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Antibacterial activities of combinations of areca nuts (*Areca catechu*), cardamon seeds (*Amomum compactum*), and green betel leaves (*Piper betle*) ethanol extracts against *Staphylococcus aureus*

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Abstract. *Prasetya AA, Etikawati N, Susilowati A. 2024. Antibacterial activities of combinations of areca nuts (*Areca catechu*), cardamon seeds (*Amomum compactum*), and green betel leaves (*Piper betle*) ethanol extracts against* Staphylococcus aureus*. Asian J Nat Prod Biochem 22: 67-73.* Areca nut (*Areca catechu* L.), Javanese cardamom seed (*Amomum compactum* Soland. ex Maton), and betel leaf (*Piper betle* L.) are well-known as the formulas for betel quid chewing in Indonesia. According to tradition, betel quid chewing can prevent tooth decay, gum diseases, and lousy breath. This study investigated the antibacterial activity of the single extract with various concentrations (10 mg/mL, 15 mg/mL, 20 mg/mL, and 25 mg/mL) and combined extracts with ratios (1:1:1, 2:1:1, 1:2:1, and 1:1:2) against *Staphylococcus aureus* ATCC 25923 using the disc diffusion method based on EUCAST. Data was analyzed on the inhibition zones using one-way ANOVA and Duncan multiple-range tests. The antibacterial activity of each ethanol extract has an optimal concentration of 20 mg/mL against *S. aureus*. The extracts of areca nut, betel leaves, and Javanese cardamom seed obtained inhibitory zone diameters of 8.70±0.570 mm, 7.50±0.395 mm, and 7.35±0.379 mm, respectively. The combinations of three extracts obtained inhibitory zone diameters of 9.60±0.379 mm and 9.90±0.675 mm in 1:1:1 and 2:1:1 ratios, demonstrating a significant effect of the inhibition zone ($p<0.05$) compared to other ratio combinations and single extracts. These combinations of extracts also show a synergistic effect against *S. aureus*.

Keywords: *Amomum compactum*, antimicrobial agents, *Areca catechu*, combinations extract, dental plaque, *Piper betle*

INTRODUCTION

Currently, one potential means of overcoming problems during dental infections is to do it chemically through antibiotics and antimicrobials in the form of mouthwash containing chlorhexidine. According to Cock et al. (2023), oral healthcare is the fourth most expensive disease to treat; some factors, like the limited oral health facilities in rural areas, also lower the investment in oral healthcare. However, people also tend to rely on traditional medicine for problems related to treating dental disease. At least 80% of the world's population uses traditional medicine for primary health care (WHO 2023). Medicinal plants are often considered easy to obtain, cheap, efficient, and rarely accompanied by side effects. The medicinal plants selected for use for thousands of years by society are the most apparent starting point for potential ingredient candidates in developing new and effective antimicrobial agents (Besra and Kumar 2018). Traditional medicine is often an accumulation of various values and knowledge or practices based on theory, beliefs, and culture. Based on regional and cultural factors, conventional medicines are used as single formulations containing part of a plant or as combination formulations with another plant as active ingredients (Che et al. 2013).

The habit of "*nginang*" (betel chew) is an example of an herbal combination in Indonesia; it's usually used for medicinal purposes, social purposes, and religious ceremonies. Betel chewing is often associated with and believed to prevent various complaints related to oral health, such as avoiding bad breath, strengthening teeth, and maintaining healthy gums (Sari et al. 2020). According to Verawati et al. (2017), betel chews also benefit dental health. The standard formulation for betel chew in Indonesia consists of two main ingredients: areca nuts and betel leaves. Areca nuts and other ingredients are wrapped and folded in betel leaves during the preparation procedure. Sanskrit literature recorded several additional ingredients for betel chew in Indonesia, including cloves, nutmeg, and cardamom. According to Portal Informasi Indonesia (2019), the development of other ingredients, such as injet (betel lime) and gambir (*Uncaria gambir* (W.Hunter) Roxb.), has become a common additional ingredient used by people in betel chewing activities.

Staphylococcus aureus is a commensal bacterium and is the primary pathogen of various clinical infections, including abscesses and wound infections. It is found in the skin, skin glands, and mucous membranes, mainly in the nasal passages and oral cavity of humans (Brooks et al. 2013). *Staphylococcus* spp. were reported to colonize 90.4% of the mouths of healthy adults, while *S. aureus* was found to colonize about 24% (Jackson 2000). According to Ohara-Nemoto et al. (2008), *S. aureus* found about 33.9% in dental plaque and 46.4% in the saliva of healthy adults. The *S. aureus* in the oral cavity did not directly cause infection and disease. Furthermore, in advantageous

conditions like an open wound, oral hygiene, and an imbalanced oral microbiome, these oral pathogens may develop into an infection or some dental disorder in the oral cavity of humans (Dewhirst et al. 2010). Huang et al. (2017) reported that *S. aureus* was the most frequently isolated from acute dental abscesses in children and considered an essential microorganism in the etiology of infection in the oral cavity of humans (Ibrahim et al. 2021).

In our comprehensive study, we evaluated the antibacterial activity of each plant and the combined extract of betel chew formulations commonly used in Indonesia. This included areca nuts, cardamom seeds, and betel leaves ethanol extract. Our aim was to determine the effect of this combined plant extract against *S. aureus*, a model bacterium, providing a detailed understanding of the potential antibacterial properties of these formulations.

MATERIALS AND METHODS

Material

Areca nut, cardamom seeds, and betel leaves were collected from Ponorogo District, East Java, Indonesia. The plant samples were dried at 50°C for 48 h. The dried samples were then powdered and stored at 4°C in the refrigerator. Chlorohexidine gluconate 2%, distilled water (aquadest), Mueller Hinton Agar (MHA) (Merck), Nutrient Agar (NA) (Merck), and technical ethanol (70%) were provided by the laboratory of the Department of Biology, Universitas Sebelas Maret, Indonesia. The bacteria strain *S. aureus* (ATCC 25923) was obtained from the microbiology laboratory, Faculty of Medical, Universitas Sebelas Maret, and stored at 4°C in the refrigerator.

Procedures

Solvent extraction of areca nuts, cardamon seeds, and betel leaves

Areca Nuts (AN), Betel Leaves (BL), and Cardamom Seeds (CS) were subjected to a rigorous process of drying, powdering, and extraction by maceration using a 70% ethanol solvent. Total 50 g of each plant sample was mixed with 550 mL of 70% ethanol 1:11 (w/v) in a sample bottle (Zhang et al. 2014). The extraction process was carried out for 3 days, during which the extracts were filtered through Whatman No. 1 every 24 hours and re-macerated three times with 70% ethanol. The ethanol solvent was evaporated using a water bath at 50°C until a thick extract was obtained. After that, the thick extract was stored at 8°C in the refrigerator to maintain the integrity of the samples (Zhang et al. 2014).

Medium preparation

First, the NA medium was prepared by measuring 10 mg of agar and dissolved in 100 mL of distilled water (the manufacturer's protocol for solubility was 20 g/L). The MHA was prepared by measuring 17 g of agar and dissolved in 500 mL of aquadest (the manufacturer's protocol for solubility was 34 g/L). The sterilization process of the medium used an autoclave at 121°C for 15 min. After that, warm agar was dispensed in Petri dishes to achieve a depth of 4 mm (EUCAST 2022). Then, a petri dish containing MHA medium was incubated for 24 hours at 37°C. The incubation process is to make the surface of the agar dry before use, with no visible drops of water visible on the surface of the agar. This is to prevent excess moisture, which could cause problems with zone edges and haze within zones (EUCAST 2022).

Preparation of bacterial suspension and inoculation of agar plates

The *S. aureus* ATCC 25923 bacteria strain was prepared after 24 h of incubation on a non-selective medium (NA) with an inoculation loop. One inoculation loop of *S. aureus* culture was added to 0.85% NaCl and visually measured with the 0.5 McFarland turbidity standard. The density of 0.5 McFarland corresponded to approximately $1\text{-}2\times10^8$ CFU/mL of bacteria. A sterile cotton swab is dipped into the bacterial suspension, and the excess fluid is removed by turning the swab inside the tube to avoid over-inoculation. The inoculum was spread over the agar surface with manual swabbing in three directions (EUCAST 2022).

Antibacterial activity assessment of single and combination extracts

The antibacterial activity of a single extract of areca seeds, cardamom seeds, and betel leaves against the Gram-positive bacteria *S. aureus* ATCC 25923 was evaluated using the disk diffusion method by EUCAST (EUCAST 2022). Each plant extract was concentrated at 10 mg/mL, 15 mg/mL, 20 mg/mL, and 25 mg/mL with aquadest used as the diluted solvent. After that, the plant extract was sterilized with a syringe method with a 0.45 μm filter.The 20 μL of sterilized AN, BL, and CS extracts were put in to 6 mm disk and applied on the agar surface after 15 min of bacteria inoculation. The maximum number of disks applied was six on 90 mm plate dishes (EUCAST 2022). After 15 min of disk application, the plate dish was incubated at 37°C for 24 h. Chlorhexidine gluconate 2% was used as the positive control, and aquadest was used as the negative control.

The antibacterial test of the combinations of *Areca catechu* L. nuts, *Piper betle* L. leaves, and *Amomum compactum* Soland. ex Maton seeds ethanolic extracts was evaluated using a modified antibacterial test of herb combination procedure according to Widiyastuti et al. (2012) and Verawati et al. (2017). The ratio concentration of the combination dilution range of 20 mg/mL was used as the final concentration, which was based on the optimum concentration to inhibit the growth of *S. aureus* in each single extract, as shown in Table 1.

Measurement of inhibition zone

After 24 hours of incubation, an inhibition zone was determined where no *S. aureus* grew by unaided eye when plates were held about 30 cm from the eyes. The diameters of the inhibition zone were measured with Vernier calipers (mm) from the back of the plate dish with a dark background.

Table 1. Combination treatment was based on the best concentration of *Staphylococcus aureus* ATCC 25923 inhibition in single-extract testing

Ratio	Mass (g)			
	A. catechu nuts (AN)	*P. betle* leaves (BL)	*A. compactum* seeds (CS)	Final concentration mg/mL
1:1:1	6.6	6.6	6.6	20
1:1:2	5	5	10	20
1:2:1	5	10	5	20
2:1:1	10	5	5	20

Data analysis

Each antibacterial test was conducted in quintuplicate. The normality of data was carried out by the Shapiro-Wilk test, and the differences of the inhibitory zone diameter were determined by One-Way Analysis of Variant and Duncan multiple range tests at a significant level of $p<0.05$ using SPSS v. 16.0. The combination extract is called a synergy effect if the diameter of the inhibitory zone is significantly greater than the single extract of each plant at 20 mg/mL.

RESULTS AND DISCUSSION

Antibacterial activity of each plant extract against *S. aureus*

This study showed the susceptibility of *S. aureus* ATCC 25923 to the extracts of *A. catechu* nuts (AN), *P. betle* leaves (BL), and *A. compactum* seeds (CS). Zones of inhibition diameter produced by various concentrations of 10 mg/mL, 15 mg/mL, 20 mg/mL, and 25 mg/mL were measured using the disk diffusion method by EUCAST (2022). Table 2 shows the antibacterial activity of the AN, BL, and CS extracts; as expected, the results greatly varied for each plant extract. As shown in Table 2, the antibacterial activity of the AN increased with concentrations (Figure 1.A). However, there was no significant difference between the concentration of 20 mg/mL and the highest concentration of 25 mg/mL ($p>0.05$), with the average diameter of the inhibition zone obtained being 8.70 mm and 9.30 mm, respectively. It was also shown that an increase in the concentrations of BL extract led to an increase in inhibition of *S. aureus* growth, as indicated by the formation of a larger inhibitory zone diameter (Table 2 and Figure 1.B). The BL extract at the highest concentration of 25 mg/mL obtained an average inhibitory zone diameter of 7.90±0.379 mm; nevertheless, these results were not significant ($p>0.05$) compared to 20 mg/mL, which obtained an average of 7.50±0.395 mm. Additionally, compared to 10 mg/mL and 15 mg/mL, the inhibition diameter was significantly larger at a concentration of 20 mg/mL. As shown in Table 2, the antibacterial effect of CS also increased with concentration, as indicated by the larger formation of the inhibitory zone (Figure 1.C). It was shown that CS extracts at 25 mg/mL obtained an average inhibitory zone diameter of 7.75±0.395 mm and at 20 mg/mL obtained an average of 7.35±0.395 mm; nevertheless, these results were not significant ($p>0.05$). Statistically, a concentration of 20 mg/mL of AN, BL, and CS extracts was obtained as the optimal concentration for inhibiting the growth of *S. aureus*. Furthermore, we found that AN extract was more effective against *S. aureus*, as indicated by the greater inhibition zone diameter than BL and CS extracts at the same concentration. The inhibitory diameter zones produced by the positive control (chlorhexidine gluconate 2%) (23.40 mm) were significantly greater than the plant extracts.

Antibacterial activity of the combined extract against *S. aureus*

The inhibitory zone diameter obtained from the combined extract ranged from 9.00 mm to 9.90 mm against *S. aureus*, as shown in Table 3 and Figure 2. The 2:1:1 ratio (9.90±0.675 mm), dominated by a high concentration of AN extract, exhibited the largest diameter of the inhibitory zone, which was significant at a p level<0.05 compared to the 1:1:2 (9.00±0.586 mm), 1:2:1 (9.40±0.720 mm) ratios, and the three (single) extracts at the same concentration (20 mg/mL). However, the combination ratios 1:1:2 and 1:2:1 also demonstrated good activity when combined, compared to weaker effects when BL and CS extracts were tested independently (Tables 3 and 4). The key finding of this study is the comparison between the combined and single extracts, which is detailed in the Duncan analysis at a p level of 0.05 shown in Table 3.

Table 2. Antibacterial activity of various concentrations of areca seed, betel leaf, and cardamon seed ethanol extracts against *Staphylococcus aureus* ATCC 25923

Extracts	Concentration (mg/mL)	Inhibitory zone (mm) ± SD
Aquadest (control -)		6.00±0.000[a]
Areca nuts	10	6.70±0.410[a]
Areca nuts	15	7.50±0.353[b]
Areca nuts	20	8.70±0.570[c]
Areca nuts	25	9.30±0.570[c]
Chlorhexidine gluconate 2% (control +)		22.40±0.894[d]
Aquadest (control -)		6.00±0.000[a]
Betel leaves	10	6.45±0.209[a]
Betel leaves	15	6.70±0.209[a]
Betel leaves	20	7.50±0.395[b]
Betel leaves	25	7.90±0.379[b]
Chlorhexidine gluconate 2% (control +)		23.40±1.140[c]
Aquadest (control -)		6.00±0.000[a]
Cardamon seeds	10	6.30±0.273[a]
Cardamon seeds	15	6.60 ±0.285[a]
Cardamon seeds	20	7.35±0.379[b]
Cardamon seeds	25	7.75±0.395[b]
Chlorhexidine gluconate 2% (control +)		23.35±0.335[c]

Note: a, b, c, d: The mean that had common alphabet in a row are not significantly different at level of $p<0.05$

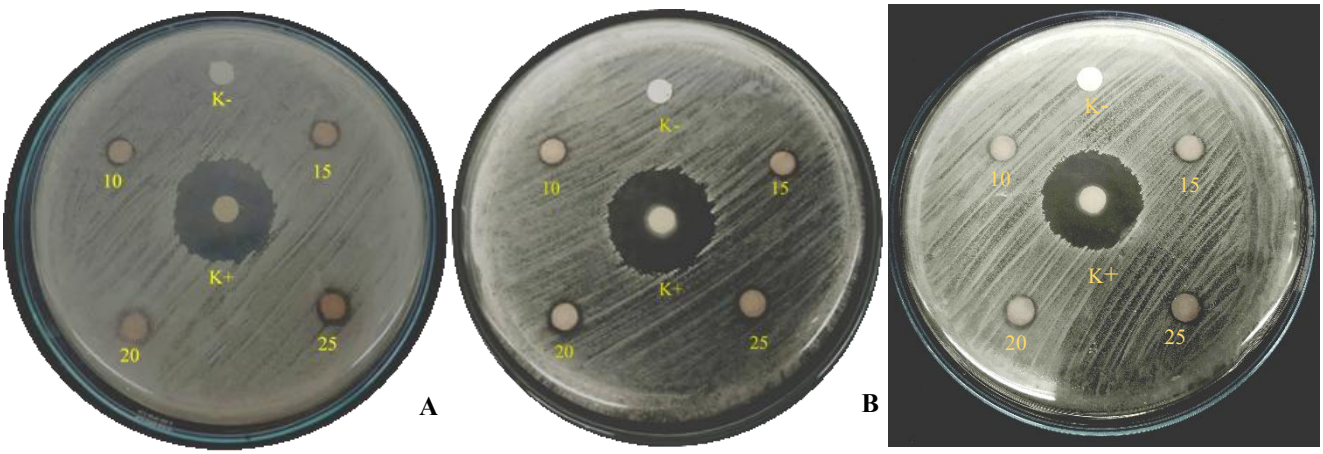

Figure 1. Antibacterial activity of various concentrations of single ethanol extract of A. *Areca catechu*, B. *Piper betle*, and C. *Amomum compactum*. Note: K-: Aquadest; K+: Chlorhexidine gluconate 2%; 10, 15, 20, 25 mg/mL concentration of each extract

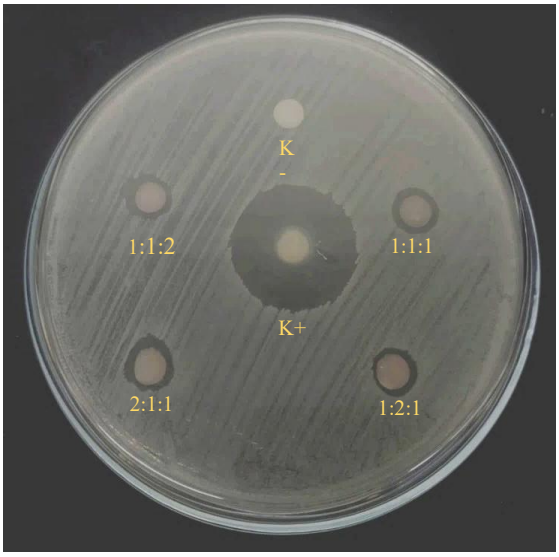

Figure 2. Antibacterial activity of various ratios of combination ethanol extract of *A. catechu*, *P. betle*, and *A. compactum*. Note: K-: Aquadest; K+: Chlorhexidine gluconate 2 %; 1:1:2 (5 mg AN, 5 mg BL, 10 mg CS); 1:1:1 (6.67 mg AN, 6.67 mg BL, 6.67 mg CS); 2:1:1 (10 mg AN, 5 mg BL, 5 mg CS); 1:2:1 (5 mg AN, 10 mg BL, 5 mg CS)

The comparison of inhibitory zone diameters between various ratio combinations can be seen in Figure 2. Based on statistical analysis, it was found that combining the three ethanol extracts at 1:1:1 and 2:1:1 ratios will result in a much larger inhibitory zone diameter than the optimal concentration of each extract, as shown in Table 3. It seems that the combination of AN, BL, and CS had a significant effect on the inhibition zone diameter against *S. aureus* compared to the respective single extract tests, so it can be said that the combination treatment of the three extracts also shows an effect, which is synergistic.

Discussion

The antibacterial activity was assessed by the development of a clear inhibition zone in the petri dish. The active compounds of three plant extracts, which were used to inhibit the growth of the tested bacteria, are detailed in Table 4. The results of our study show that the inhibition zone of *S. aureus* increased with the concentration of each plant extract. In terms of single extracts, the AN extract demonstrated the highest antibacterial properties against *S. aureus*, outperforming BL and CS (Table 2). The combination of the three extracts, with the AN extract dominating the proportion (2:1:1), exhibited significantly better antibacterial activity ($p<0.05$) against *S. aureus*, indicating a synergistic effect (Table 3; Figure 2).

Table 3. Antibacterial activity of various ratios of combination extract and effective concentration of single extract against *Staphylococcus aureus*

Extracts	Ratio	Concentration (mg/mL)	Inhibitory zone (mm) ± SD
Cardamon seed (CS)	Single extract	20	7.35 ± 0.379^{a}
Betel Leaves (BL)	Single extract	20	7.45 ± 0.480^{a}
Areca Nuts (AN)	Single extract	20	8.70 ± 0.570^{b}
Areca nuts: Betel leaves: Cardamon seeds	1:1:2 (combined)	20	9.00 ± 0.586^{bc}
Areca nuts: Betel leaves: Cardamon seeds	1:2:1 (combined)	20	9.40 ± 0.720^{bc}
Areca nuts: Betel leaves: Cardamon seeds	1:1:1 (combined)	20	9.60 ± 0.379^{cd}
Areca nuts: Betel leaves: Cardamon seeds	2:1:1 (combined)	20	9.90 ± 0.675^{d}

Note: a, b, c, d: The mean that had common alphabet in a row are not significantly different at level of $p<0.05$

Table 4. Major phytochemical content of areca nuts, betel leaves, and cardamom seeds with reference

Materials	Major phytochemical content				
	Alkaloid	**Phenolic**	**Tannin**	**Flavonoid**	**Reference**
A. catechu nuts	Arecoline Arecaidine Guvacolin Guvacine		Catechin Epicatechin		Gupta et al. (2020); Sari et al. (2020); Wang et al. (2021); Hugar et al. (2024)
P. betle leaves		Allylpyrocatechol (hydroxychavicol) Eugenol 4-chromanol			Muruganandam et al. (2017); Almasyhuri and Sundari (2019); Nayaka et al. (2021)
A. compactum seeds		Kaempferol 2,2'-metilen bis[6-(1,1-dimetiletil)-4-etil]		Quercetin Hesperetin	Sukandar et al. (2015); Dinata et al. (2021); Cai et al. (2021); Nurcholis et al. (2021, 2022)

Combinations of different medicinal plants in herbal formulations have become common in some traditional medicine systems. According to Ncube et al. (2012), plant extract combinations may offer potential prospects for treating diseases caused by bacteria in conventional medicine. However, Yang et al. (2014) explained that the primary interaction mechanism between plant extract combinations is that the active compounds with different or exact targeting will interact synergistically or antagonistically. Synergistic antibacterial potential is defined as the combinations of plant extracts that strengthen the antibacterial activity of each other, making combined extracts more efficient compared to single extracts (Hussain et al. 2024). In this study, the 2:1:1 ratio's combination of 10 mg AN, 5 mg BL, and 5 mg CS extracts showed a synergistic effect, increasing the diameter of the inhibitory zone against *S. aureus*. While the other ratios of combination 1:2:1 (5 mg AN, 10 mg BL, and 5 mg CS) and 1:1:2 (5 mg AN, 5 mg BL, and 10 mg CS) did not significantly increase compared to 20 mg/mL AN single extract, we suspected that AN ethanolic extract was the most effective plant extract among betel chew formulations against *S. aureus* because the diameter of the inhibitory zone increased when using the high concentration of AN ethanolic extract. Gharbani et al. (2023) also reported that the ethanol extract of AN produced a greater inhibitory zone diameter against *S. aureus* with an increase in concentration. The phytochemical content of AN ethanolic extract may have played the most crucial role in the antibacterial activity against *S. aureus*.

Ethanol extracts of AN, BL, and CS found that all three plants were able to inhibit *S. aureus* bacteria at the lowest test concentration of 10 mg/mL, with areca nut seeds producing a significantly larger average diameter of the inhibition zone compared to BL and CS. According to Hussain et al. (2024), the antibacterial potencies between plant extracts varied based on the phytochemical composition of each plant extract. Previous research by Xin et al. (2021) shows that the ethanol extract of areca nut seeds is potent in inhibiting *S. aureus*. This shows the possibility that the phytochemical content of AN extract is very effective in inhibiting *S. aureus*. The content of active compounds in AN, BL, and CS is briefly summarized in Table 4.

Xin et al. (2021) reported that AN ethanolic extract showed potent antibacterial activity against *S. aureus* strains Methicillin-Sensitive *S. aureus* (MSSA) and Methicillin-Resistant *S. aureus* (MRSA) with minimum Inhibitory Concentration (MIC) at 0.4 mg/mL. Rialita et al. (2019) suggested that the phenolic compound was related to the bacterial activity of the plant extract. Wang et al. (2021) also confirmed that the AN ethanolic extract has a high content of phenolic compounds and showed strong antibacterial activity, especially against Gram-positive bacteria. According to some previous studies, AN ethanolic extract contains alkaloids and catechin at high concentrations, especially in unripe conditions (Sari et al. 2017; Wu et al. 2019; Chen et al. 2021). Another report also showed that catechin and epicatechin were the main compounds in the unripe areca nuts (Sari et al. 2020; Hugar et al. 2024). Catechin was reported to be an effective compound against Gram-positive bacteria such as *Propionibacterium acnes*, *S. epidermidis*, and *S. aureus* (Verawati et al. 2017; Alkufeidy et al. 2024). The mechanism of action of catechin disturbed the cell walls' integrity, increasing cell membrane permeability. In Gram-positive bacteria lacking an outer membrane and thicker cell walls, catechin will mediate the release of lipoteichoic acid from the bacterial wall, weakening the bacterial cell wall (Wu and Brown 2021). Furthermore, arecoline is the primary alkaloid compound isolated from AN, and the concentration of arecoline is estimated to be around 0.30-0.60% in fresh AN (Wu et al. 2019). According to Liu et al. (2016), arecoline has various activities, such as antimicrobial, antiparasitic, and pharmacological effects on the cardiovascular, digestive, and nervous systems. Luo et al. (2010) also confirmed that arecoline had antibacterial activity against *Bacillus proteus* and *B. anthracis* with an MIC value of 0.8 mg/mL. Still, the mechanism of action needs to be better understood.

In the current report by El-Sawy et al. (2024), combinations of ethanolic extracts of cinnamon bark, chamomile flowers, marigold flowers, and sage leaves were more effective against *S. aureus* than each single extract. A previous study by Ncube et al. (2012) also showed that a combination ratio of 1:1 of phenolic and saponin-rich *Hypoxis hemerocallis* corn and leaf extracts

resulted in a synergistic interaction effect against *S. aureus*. Gharbani et al. (2023) also reported that a combination ratio of 1:1 of *A. catechu* and *Punica granatum* L. ethanol extracts had a synergistic effect against *S. aureus*. It has also been reported by Gharbani et al. (2023) that polar and non-polar compounds present in both extracts strengthen each other through a synergistic effect. Jeong et al. (2023) also reported that the combination of *Sanguisorba officinalis* L. and *U. gambir* extracts has shown synergism against MRSA as well as enhanced antibacterial inhibition with bactericidal effects. According to Hemeg et al. (2020), the potent antibacterial activity of extracts is affected by the bioactive compound, its concentration, and the possibility of interaction with another compound. Combining different extracts can cause different interactions between compounds because of the various compounds contained in the extracts. According to Vaou et al. (2022), in the combination of medicinal plants, there is a unique synergistic interaction between bioactive compounds; for example, phenolic compounds contained in an extract will function to damage the cytoplasmic membrane, causing loss of bacterial cell integrity. This will potentially bring in other compounds that have a mechanism of action by directing the cells, such as targeting DNA replication.

In conclusion, our research demonstrated that the inhibitory zone diameter affected by various ratio combinations of *A. catechu* nuts (AN), *P. betle* leaves (BL), and *A. compactum* seeds (CS) ranged from indifference to synergism compared to each plant extract against *S. aureus* ATCC 25923. Previous studies have explored the antibacterial properties of AN, BL, and CS ethanol extracts and identified active compounds, including alkaloids, phenolics, and tannins. Therefore, the proper antibacterial and toxicity test methods for combinations of extracts of AN, BL, and CS should be optimized in future studies.

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