ertapenem, Meropenem, Ciprofloxacin Sulfamethoxazole</i>
ESβL + CRE, <i>K. pneumoniae</i> ssp <i>pneumoniae</i>	60 / female, bronkus	<i>Ampicillin, Sulbactam, Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Ertapenem, Meropenem, Gentamicin, Ciprofloxacin, Sulfamethoxazole</i>
MRSA	53 / female, pleura	<i>Benzylpenicillin, Oxacillin, Gentamicin, Ciprofloxacin, Levofloxacin, Erythromycin, Clindamycin, Tetracycline, Rifampicin, Sulfamethoxazole</i>
ESβL + CR <i>Pseudomonas aeruginosa</i>	41 / female, sputum	<i>Ampicillin, Sulbactam, Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Meropenem, Amikacin, Gentamicin, Ciprofloxacin, Tigecycline, Nitrofurantoin, Sulfamethoxazole</i>

ESβL: extended spectrum beta-lactamase, CRE: Carbapenem-resistant Enterobacteriaceae CR: Carbapenem-resistant, MRSA: Methicillin-resistant *Staphylococcus aureus*

Antibacterial susceptibility assays

The antibacterial activity from various extracts of bilimbi was evaluated using agar well diffusion assay (Andleeb et al., 2020). In this method, 100 μL of each test organism which was equivalent to a 0.5 McFar-

land standard was inoculated on the Muller Hilton Agar (MHA). Then it was spread onto the surface of the agar using a sterilized glass spreader. After 10 minutes of inoculation, the wells were prepared using sterilized steel cork borer (1cm diameter). Wells were

made in each plate, out of which five wells were loaded with each extracts (200 µg or 200 µL from 1000 µg/mL). All the plates were then incubated aerobically at 35 ± 2 °C for 24 h. Antibacterial activity of the extracts was determined by measuring the diameter of inhibition zone in mm against the test organism.

Minimum bactericidal concentration (MBC) of the bilimbi fruits extracts.

The MBC of bilimbi fruit extract was determined using Mueller–Hinton broth microdilution (CLSI, 2018). MBC determination was performed by a serial dilution technique using 12-well microtiter plates. Bilimbi fruit extract (100 mL) was placed into the well/plate. Then, 100 mL of bacterial cell suspensions (0.5 McFarland) were placed in each well/plate. Microplates were incubated for 24 h at 35 ± 2 °C. The MBC of bilimbi fruit extract was determined following the methods described by Parvez et al. (2019) with slight modifications. The bacteria in wells were subcultured using a 10 mL inoculating loop onto a 5% sheep BAP at (35±2) °C for 16–20 h incubation. The MBC was defined as the lowest concentration of the

extract that did not any growth of bacterial colony on 5% sheep BAP. Mueller–Hinton broth as negative control. Cefazolin and meropenem were used as positive controls for ESBL–producing bacteria. Cefazolin and tigecycline were used as positive controls for ESBL + Carbapenem resistance (CR), while Sulfamethoxazole and vancomycin were used as positive controls for MRSA

RESULTS AND DISCUSSION

Antibacterial susceptibility assay

The antibacterial susceptibility assays were initially performed to determine the antibacterial activities of the various extracts of bilimbi fruit against MDR bacteria, namely, ESβL *K. pneumoniae* ssp *pneumonia*, ESβL *E. coli*, ESβL + CRE, *E. coli*, ESβL + CRE, *K. pneumoniae* ssp *pneumonia*, MRSA, ESβL + CR *P. aeruginosa*. The extract of bilimbi fruit with various solvents (methanol, ethanol, chloroform, n-Hexane and water) showed inhibition of bacterial growth against some or all of the test organisms (Figure 2 and Table 2).

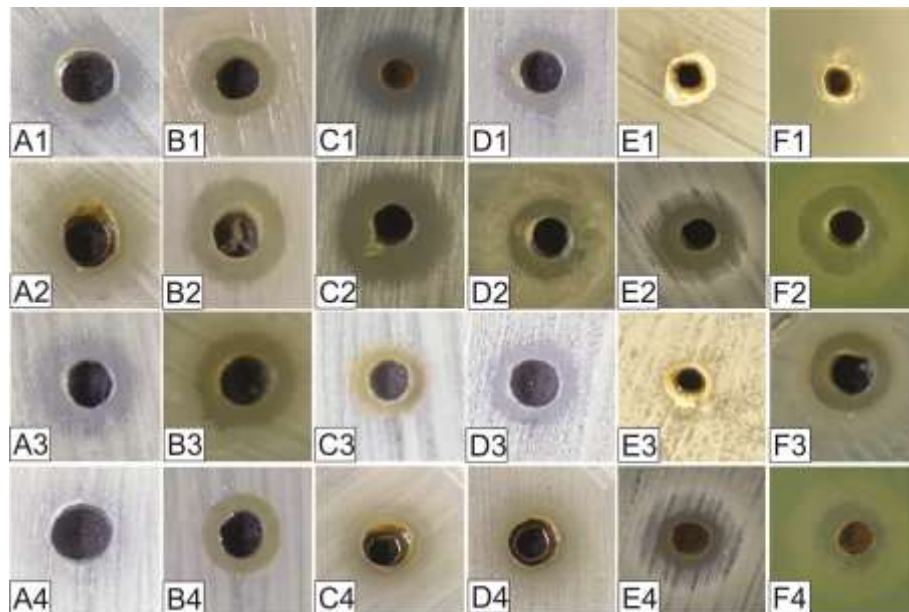


Figure 2. Inhibition zone of bilimbi extracts against MDR bacteria: (A) ESβL *K. pneumoniae* ssp *pneumonia*; (B) ESβL *E. coli*; (C) ESβL + CRE, *E. coli*; (D) ESβL + CRE, *K. pneumoniae* ssp *pneumonia*; (E) MRSA; (F) ESβL + CR *P. aeruginosa*, and solvent (1) chloroform; (2) ethanol; (3) methanol; (4) water

Table 2. Diameters of inhibition zone of bilimbi fruit extract (100 mg/mL) with various solvents against clinical isolates of MDR bacteria (mm)

Solvent	ESβL <i>K. pneumoniae</i> ssp. <i>pneumoniae</i>	ESβL <i>E. coli</i>	ESβL + CRE <i>E. coli</i>	ESβL + CRE <i>K. pneumoniae</i> ssp. <i>pneumonia</i>	MRSA	ESβL + CR <i>Pseudomonas aeruginosa</i>
Chloroform	6	10.5	11	8.5	-	-
Ethanol	10	10.5	14.5	10	11.5	13.5
Methanol	8	10.5	7	10	-	8.5
Water	-	9	9.5	8.5	10	11.5

The extracts exhibited inhibition zone ranging from 6 mm to 14.5 mm diameter, with the most noteworthy results shown by ethanol solvent. The ethanolic extract of the bilimbi fruit showed inhibition zones ranging from 10–14.5 mm against the clinical strains of all strain test. These results are in accordance with the research report bay Valle-Jr et al. (2015) that, the ethanolic extract *P. betle* leaf demonstrated inhibition zones against MRSA, ESBL-*Enterobacteriaceae* and non *Enterobacteriaceae*. The methanolic extract of bilimbi fruit demonstrated inhibition zones of 7–10.5 mm against the Gram-negative bacteria of all strain test, but not at MRSA (Gram positive). In other study, the methanolic extract of *Albizia adianthifolia*, *Alchornea laxiflora*, *Laportea ovalifolia* demonstrated inhibition zones against MDR Gram-negative bacteria (Tchinda et al., 2017). The water extract of bilimbi fruit demonstrated inhibition zones of 8.5–11.5 mm against all strain test, but not at *K. pneumoniae*. The growths of *K. pneumoniae* were not inhibited by all the extracts of *Ocimum gratissimum*, *Vernonia amygdalina* and *Aframomum melegueta* with water solvent that inhibit the other *Enterobacteriaceae* (Alo et al., 2012). The chloroform extract of Bilimbi fruit demonstrated inhibition zones of 6–11 mm against all of strain test, but not at *S. aureus* and *P. aeruginosa*. In other study, the chloroform extract of *Streptomyces* sp. strain Al-Dhabi-97 isolated from the marine could not inhibit *S. aureus* (Al-dhabi et al., 2020) and the chloroform extract of seed of *Callistemon lanceolatus* could not inhibit *P. aeruginosa* (Khavitha and Satish, 2014). Results of the antibacterial assays showed that the most commonly inhibited bacteria by the ethanolic plant extracts were the clinical ESBL + CRE, *E. coli*, ESBL + CR *P. aeruginosa*, MRSA, ESBL *E.*

coli, ESBL *K. pneumoniae* ssp *pneumoniae*, ESBL + CRE, *K. pneumoniae* ssp *pneumoniae*

Minimum bactericidal concentration (MBC) of the fruit extracts.

The antibacterial activity of the four extracts were assayed in vitro by the agar microdilution method against six resistant bacteria. The antibacterial activity against each bacterium was observed to be varied. Table 3 shows that the all bilimbi extracts exhibited inhibition of bacterial growth against MDR bacteria (12.5-50 mg/mL). Table 3 shows that all bilimbi extracts exhibited lower MBC values than Cefazolin against ESBL strains and lower than Sulfamethoxazole against MRSA. This showed that extract of bilimbi have the potential to be developed as antibacterial agents for MDR bacteria. The microdilution method was used in the present study because it is a quantitative reference method routinely used in clinical laboratories.

Furthermore, all extracts showed highly varying MBC values against MDR bacteria, but the lowest MBC value belonged to water extract (12.5 mg/mL) against ESBL + CR *P. aeruginosa*. Aqueous extract of the truffle *Terfezia clavaryi* contains a potent antimicrobial agent toward *P. aeruginosa* (Janakat et al., 2005). In the present study, favorable antagonistic activities against various MDR bacteria were exhibited by the all extract of bilimbi. However, this study resolutely established the ethanolic fruit extract of bilimbi exhibiting wide zones of growth inhibition against MDR bacteria tested. Preliminary phytochemical studies of the fruit extracts using chemical methods and thin layer chromatography (TLC) revealed the presence of flavonoids, tannins, and terpenes (Hasanuzzaman et al., 2013; Patil et al., 2013).

Table 3. MBC of of bilimbi fruit extract against clinical isolates of MDR bacteria (mg/mL).

Solvent	ESBL <i>K. pneumoniae</i> ssp <i>pneumoniae</i>	ESBL <i>E. coli</i>	ESBL + CRE, <i>E. coli</i>	ESBL + CRE <i>K. pneumoniae</i> ssp <i>pneumoniae</i>	MRSA	ESBL + CR <i>Pseudomonas aeruginosa</i>
Chloroform	50	25	50	50	25	25
Ethanol	25	50	50	25	25	25
Methanol	50	50	50	50	25	25
Water	25	25	50	50	50	12.5
Cefazolin	64	64	64	64	-	64
Meropenem	0.25	0.25	-	-	-	-
Tigecycline	-	-	0.5	1	-	8
Sulfamethoxazole	-	-	-	-	320	-
Vancomycin	-	-	-	-	1	-

The antibacterial activity of the plant can be attributed to its phytochemical compounds. Phytochemical compounds act as shields against disease infec-

tions. The most important phytochemicals are tannins, flavonoids, alkaloids, and terpenes (Kumar et al., 2013). Flavonoids (Khalid et al., 2019), tannins

(Shahat and Marzouk, 2013), and terpenes (Broniatowski and Mastalerz, 2015) have been recognized to exhibit quite potent antibacterial activity. The mechanism of antibacterial activity of flavonoids, tannins, and terpenes compound in the bilimbi is unknown. However, according to (Abuga et al., 2020), phytochemical compounds can inhibit the growth of bacteria by destruction of the bacterial cell wall. The fruit of bilimbi was shown to be potentially developed as antibacterial agents, especially for MDR strains. Further in vivo research and discovery of action modes are needed to shed light on its antibacterial effects. so that potential clinical drug and health products could be developed. This study can provide new information about the benefits of bilimbi as a source of natural antibacterial against MDR bacteria.

CONCLUSION

The fruit of bilimbi was shown to be potentially developed as antibacterial agent, especially for MDR strains. The ethanolic extract showed the highest antibacterial activity against the all MDR bacterial test in the agar well diffusion assay (10-14.5 mm inhibition diameter). The MBC of bilimbi water extract against ESBL + CR *Pseudomonas aeruginosa* showed the best antibacterial activity (12.5 mg/mL).

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