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SINERGISTIC COMBINATION OF ALOE VERA AND KERSEN LEAF EXTRACTS FOR INHIBITION OF THE GROWTH OF *Staphylococcus aureus* 25923

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ABSTRACT

Aloe vera leaf (Aloe vera L.) and kersen (cherry) leaf (Muntingia calabura L.) contain bioactive compounds that serve as the potential antibacterial agent. The combination treatment of multiple extracts can produce a stronger effect than a single compound. This study aims to determine the synergistic combination of aloe vera leaf extract (EDLB) and cherry leaf extract (EDK) as an antibacterial agent against Staphylococcus aureus ATCC 25923. The extract was obtained by maceration using 96% ethanol. Chemical content in the extract was analyzed by tube test and thin layer chromatography (TLC). Antibacterial activity against S. aureus ATCC 25923 was measured by the checkerboard microdilution method on a 96 well microplate. The effect of the combination of extracts on antibacterial activity was based on the fractional inhibitory concentration index (FICI) value. EDLB and EDK contain flavonoids, tannins, alkaloids, and saponins. The second combination showed a synergistic effect with a FICI value of 0.5 in inhibiting the growth of S. aureus with minimal inhibitory levels in a mixture of 0.625 mg/mL EDLB and 2.5 mg/mL EDK.

Keywords: Antibacterial; Aloe vera (Aloe vera L.); Cherry (Muntingia calabura L.); Synergistic

1. INTRODUCTION

Staphylococcus aureus bacterium can cause various infectious diseases both on the skin and in body organs (Erikawati et al., 2016). At a global scale, there has been an increasing rate of infections attributed to S. aureus in last two decades, with a prevalence of 18-30% in the United States and Europe, and almost the same infection rate between *S. aureus* and *Pseudomonas aeruginosa* in Asia (Mehraj et al., 2014). *S. aureus* was also found to be resistant to various types of antibiotics. A study by the East Kalimantan Provincial Health Laboratory showed that resistance to penicillin was found in 79.5% of isolates, 34.6% of gentamicin, and 33.3% of ciprofloxacin (Nuryah et al., 2019). Resistance to these antibiotics can cause various problems in the treatment of infectious diseases, and thus alternative steps are needed to overcome infection by developing antibacterial substances and reducing the number of resistance to antibiotics (Azubuike et al., 2015).

The use of a combination of antibacterial compounds serves as an alternative to overcome antibiotic resistance. The synergistic combination of antibacterial compounds is more effective in overcoming multicausal bacteria and multidrug resistance compared to a single compound (Olajuyigbe & Afolayan, 2013). Aloe vera and kersen (cherry) plants contain antibacterial activity and antibacterial bioactive compounds, such as saponins, flavonoids and tannins (Karina et al., 2019; Kumar et al., 2012). Flavonoids are reported to inhibit nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism. In addition, flavonoids can inhibit the formation of

biofilms (Matilla-Cuenca et al., 2020). Tannins can penetrate cell walls into cell membranes, thus disrupting cell metabolism and causing bacterial cell death (Kaczmarek, 2020). Saponins lead to cell wall damage, disruption of cytoplasmic membranes, and membrane proteins, which can leak bacterial cells (Dong et al., 2020).

From the compounds contained in aloe vera and cherry leaves, combining the two types of leaves as antibacterial is very prospective. Therefore, this study aims to determine the antibacterial activity of the combination of the two types of leaves. To date, there have been no studies on the combination of these two plants. This study aims to test the presence or absence of a synergistic antibacterial effect on the combination of aloe vera and cherry leaves using the checkerboard microdilution method. This method can determine the antibacterial activity of aloe vera and cherry leaves, either individually or in combination (Bellio et al., 2021).

2. METHOD

2.1. Materials

Aloe vera leaves were taken in Sorosutan, Umbulharjo Yogyakarta, and cherry leaves were taken in Pelem Sari village, Kotagede, Yogyakarta. Both were identified at the Biology Laboratory, Ahmad Dahlan University with code 122/Lab.Bio/B/VII/2019. S. aureus ATCC 25923 bacteria were purchased from the Central Yogyakarta Health and Calibration Laboratory. Other ingredients were 96% ethanol and 0.9% NaCl obtained from PT Brataco, Brain Heart Infusion (BHI) (Oxoid) media, Mueller Hinton Agar (MHA) (Oxoid) media, Dimethyl Sulfoxide (DMSO) (Merck) and Silica gel GF254 (Merck).

2.2. Procedures

2.2.1. Extraction of aloe vera and cherry leaves

Aloe vera and cherry leaves which had previously been dried and powdered were extracted by maceration using 96% ethanol. The sample was macerated in 96% alcohol with a ratio of 1:10, then stirred using an electric stirrer for ±3 hours. Afterwards, it was allowed to stand for 24 hours and then filtered. The filtrate was then evaporated using a rotary evaporator at a temperature of 50°C. It was then concentrated on a water bath until the extract thickens (Syarifuddin, Sulistyani, et al., 2019).

2.2.2. Determining drying shrinkage of aloe vera and cherry leaves extracts

The drying shrinkage of the extracts was determined using a Halogen Moisture Anaylzer.

2.2.3. Phytochemical screening of extracts using test tube method

The test tube was carried out using the same method as applied by (Pooja & Vidyasagar, 2016). Tests were carried out to identify alkaloids, flavonoids, tannins, terpenoids, steroids, and saponins. Alkaloids were identified with Dragendorf and Mayer reagents, flavonoids with Mg and 2N HCl powder, tannins with 1% $FeCl_3$, terpenoids and saponins with Liebermann Burchard reagent. Saponins were identified by way of shaking and HCl was detected from foam stability of acid solution.

2.2.4. Phytochemical screening of extracts by TLC method

The extract was dissolved in 96% ethanol to obtain a concentration of 5% w/v. The extract solution was then spotted on a TLC plate with silica gel GF254 as a stationary phase, which had been activated by means of an oven at 100°C for 1 hour, then eluted using chloroform: methanol: ethyl acetate mobile phase in a ratio of (8.0: 1.5: 0.5). The results of the separation were then viewed under UV light 254, 366, and sprayed using Dragendroff, Sitroborat, Lieberman-Burchard, and $FeCl_3$ reagents.

2.2.5. Preparation of bacterial suspension of Staphyloccocus aureus

One loop of bacteria suspended into a sterile BHI DS was scraped then incubated at 37 °C for 2 hours (Syarifuddin, Kamal, et al., 2019). Then, it was diluted to the same turbidity as the 0.5 standard of Mc Farland to make the total bacterial density equivalent to about 1 to $2x10^8$ CFU/mL (Clinical And Laboratory Standards Institute, 2015). Furthermore, it was diluted to a concentration of 10^6 which was used in testing the antibacterial activity using the checkerboard microdilution method and referred to as bacterial suspension.

2.2.6. Antibacterial test of the combination of cherry leaf extract and aloe vera leaf extract using the checkerboard microdilution method

The test solutions of each extract (EDLB and EDK) were made with a concentration of 10; 5; 2.5; 1.25; 0.625; 0.3125; and 0.156 mg/mL in 5% DMSO. The activity of the combination of extracts was tested using a microplate checkerboard. A total of 100 µL of extract solution in the concentration series was added with 100 µL of *S. aureus* bacterial suspension, then incubated for 18-24 hours at 37°C to determine the minimum inhibitory levels of single extracts of both EDLB and EDK. To determine FICI, 50 µL of each concentration of EDLB solution was added with 50 µL of EDK solution in the concentration series that had been made, then added with 100 µL of bacterial suspension and then incubated for 18-24 hours at 37°C. Thus, there were 49 combinations of EDLB and EDK extracts that were tested for their antibacterial activity on microplates. Antibacterial activity test was also carried out on the control solvent (DMSO 5%). The minimum inhibitory concentration (MIC) of the extract combination was determined based on the lowest concentration indicating the clarity of the test solution at the end of the incubation process. The test for determining the minimum killing rate (MBC) was carried out on a mixed series of extract concentrations that showed the presence of MIC (Clinical And Laboratory Standards Institute, 2015).

2.2.7. Data Analysis

The MIC value was analyzed to identify its synergism using the Fractional Inhibitory Concentration Index (FICI) calculation, which indicates the combined effect of aloe vera leaf extract (EDLB) and cherry leaf extract (EDK). FICI was calculated using the following equation:

$$FICI = \frac{MIC\,(EDLB) in\ combination}{MIC\,(EDLB)} + \frac{MIC\,(EDK) in\ combination}{MIC\,(EDK)} \quad (1)$$

The FICI value of 0.5 indicates a synergistic effect. The value of 0.5 less than < FICI 1, indicates an additive effect. The FICI value of > 1 and 4 indicates no different effect, and the FICI value of > 4 shows an antagonistic effect (Olajuyigbe & Afolayan, 2013).

3. RESULTS AND DISCUSSION

3.1. Simplicia Extract

The yield, drying loss and organoleptics of the extracts are presented in **Table 1**.

Table 1. Yield, drying loss and organoleptic of cherry leaf (EDK) and aloe vera leaf (EDLB) extracts

Extracts	Yield (%)	Drying Loss (%)	Organoleptic
EDK	6.17	3.32	Dark brown, having the characteristic smell of extract, distinctive taste slightly bitter
EDLB	8.43	4.10	Dark green, having the characteristic smell of extract, distinctive taste slightly bitter

From the determination of drying loss (Table 1), it is conclusive that both aloe vera leaf extract and cherry leaf extract met the requirements of less than 10%. The drying shrinkage test

using a moisture analyzer revealed that the overall liquid content, which was also analyzed, indicated that each of the water content of Aloe Vera leaf extract was < 3.32% and the water content of cherry leaf extract was < 4.10%. Yield becomes a measure of solvent capacity in extracting chemical content. The yield of EDLB in this study was lower than that resulted by a previous study (22.32% yield), although both used simplicia powder as an extraction material and 96% ethanol solvent (Yasir et al., 2021). Meanwhile, other study obtained a low yield (1.20%) when using fresh aloe vera and extracted with 96% ethanol , but other researchers obtained a yield of 12.28% because the extraction technique was different even though using fresh aloe vera (Martono & Suharyani, 2018). Other researchers obtained higher yields (20.1 and 11.14%) than EDK yields in this study Syahara & Siregar (2019). The amount of yield is influenced by the type and method of preparation of the extracted material, the solvent, as well as the extraction method and technique. In addition, the extraction time, conditions and storage time of the extracted material also affect the amount of yield (Salamah & Widyasari, 2015).

3.2. Phytochemical Screening

Phytochemical screening was carried out to determine the content of chemical compounds in the extract qualitatively. Secondary metabolite compounds generally have bioactivity capabilities so that they are widely used as traditional medicines. The phytochemical compounds of most of the medicinal plants have been identified. Phytochemical screening tests were carried out by tube test (**Table 2**) and KLT (**Table 3**).

Table 2 shows that both EDLB and EDK contain flavonoids, tannins, alkaloids, and saponins. These results are in line with previous research, which stated that both EDLB and EDK (Sentat & Pangestu, 2017) contain flavonoid compounds, tannins, alkaloids, and saponins. The results of previous studies detected steroid compounds in EDLB and EDK (Kurniawan, 2015; Widjaya et al., 2019), but steroids were not detected in the results of this study, presumably due to the lack of steroid compounds contained in the current extract. As a result, the occurring changes, namely the formation of precipitates during testing, could not be seen visually. In this study, new steroids were detected in the EDLB and EDK from TLC results. The presence of a separation process in TLC made it easier to detect the presence of steroids.

Table 2. Phytochemical screening of cherry leaf (EDK) and aloe vera leaf (EDLB) extracts by test tube

No	Testing	Reagents	Description	EDK Result	EDLB Result
1	Flavonoids	Mg powder and 2 mL HCl 2N	Orange to red	+	+
2	Tannins	FeCl3 1%	Green or blue color is formed	+	+
3	Alkaloids	Dragendroff	Red precipitate	+	+
4	Saponins	Water and shaken + HCl 2N	Formation of foam and stability	+	+
5	Steroids	Lieberman Burchard	No green precipitate is formed	-	-
6	Terpenoid	Lieberman-Burchad	Red precipitate	-	-

In the identification of flavonoids using concentrated Mg and HCl, an orange to red color was produced due to the formation of a Mg^{2+} complex with phenoxy ions of flavonoid compounds. The color formed depends on the type of flavonoid in the sample being analyzed. Tannins can be detected with $FeCl_3$ reagent which is used to test phenolic compounds (Oktavia & Sutoyo, 2021). The use of these reagents can produce different colors, including red, purple, blue or black depending on the type of phenolic compound (Habibi et al., 2018). The appearance of a greenish black color indicates the presence of tannins due to a complex formation reaction between Fe^{3+} ions and tannins (Oktavia & Sutoyo, 2021). In the identification of alkaloids, Dragendroff's reagent can produce an orange to brownish red precipitate. This precipitate is due to the formation of potassium-alkaloid complexes (Habibi et al., 2018). Saponins are lower

surface tension and thus they will produce foam when shaken with water. Saponins which are glycosides are able to form stable foam (30 seconds) and undergo hydrolysis (Oktavia & Sutoyo, 2021). The reaction between Liebermann Burchard (LB) reagent and steroids will produce a blue green color, while with triterpenoids it will be purple (Pooja & Vidyasagar, 2016; Setyawaty et al., 2020). The LB reagent causes the oxidation of steroid compound to form a conjugated double bond (Oktavia & Sutoyo, 2021).

Table 3. Phytochemical screening of cherry leaf (EDK) and aloe vera (EDLB) extracts by thin layer chromatography

No	Testing	Spray Reagent	Color of result	EDK Result	EDLB Result
1	Flavonoids	Citroborate	Yellow	+	+
2	Tannins	$FeCl_3$	Black	+	+
3	Alkaloids	Dragendroff	Yellow – Orange	+	+
4	Steroids	Liebermann-Burchard	Bluish green	+	+
5	Terpenoids	Liebermann-Burchard	Reddish purple	+	+

The content of compounds in each extract may have an antibacterial effect. In the TLC test, EDLB produced the highest number of spots in the form of tannins (polyphenols). Tannins can work as antibacterial agent due to its ability to activate adhesion enzymes and protein transport of cell envelope. Tannins also form polysaccharide complexes that can damage bacterial cell walls. As a result, bacterial metabolism is disrupted and causes bacterial death (Salamah & Widyasari, 2015). The most TLC spots on EDK were flavonoids and alkaloids. The significant difference in the secondary compounds in each extract may have a synergistic effect on the antibacterial activity test against *S. aureus*.

3.3. Synergism of Antibacterial combination of EDLB and EDK

The FICI calculation begins with determining the minimum inhibitory level (MIC) in both single extracts and combinations of extracts using the checkerboard microdilution method. The MIC test using the microdilution method consisted of a combination of aloe vera leaf extract (EDLB) and cherry leaf extract (EDK) as well as *S. aureus* bacteria with a ratio of 1:1 in the test solution and the test bacteria. The MIC value was determined based on the lowest extract concentration that did not show any turbidity in the test solution. **Table 4** illustrates that the EDLB had the MIC value against *S. aureus* at a concentration of 2.5 mg/mL, while the EDK was at 10 mg/mL. After the MIC was combined, the EDLB-EDK combined extract occurred in a mixture of EDLB with a concentration of 0.635 mg/mL and EDK 2.5 mg/mL. The amount of MIC of the two combined extracts showed a reduction to a quarter of the concentration of each extract before being combined. This indicates a beneficial combination, because to produce the same effect, each requires a smaller concentration when combined.

To determine the existence of a synergistic effect, it is necessary to calculate the FICI value based on the MIC extract data, either singly or in combination. The combination of extracts showed synergism when the FICI value obtained was 0.5. The results of the FICI calculation are:

$$FICI = \frac{0.625mg/mL}{2.5mg/mL} + \frac{2.5mg/mL}{10mg/mL} = 0.5 \quad (2)$$

The FICI value of the antibacterial activity of the EDLB-EDK combination against *S. aureus* was 0.5, indicating that the combination of the two extracts produced a synergistic effect. A synergistic effect can occur because the two extracts both contain flavonoids, alkaloids, tannins and saponins, each of which has the potential to produce antibacterial activity through the same or different mechanism of action. Flavonoids can inhibit cell wall synthesis, protein synthesis and damage the integrity of the cell wall thereby increasing the bacterial cell membrane. Alkaloids, tannins and saponins can also damage the integrity of the cell wall so that they synergize in

causing bacterial cell lysis (Putra et al., 2021; Stefanović, 2018). The combination of antibacterials that show a synergistic effect in vitro against pathogenic bacteria can increase the success of treatment. In vitro evidence of synergism is useful in selecting the most beneficial antibacterial combination and allowing a reduction in the dose of the combined antibacterial (Freitas et al., 2013).

Table 4. Values of MIC and MBC and FICI of the EDLB-EDK combination against S. aureus

Replication	KHM (mg/mL)			FICI	KBM (mg/mL)		
	EDLB	EDK	Combination (EDLB:EDK)		EDLB	EDK	Combination (EDLB:EDK)
1	2.5	10	0.625;2.5	0.5	5	10	0.625;5 dan 1.25;2.5
2	2.5	10	0.625;2.5	0.5	5	10	0.625;5 dan 1.25;2.5
3	2.5	10	0.625;2.5	0.5	5	10	0.625;5 dan 1.25;2.5

The MBC value (Table 4) delineates that the single extract of aloe vera leaf has a minimum killing concentration (KBM) of 5 mg/mL, while the single extract of cherry leaf has a minimum killing concentration (KBM) of 10 mg/mL. In the combination of the two extracts, vertical and horizontal streaking were carried out to obtain 2 minimum killing concentrations, namely in the horizontal combination of 1.25 mg/mL (EDLB); 2.5 mg/mL (EDK) and in the vertical combination of 0.625 mg/mL (EDLB); 5 mg/mL (EDK).

4. CONCLUSION

Aloe vera leaf extract and cherry leaf extract contain compounds belonging to the group of alkaloids, flavonoids, tannins, steroids and saponins. The minimum inhibitory level (MIC) of cherry leaf extract (EDK) was 10 mg/mL, while aloe vera leaf extract (EDLB) was 2.5 mg/mL. Combined MICs were shown in a mixture of 2.5 mg/mL EDK and 0.625 mg/mL EDLB. The minimum killing rate (KBM) was EDK 10 mg/mL, while the EDLB was 5 mg/mL. Combined MBC occurred in two combinations, namely EDK 5 mg/mL with EDLC 0.625 mg/mL and EDK 2.5 mg/mL with EDLB 1.25 mg/mL. The combination of the two resulted in synergistic antibacterial activity against *Staphylococcus aureus* with an FICI value of 0.5. The results of this study may be further developed by making pharmaceutical preparations using a combination of EDK and EDLB and retesting their antibacterial activity.

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6. CONFLICT OF INTEREST

The authors declare that there is no conflict of interest to disclose.

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