Anti-methicillin resistant *Staphylococcus aureus* (MRSA) activity of fermented apple peels kombucha and their chemical content

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Abstract. The production of kombucha using local apple peels is expected to reduce the production of apple peel waste while increasing the nutritional and functional value of kombucha products. This study aimed to determine the active compounds and antibacterial activity of kombucha apple peels against MRSA (Methicillin Resistant *Staphylococcus aureus*). This research was carried out experimentally in a completely randomised design with two factors: the apple varieties (Anna, Manalagi, and Romebeauty) and the concentration of the apple peel substrate (2.5%, 7.5% and 12.5%). The analyses comprised phytochemical screening and antimicrobial activity testing, both qualitative and quantitative. The results showed that the kombucha apple peel contains tannins, saponins, flavonoids, polyphenols, and organic acids. The average total phenolic, total acid, and antibacterial activities of Kombucha apple peels were significantly ($\alpha \leq 0.05$) differently affected by apple cultivar and substrate concentration. Antimicrobial activity against MRSA showed a range of weak to moderate inhibition, with the highest inhibition zone diameter value of 6.58 ± 0.52 mm in the Anna variety treatment at 12.5% substrate concentration. The MIC of the best treated kombucha was found at a concentration of 85%, while the MBC could not be determined.

1. Introduction

Apples (*Malus sylvestris* Mill.) are one of the fruit commodities with high production rates in Indonesia, especially in East Java Province [1]. Indonesia has produced apples up to 509,544 tons/year in 2021 [2]. The most commonly cultivated local apple varieties are Anna, Manalagi, and Rome beauty [1,3]. These three varieties are not only in high demand as consumption fruit in fresh form but are also widely used as ingredients for processed products [1]. High production of local apples also triggers an increase in apple peel waste [4]. The peels contain higher levels of total phenolics than the flesh [5]. It contains catechins, phloretin glycosides, procyanidins, caffeic acid, chlorogenic acid, phloridzin, quercetin glycosides and cyanidin glycosides [6]. Apple peels also contain active compounds of alkaloids, saponins, terpenoids [7], and tannins [8]. These active compounds have potential as an antibacterial due to their biological activity [8].

Processing apple peels into fermented products is one of the functional food innovations that is gaining popularity due to its good health benefits, including kombucha [9]. Kombucha is a beverage product resulting from the fermentation of tea or other alternative ingredients (such as fruits and vegetables) for 7-14 days using a symbiotic culture of bacteria and yeast or SCOBY (Symbiotic Culture of Bacteria & Yeast) [10]. The beneficial effect of kombucha fermentation is the active compounds produced, such as amino acids, polyphenols, sugars, organic acids, ethanol, water-soluble vitamins, and

various micronutrients that are potentially good for health [11]. Apple peels processed into kombucha products are expected to be an alternative high nutritional beverage that are effective in treating various health problems.

One of the serious health problems in the last decade is bacterial infection caused by *Staphylococcus aureus* (S. *aureus*) [12]. S. *aureus* bacteria are opportunistic pathogens that can cause various types of diseases, ranging from mild skin infections to life-threatening diseases such as meningitis, bacteremia, and sepsis due to the virulence factor of these bacteria [12,13]. The S. *aureus* strain that is resistant to various antibiotics, including methicillin and beta lactam antibiotics known as Methicillin Resistant *Staphylococcus aureus* (MRSA) strains, has become a new threat [14]. Recent data from the World Health Organization (2022), there was an increase in the median percentage of MRSA cases globally from 16.6% in 2017 to 18.3% in 2020 [15]. Drug-resistant strains can spread rapidly, causing high mortality rates in infected patients [14].

The potential antibacterial activity of apple peel kombucha can be influenced by the phytochemical content in the raw material itself [16]. Each apple variety has a different concentration of active compounds [1;3]. The percentage of apple peel will affect the total active compounds produced during kombucha fermentation [16]. This study aims to explore the potential of local apple peel kombucha (M. *sylvestris* Mill.) as an alternative MRSA antibacterial agent.

2. Methods

2.1. Kombucha Preparation

Sample preparation was carried out by the peels of Anna, Manalagi, and Rome Beauty apples were sorted, washed, air-dried, cut into small pieces, and then baked at ±60ºC for 5 hours ±5 minutes [4] to obtain a crispy texture and dark brown color. Furthermore, kombucha was made by the dried apple peel brewed in hot water according to the percentage of 2.5% [17], 7.5% [16], and 12.5%, then allowed to stand for ± 20 minutes [18]. The brewed product was filtered, added 12.5% sugar [16;17], homogenized, then put into a glass jar and allowed to stand until it reached room temperature [18]. The starter kombucha was inoculated in the amount of 10% (v/v) mother kombucha liquid [16;17;18] and 3% (b/v) SCOBY. The glass jar was covered with cloth/tissue and tied tightly and then incubated in a fermentation room at room temperature (±25˚C) for 14 days [18]. The success of fermentation was indicated by the formation of a new SCOBY layer on the surface of the kombucha liquid [19].

2.2. Phytochemical Test of Kombucha

2.2.1. Tannin Test. A total of 3 ml of kombucha sample was put into the test tube, then 2-3 drops of FeCl3 1% were added, then homogenized. Positive results for tannins are indicated by the appearance of a blue-black or brownish-green colour [20].

2.2.2. Saponin Test. A total of 5 ml of kombucha sample was put into the test tube, 1-2 drops of 2N HCl solution were added, then shaken vigorously for ± 30 seconds. Positive results of saponins are recorded if stable foam is formed for ± 30 seconds [21].

2.2.3. Flavonoid Test. A total of 5 ml of kombucha sample was heated for ± 5 minutes and then filtered. The filtrate obtained was added 2-3 drops of ethanol, Mg powder, 5M HCl and amyl alcohol. Positive results of flavonoids are indicated by the presence of yellow to orange colour in the amyl alcohol layer [22].

2.2.4. Alkaloid Test. A total of 5 ml of kombucha sample was put into a test tube, added 5 ml of HCl 2N, then divided the solution into 3 tubes, each reacted with Mayer, Wegner, and Dragendorff reagents. Positive results of alkaloids are indicated by the presence of a precipitate from the reaction of Mayer, Wagner, Dragendorff [21].

2.2.5. Total Phenol Test. Determination of total phenols is carried out by put 1ml of kombucha sample into a 10 ml volumetric flask, added distilled water until the limit mark, then homogenized. The kombucha sample of each treatment was treated in the same way as gallic acid solution, so that the total phenol results were equivalent to gallic acid or GAE (gallic acid equivalent) [23].

2.2.6. Total Acid Test. Determination of total acid is carried out by putting 1 ml of kombucha sample into a 100 ml volumetric flask, adding distilled water to the limit mark, then homogenized. After that, 2-3 drops of PP indicator were added, then titrated with 0.1 N NaOH solution.

2.3. Antibacterial Activity Test

MRSA bacterial isolates from Nutrient Agar (NA) media were suspended into a test tube containing 10 ml of Nutrient Broth (NB) media, homogenized using a vortex, then adjusted for turbidity with a McFarland standard of 0.5 which means bacterial colony density of 10^6 CFU/mL [24]. MHA media was poured into a sterile petri dish as a base layer and allowed to solidify. Next, MHA media that had been added with 8µl of bacterial suspension as a seed layer was poured on top of the base layer media [25]. After that, wells were made with a distance arranged in such a way. Kombucha samples from each treatment were put into each well of 30 µL with a positive control of vancomycin 30 µg/ml [26], and a negative control of sterile distilled water. The culture medium was incubated at 37˚C [25]. After 24 hours of incubation, each inhibition zone was measured and recorded in millimetres (mm). The results were interpreted as Zone of Inhibition criterion: 0-5 mm = no inhibition (-); 6-9 mm = moderate inhibition (+); 10-14 mm = strong inhibition (++); and ≥ 15 mm = very strong inhibition (+++) [38].

2.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Tests

2.4.1. Minimum Inhibitory Concentration Test. All test samples of kombucha solution with concentrations of 100%, 85%, 70%, 55%, 40%, 25%, 3% vancomycin (30µg/ml) (Positive control), and sterile distilled water (Negative control) were placed in a tube containing 9 ml of NB media, each amounted to 0.5ml. Furthermore, each treatment was added 0.5 ml of MRSA $1x10^6$ CFU/ml test bacterial suspension, then vortexed. Blank control was carried out by repeating each treatment without adding test bacteria. All solutions from the treatment tubes were read for absorbance value by UV-Vis spectrophotometry λ 620nm. Incubation was carried out at 37˚C for 24 hours, then the absorbance value was read again. The MIC value was determined based on the optical density (OD) value after incubation minus the OD value before incubation. MIC value was determined at the lowest concentration of antibacterial that has $\Delta OD \leq 0$ [8].

2.4.2. Minimum Bactericidal Concentration Test. MHA media was poured into sterile petri dishes and allowed to solidify. Then, 0.1 ml of the test sample suspension determined as MBC was inoculated on MHA media by the spread plate method. The positive control was vancomycin (30µg/ml) and the negative control was sterile distilled water. Furthermore, the test media was incubated at 37˚C for 24 hours. Determination of MBC was carried out by observing the growth of bacteria on the test media and counting the number of colonies with a colony counter. The MBC value was determined at the lowest concentration of antibacterial that is overgrown with bacterial colonies ≤ 0.1% of the bacterial cell density in the negative control [8].

3. Results and Discussion

Kombucha apple peels contains active compounds of tannin, saponin, and flavonoids (Table 1). The active compounds in kombucha are basically derived from apple peel raw materials and the results of fermentation by bacteria and yeast. The difference in results is due to differences in the levels of active compounds from each fruit varieties as raw materials for kombucha. The phytochemical content of apple peels can be influenced by several factors, especially fruit varieties [27].

Table 1. Phytochemical screening of local apple peels kombucha (*Malus sylvestris* Mill.)

Tests	Apple Peels Kombucha								
	Anna			Manalagi			Romebeauty		
	2.5%	7.5%	12.5%	2.5%	7.5%	12.5%	2.5%	7.5%	12.5%
Tannin	+	+	+	+	+	+	++	++	++
Saponin	+	++	+++	+	++	+++	+	++	+++
Flavonoid	-	+	+	-	+	+	-	+	+
Alkaloid									
a. Meyer	-	-	-	-	-	-	-	-	-
b. Wegner	-	-	-	-	-	-	-	-	-
c. Dragendorff	-	-	-	-	-	-	-	-	-
Total Phenol (µgGAE/ml)	490.48c	1168.26h	1523.33i	295.31a	689.03e	612.71d	397.73b	784.20f	933.48g
Total Acid (%)	1.277	1.460	1.551	1.095	1.095	1.186	1.003	1.277	1.460

Notes: (+) = discolored / less foam; (++) = discolored quite concentrated / quite a lot of foam; (+++) = discolored concentrated / a lot of foam; (-) = not discolored / no foam; letter notation indicates significant differences based on the Least Significant Different test.

The data shows that apple peel kombucha from each treatment combination has a significantly different average value of total phenols with the highest value (1523.33 µgGAE/ml) in the Anna variety treatment (12.5%) (Table 1). Phenolic components of apple peel kombucha can come from the content of apple peel compounds, such as quercetin glycosides, phloridzin, catechins, chlorogenic acid and anthocyanins [28]. The difference in the phenolic component of kombucha in this study can be influenced by the total sugar content of apple peels, because during the fermentation process there is microorganism activity that utilizes sugar to produce phenol compounds. The high total phenolics of Anna apple skin kombucha is thought to be due to its higher sugar content compared to the other two varieties. In 100 grams of apple fruit, Anna variety has a total sugar content of 11.50g, while Manalagi is 8.29g and Romebeauty is 9.79g [28].

The high average total phenol in the 12.5% treatment shows that the more materials added, the higher the total phenol of kombucha. The amount of sugar accumulation from raw materials in making kombucha will be utilized by bacteria and yeast to produce phenol compounds [16]. Phenol compounds in kombucha can also be influenced by flavonoids contained in each ingredient [29]. During the fermentation process, catechins in apple peels will be degraded, causing an increase in phenols [29].

Apple peels kombucha from each treatment combination has a different average value of total acid with the highest value (1.551%) shown by the Anna variety treatment with a proportion of 12.5%. The high total acid in the Anna variety treatment which provides a significant difference to the other 2 varieties is possible due to the content of total acid and total sugar in it which is more. The total acid content in 100g of Anna apple is 0.61g, Manalagi 0.32g, and Romebeauty 0.35g [28]. The total sugar content in 100g of Anna apple is 10.50g, Manalagi 9.79g, and Romebeauty 8.29g [28]. The sugar content in kombucha can be utilized by bacteria and yeast in producing glucose and fructose which are then converted into organic acids [16]. The increase in concentration treatment which is directly proportional to the total kombucha acid can be attributed to the activity of utilizing phytochemicals dissolved apple peel in kombucha by microbes to produce organic acids [16]. The higher the amount of organic acids produced during fermentation, the higher the total acidity [30].

The results of the antibacterial activity test of apple peel kombucha against MRSA bacterial strains shows that the average value of the diameter of the antibacterial inhibition zone of apple peel kombucha shows a weak to moderate inhibition category (Table 2, Figure 1). The highest average ZOI value of the entire apple peel kombucha treatment was shown by the Anna variety with 12.5% concentration of apple peel, which was 6.58 ± 0.520 mm. This was classified as moderate inhibition. The positive control

Vancomycin 3% (30µg/ml) showed a strong category with a mean ZOI value of 11.50mm. The negative control of sterile distilled water showed no antibacterial activity.

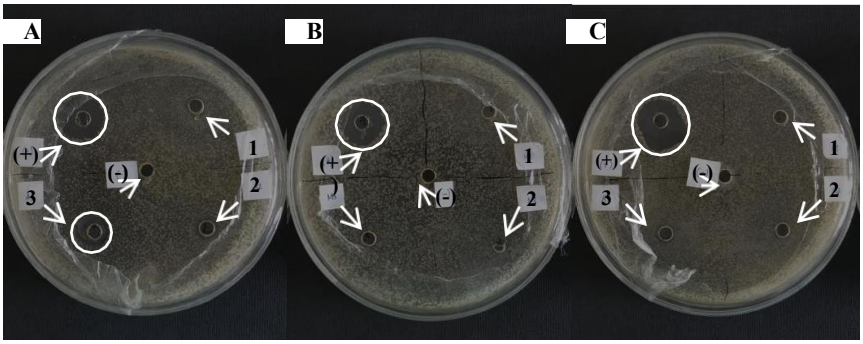

Figure 1. Zone of Antibacterial Inhibition against MRSA (A = Anna variety; B = Manalagi; C = Romebeauty; (1) = concentration of apple peel 2.5%; (2) = 7.5%; (3) = 12.5%; (+) = positive control vancomycin 3% (30µg/ml); (-) = negative control sterile distilled water.

Table 2. Diameter of Zone of Inhibition (ZOI) Antibacterial Activity

Treatments	Concentration	Average of ZOI (mm)	Category
Anna	2.5%	2.17±0.58	(-)
	7.5%	4.50±1.09	(-)
	12.5%	6.58±0.52	(+)
Manalagi	2.5%	0.50±0.87	(-)
	7.5%	1.17±1.13	(-)
	12.5%	2.42±0.52	(-)
Romebeauty	2.5%	0.58±0.52	(-)
	7.5%	2.50±0.25	(-)
	12.5%	3.17±0.52	(-)
Control (+)	Vancomycin 3%	11.50±1.15	(++)
Control (-)	Sterile distilled water	0.00	(-)

Notes: Zone of Inhibition criterion: 0-5 mm = no inhibition (-); 6-9 mm = moderate inhibition (+); 10-14 mm = strong inhibition (++); and ≥ 15 mm = very strong inhibition (+++)

The presence of antibacterial activity in local apple peel kombucha against MRSA bacterial strains indicates the presence of active compounds as known from the results of phytochemical screening, such as tannins, saponins, flavonoids, polyphenols and organic acids. Tannin compounds have antibacterial activity by inhibiting protein synthesis in the formation of bacterial cell walls [31]. Saponins are able to suppress bacterial growth by reducing the surface tension of the cell wall, cell metabolism is disrupted [32]. Flavonoids are able to inhibit bacterial growth by disrupting the permeability of bacterial cell membranes [33]. Various organic acid compounds formed during fermentation cause the acidity level of kombucha to be relatively high so that it can inhibit the growth of other microorganisms [34]. The highest average value of ZOI in Anna apple peel kombucha with a proportion of 12% can be attributed to the results of its phytochemical screening which shows higher levels of total phenols and total acids than each of the other treatment factors. The higher the active compounds in a material, the effectiveness in inhibiting bacteria will increase, so that the inhibition zone formed is wider [35].

Table 3. Value of Minimum Inhibitory Concentration (MIC) Test against MRSA

Treatments		Average (nm)		
		ΔOD_U	ΔOD_B	Value (ΔOD_U-ΔOD_B)
Anna apple peels Kombucha	25%	0.370	0.147	0.223
	40%	0.553	0.239	0.314
	55%	0.462	0.178	0.284
	70%	0.460	0.202	0.258
	85%	0.371	0.374	-0.003
	100%	0.429	0.461	-0.032
Control (+)	Vancomycin 3%	-0.007	-0.001	-0.006
Control (-)	Sterile distilled water	0.382	0.003	0.379

Notes: ΔOD_U = Optical Density after - before incubation (with MRSA test microbes);
ΔOD_B = Optical Density after - before incubation (without MRSA test microbes)

The negative results of the average ΔOD value (ΔOD≤1) were shown in the 85% and 100% treatment concentrations (Table 3). These results indicate the inhibitory activity of MRSA bacteria as seen from the decrease in turbidity value in the test media. Thus, the MIC value of kombucha of Anna variety apple peel with a proportion of 12.5% against MRSA bacteria is 85%. The MIC test results showed that the positive control treatment of 3% vancomycin (30µg/ml) has bacteriostatic activity against MRSA known from the ΔOD≤0nm value, which is -0.006nm. The decrease in OD value after incubation indicates a decrease in the turbidity level of the liquid media which indicates the inhibition of the growth of the test bacteria. The mechanism of action of vancomycin against MRSA is by inhibiting the transpeptidation of the peptidoglycan layer of the bacterial cell wall by binding to the C-terminal D-Ala-D-Ala chain of the peptidoglycan pentapeptide [36]. The difference in bacterial inhibition strength can be attributed to the content of active compounds at each concentration level. The higher the active compound in a substance, the effectiveness in inhibiting bacteria will increase [35].

Table 4. Number of MRSA bacterial colonies in the MBC test

Treatments		Total colony (CFU/mL)	Notes
Apple peels Kombucha	85%	TMTC	KBM value cannot be determined
	100%	TMTC	
Control (+)	Vancomycin 3% (30µg/ml)	TMTC	
Control (-)	Sterile distilled water	TMTC	
Blank		TMTC	

TMTC = Too many to count (colonies >250 CFU/mL)

The MBC value cannot be determined because both treatments of local apple peel kombucha concentrations, 85% and 100%, show the growth of bacterial colonies >250 (CFU/mL) so that it is classified as turbidimetric or too much to count (TMTC) (Table 4). Similar to the positive control treatment of vancomycin and negative control, sterile distilled water which showed colony growth >250 CFU/ml. Vancomycin 3% as positive control in this study is enough to showed bacteriostatic characteristics against MRSA strains, not bactericidal characteristics (Figure 2). Antibacterial activity is influenced by several factors, one of which is the type of test bacteria that has a high level of susceptibility [37].

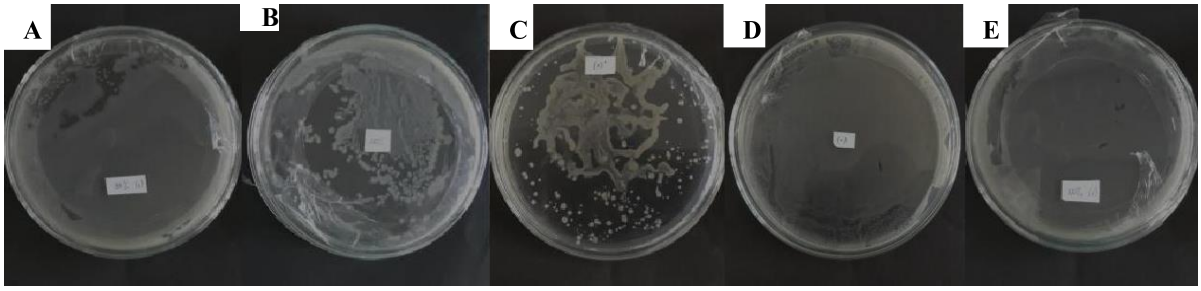

Figure 2. MBC Test against MRSA (A = 85%; B =100%; C = positive control (vancomycin 3% (30µg/ml); D = negative control (sterile distilled water); E = blank)

4. Conclusion

Active compounds contained in apple peel kombucha (*M. sylvestris* Mill.) include tannins, saponins, flavonoids, polyphenols and organic acids. The difference in apple varieties and substrate concentration gave a significantly different effect (sig ≤ 0.05) on total phenol content, total titratable acid, diameter of the bacterial inhibition zone, and the value of bacterial MIC. The interaction of the two treatment factors only showed significant differences in total phenol content. Antibacterial activity showed the strength of the inhibition zone in the range of no clear zone to moderate categories, with the highest value of 6.58±0.520 mm (moderate category) in the Anna variety with a 12.5% concentration. The MIC value of the best treatment against MRSA was 85%, while the MBC value could not be determined.

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