Synergistic Anti-Biofilm Activity of Stingless Bee Honey and *Binahong* Leaves Extract Against *Staphylococcus aureus*

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Abstract: Biofilm formation by *Staphylococcus aureus* is a major virulence factor contributing to antibiotic resistance. This study investigated the synergistic anti-biofilm activity of stingless bee honey (*Homotrigona apicalis*) combined with *binahong* leaves extract (*Anredera cordifolia*) against *S. aureus* in vitro. *Binahong* leaves were macerated using 96% ethanol. Anti-biofilm activity was assessed using a microbroth dilution crystal violet assay in 96-well plates. Five combination ratios (100% *Binahong* [K1], 100% honey [K2], 75:25 [K3], 50:50 [K4], and 25:75 [K5]) were tested at six concentrations (0.25–8%) and incubated at 37.5°C for 24, 48, and 72 hours. Phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, tannins, and terpenoids. All combinations exhibited concentration-dependent anti-biofilm activity, with the 50:50 combination (K4) showed the most stable and effective inhibition profile across all concentrations and incubation times. The minimum biofilm inhibitory concentrations for 50% and 90% inhibition ($MBIC_{50}$ and $MBIC_{90}$) were 0.25% and 8%, respectively. These results demonstrate that stingless bee honey (*Homotrigona apicalis*) and *Binahong* leaves extract exhibit strong synergistic anti-biofilm activity, highlighting their potential as alternative agents against *S. aureus* biofilms.

Keywords: Anti-biofilm, Synergistic, Honey, *Binahong*, *Staphylococcus aureus*

INTRODUCTION

The high incidence of infectious diseases, particularly bacterial infections, poses a serious public health challenge in developing countries (Savitri *et al*., 2019). Antimicrobial resistance (AMR) has emerged as a critical global issue, with bacterial biofilms playing a significant role in treatment failure. Biofilms are structured communities of microbial cells enclosed in a self-produced extracellular polymeric matrix, adhering to biotic or abiotic surfaces. This structure acts as a physical shield, increasing bacterial tolerance to antibiotics by 10-1000 times compared to planktonic cells (Kusumawati *et al*., 2023).

Staphylococcus aureus is a major pathogen in the AMR crisis, notably for its ability to form resilient biofilms, leading to persistent and difficult-to-treat infections (Kerek *et al*., 2025). Given the stagnation in new antibiotic development, exploring bioactive compounds from natural sources presents a promising alternative strategy. Natural products offer unique mechanisms of action, synergistic effects, and selective toxicity that can disrupt biofilm formation (Nuryah *et al*., 2019).

Homotrigona apicalis honey and *binahong* leaves (*Anredera cordifolia*) are Indonesian natural resources with reported antibacterial properties. *Homotrigona apicalis* honey is rich in phenolic compounds, contributing to its antibacterial potency (Rahmiati *et al*., 2023). This natural area provides habitat for many species of bees, including

stingless bees (Kustiawan *et al*., 2022). *Trigona sp*. produces bee products such as honey, propolis, and bee pollen, which have been shown to offer a variety of benefits (Kustiawan *et al*., 2023). *Binahong* leaves contain various secondary metabolites like alkaloids, flavonoids, saponins, and tannins, known for their antibacterial effects (Halim *et al*., 2022)(Mengga *et al*., 2022).

The combination of natural products, such as stingless bee honey and *Binahong* leaves extract, has the potential to enhance antibacterial efficacy through synergistic effects, targeting multiple pathways involved in biofilm formation. Previous studies have suggested that combining bioactive compounds can improve microbial inhibition compared to individual components, reduce the risk of resistance development, and provide safer alternatives to conventional antibiotics (Setiawan *et al*., 2021). Despite the documented antibacterial properties of both *Homotrigona apicalis* honey and *Binahong* leaves, research on their combined anti-biofilm activity against S. aureus remains limited. Therefore, investigating this combination could provide valuable insights into developing effective natural strategies to combat biofilm-associated infections.

METHODS

This laboratory experimental study used a quantitative approach with a true experimental design. The independent variables are combination ratio and concentration of the test samples, while the dependent variable was the percentage of biofilm inhibition against *S. aureus*. Fresh *binahong* (*Anredera cordifolia*) leaves were identified (Determination No. 140/UN17.4.08/LL/2025), washed, sliced, dried, and powdered. The moisture content of the powder was measured. The *Homotrigona apicalis* honey was sourced from a local farm in Lempake Village, Samarinda. *Binahong* leaves powder (200 g) was macerated with 96% ethanol (1:5 b/v) at room temperature for 3 x 24 hours. The filtrate was concentrated using a rotary evaporator, and the extract yield was calculated.

Phytochemical tests for alkaloids (Mayer, Bouchardat, Dragendorff reagents) (Surbakti *et al*., 2018), flavonoids (Mg-HCl test), tannins ($FeCl_3$ 1%), saponins (foam test), steroids (Liebermann-Burchard test), and terpenoids (Salkowski test) were conducted on both the *binahong* extract and *Homotrigona apicalis* honey separately, following standard procedures (Hanifah *et al*., 2022).

Five combination formulas (K1-K5) were prepared from individual stock solutions of *binahong* extract and *Homotrigona apicalis* honey (each 200 mg/mL in DMSO) by mixing them in varying volume ratios to a total volume of 1000 µL per formula: K1 contained 100% *binahong* extract; K2, 100% *Homotrigona apicalis* honey; K3, 75% *binahong* extract and 25% honey; K4, 50% *binahong* extract and 50% honey; and K5, 25% *binahong* extract and 75% honey. Each combination was then serially diluted in Brain Heart Infusion Broth (BHI-B) to yield final test concentrations of 8%, 4%, 2%, 1%, 0.5%, and 0.25%.

The assay was performed using the microbroth dilution crystal violet method (Jesus *et al*., 2020). A bacterial suspension of *S. aureus* (adjusted to 0.5 McFarland standard) was prepared in BHI-B. In a sterile 96-well plate, 50 µL of BHI-B and 10 µL of bacterial suspension were added to each well and incubated for 90 minutes at 37.5°C for initial adhesion. Then, 40 µL of each test sample (at various concentrations) was added to designated wells in triplicate. Chloramphenicol and DMSO served as positive and negative controls, respectively. The plates were incubated at 37.5°C for 24, 48, and 72

hours separately. After incubation, planktonic cells were removed, and the wells were washed with distilled water. The adherent biofilm was fixed with methanol, stained with 1% crystal violet for 15 minutes, washed, and dissolved with 96% ethanol. The optical density (OD) was measured at 595 nm using a microplate reader (Miquel *et al*., 2016). The percentage of biofilm inhibition was calculated using the formula:

$$\%\ Inhibisi = \frac{OD\ kontrol - OD\ sampel}{OD\ kontrol} \times 100\%$$

The Minimum Biofilm Inhibitory Concentration for 50% and 90% inhibition ($MBIC_{50}$ and $MBIC_{90}$) were determined. Data were analyzed statistically using One-Way ANOVA to assess the effects of concentration and incubation time, with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Based on the results of the phytochemical tests presented in Tables 1 and 2, *binahong* leaves extract and *Homotrigona apicalis* honey each contain various groups of bioactive compounds which together have great potential to support the anti-biofilm activity tested in this study.

Table 1. Phytochemical Screening Results of *Binahong* Leaves Extract

Compound Group	Phytochemical Screening	
	Result	Presence
Alkaloid	Precipitate formed	(+)
Flavonoid	Yellow color formed	(+)
Tanin	Black color formed	(+)
Saponin	Stable foam formed	(+)
Steroid	Green color formed	(+)
Terpenoid	No red color	(-)

Based on Table 1, phytochemical tests on *binahong* leaves ethanol extract showed positive results for the presence of alkaloids, flavonoids, tannins, saponins, and steroids. Terpenoid tests were negative.

Table 2. Phytochemical Screening Results of *Homotrigona apicalis* Honey

Compound Group	Phytochemical Screening	
	Result	Presence
Alkaloid	Precipitate formed	(+)
Flavonoid	Yellow color formed	(+)
Tanin	No Black color	(-)
Saponin	Stable foam formed	(+)
Steroid	No green color	(-)
Terpenoid	Red color	(+)

The phytochemical screening results (Table 2) showed that *Homotrigona apicalis* honey contains alkaloids, flavonoids, saponins, and terpenoids. Conversely, tests for the presence of tannins and steroids yielded negative results.

All five combination formulas (K1-K5) exhibited concentration-dependent antibiofilm activity against *S. aureus* at 24, 48, and 72 hours. The percentage of inhibition ranged from 59% to 92%. The highest inhibitions (>90% at 8% concentration) were consistently observed after 48 hours of incubation across all combinations. The K4 combination (50% *binahong* : 50% honey) showed the most stable and effective inhibition profile at all concentrations and time points. The $MBIC_{90}$ was achieved at 8% concentration for all combinations at 48 hours. Notably, the $MBIC_{50}$ was achieved at the lowest tested concentration of 0.25% for all combinations, indicating high intrinsic potency.

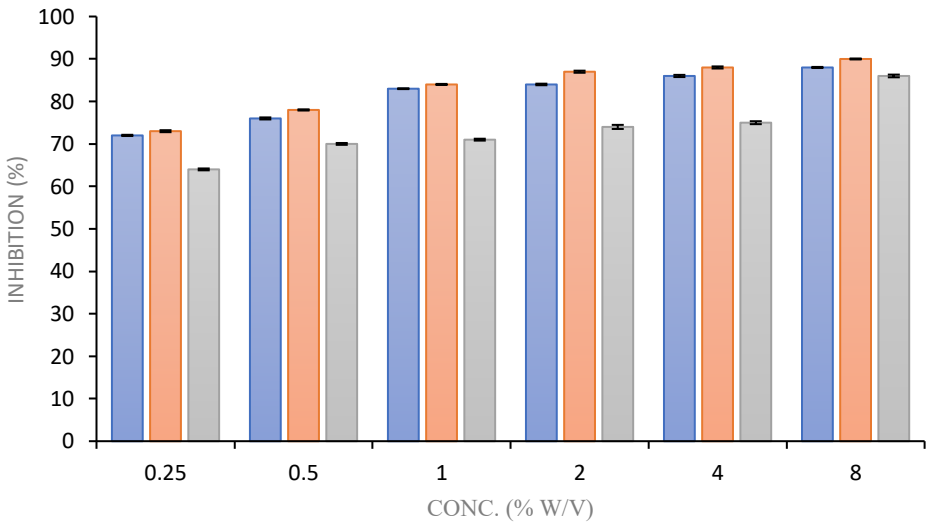

Figure 1. Inhibition of Biofilm Formation by 100% *Binahong* Leaves Extract (K1) at Different Concentrations Over Time. Time Points are Indicated as 24 h (Blue), 48 h (Orange), and 72 h (Grey)

The graph in Figure 1 of the antibiofilm activity of formulation K1 (100% *binahong* extract) illustrates the ability of the bioactive compounds from *binahong* leaves individually to inhibit *Staphylococcus aureus* biofilms. The response pattern observed indicates a concentration-dependent nature, while also providing a basis for highlighting the specific contributions of *binahong* components before they are combined.

In addition, the antibiofilm efficacy exhibited by formulation K1 is closely associated with the phytochemical profile of *binahong* leaves, which includes flavonoids, saponins, tannins, and alkaloids. These compounds have been reported to impair biofilm development by disrupting quorum sensing pathways and reducing extracellular polymeric substance (EPS) synthesis. The concentration-dependent inhibition observed suggests that increased levels of active constituents enhance their antibiofilm potency, indicating that *binahong* extract possesses intrinsic antibiofilm activity against *Staphylococcus aureus*. This result provides a strong baseline for evaluating the potential synergistic effects when *binahong* extract is incorporated into combined formulations.

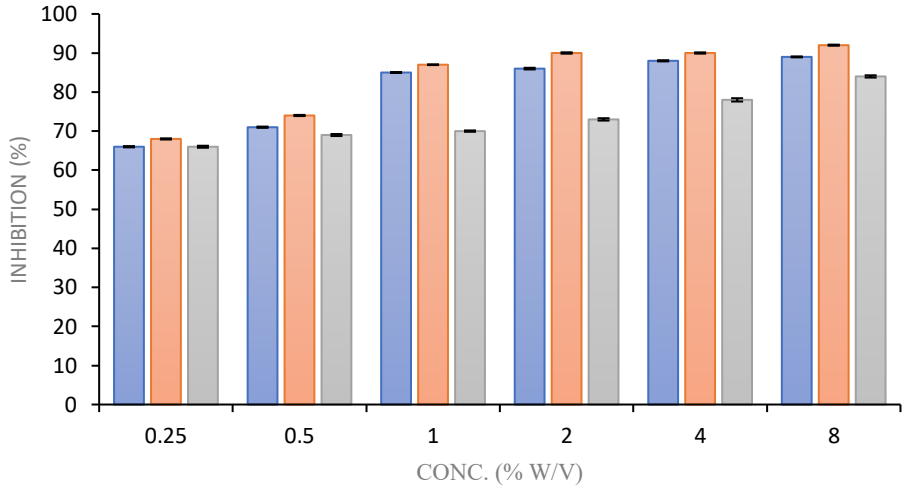

Figure 2. Inhibition of Biofilm Formation by 100% *Homotrigona apicalis* Honey (K2) at Different Concentrations Over Time. Time Points are Indicated as 24 h (Blue), 48 h (Orange), and 72 h (Grey)

The inhibition graph in Figure 2 of the K2 treatment (100% *Homotrigona apicalis* honey) demonstrates the potential of honey as a standalone anti-biofilm agent. The resulting inhibition profile serves as a reference for analyzing the role of honey's unique compounds, such as organic acids and terpenoids, in the dynamics of inhibition over the incubation period.

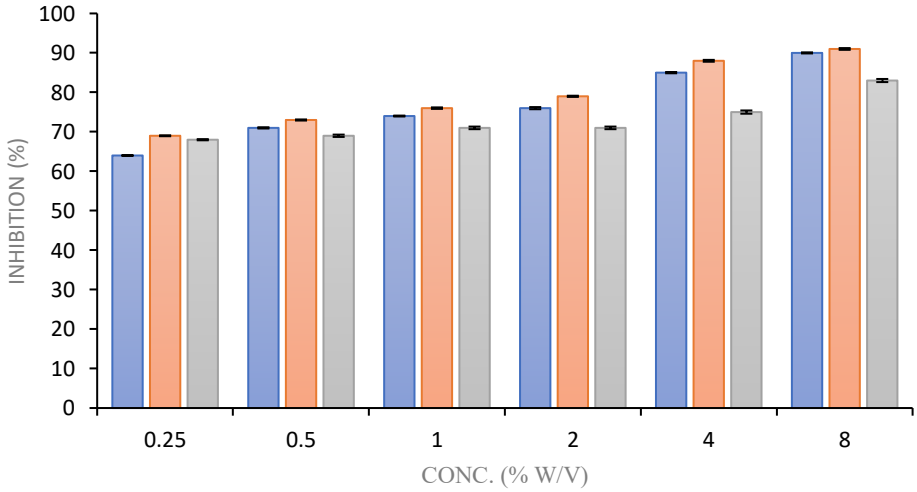

Figure 3. Inhibition of Biofilm Formation by 75% *Binahong* Leaves Extract: 25% *Homotrigona apicalis* Honey (K3) at Different Concentrations Over Time. Time Points are Indicated as 24 h (Blue), 48 h (Orange), and 72 h (Grey)

The graph in Figure 3 for the K3 combination (75% *binahong*: 25% *Homotrigona apicalis* honey) shows the effect of adding a smaller proportion of honey on the inhibition

profile. This graph allows for initial analysis of interactions between the ingredients, whether they are additive or beginning to show synergistic patterns.

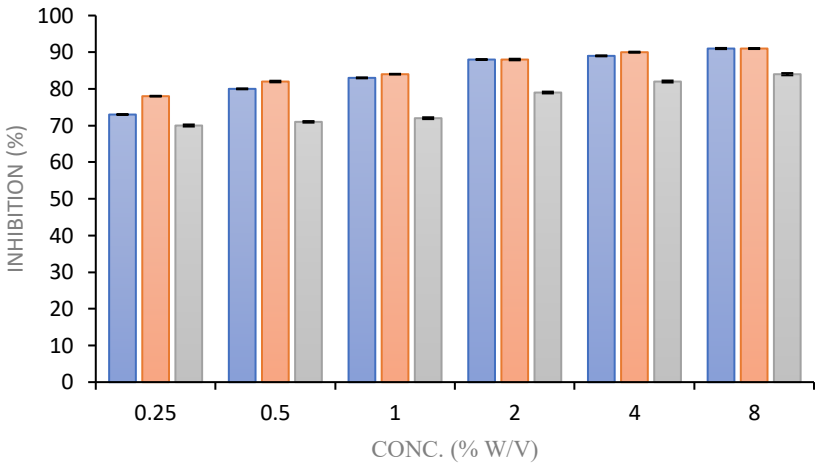

Figure 4. Inhibition of Biofilm Formation by 50% *Binahong* Leaves Extract: 50% *Homotrigona apicalis* Honey (K4) at Different Concentrations Over Time. Time points are indicated as 24 h (Blue), 48 h (Orange), and 72 h (Grey)

The graph in Figure 4 for the K4 formulation (1:1 ratio) displays the most stable and consistent inhibition profile among all treatments. This pattern strongly suggests optimal synergism between the bioactive compounds of *binahong* and honey.

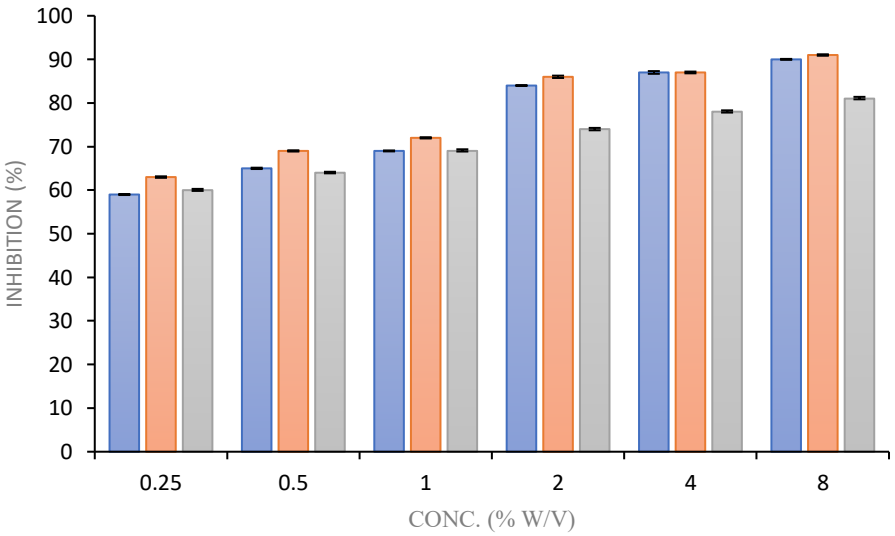

Figure 5. Inhibition of Biofilm Formation by 25 % *Binahong* Leaves Extract: 75% *Homotrigona apicalis* Honey (K5) at Different Concentrations Over Time. Time Points are Indicated as 24 h (Blue), 48 h (Orange), and 72 h (Grey)

Figure 5 shows K5 (25% *binahong*: 75% honey) experiencing a shift in the dominant effect toward the honey component. Analysis of this graph indicates a

proportion limit where the *binahong* contribution remains significant in maintaining high inhibitory activity. For comparison, the positive control graph in Figure 6 (chloramphenicol) shows a strong and consistent standard pattern of inhibition. This profile serves as a benchmark for the validity of the test method and as a basis for comparison to assess the relative effectiveness of the five test sample formulations.

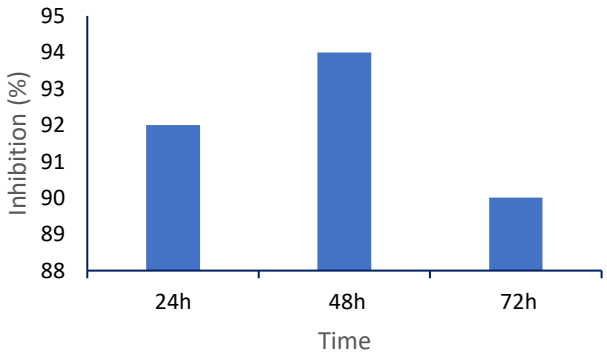

Figure 6. Inhibition Activity of Chloramphenicol as Positive Control Against *S. aureus*

The significant antibiofilm activity observed aligns with the presence of diverse bioactive compounds (Azzahra *et al.*, 2025). Flavonoids from both materials are known to inhibit initial bacterial adhesion and quorum sensing, a key regulator of biofilm maturation (Suryani *et al*., 2019). Saponins, acting as natural surfactants, can disrupt cell membrane integrity (Putri *et al*., 2023). Tannins from *binahong* may bind to and denature biofilm matrix proteins (Nabil *et al*., 2024), while terpenoids from honey contribute additional antimicrobial effects (Darmawansyah *et al*., 2023). The 1:1 ratio (K4) likely represents an optimal synergistic balance, where the complementary compounds from each source enhance each other's efficacy without interference, as suggested by the superior and consistent performance of K4 compared to single components (K1, K2) or imbalanced ratios (K3, K5). The peak activity at 48 hours corresponds to the maturation phase of biofilm development (Nurlita *et al*., 2024), where the test compounds effectively penetrated and disrupted the complex three-dimensional structure (Homenta, 2016). These bacteria can activate resistance genes, increase the production of protective matrices, and alter metabolism to survive the pressure of antimicrobial compounds (Majid, 2019). The decrease in inhibition at 72 hours may be due to compound degradation or bacterial adaptive responses within the biofilm (Kurniawati *et al*., 2019).

Statistical analysis (One-Way ANOVA) confirmed that the concentration factor had a highly significant effect on inhibition percentage ($F = 572.009$; $p < 0.001$), while the incubation time factor (24, 48, 72 hours) did not show a statistically significant difference ($p = 0.524$), despite the observable numerical peak at 48 hours (Anam, 2020). This indicates that concentration is the primary driver of activity, and the formulation's effect remains relatively stable over the 72-hour period.

These findings further suggest that the stability of antibiofilm activity across different incubation periods reflects the sustained bioactivity of the formulation, particularly at the optimal 1:1 ratio. Although numerical variations were observed over time, the absence of statistically significant differences among incubation durations

indicates that the active compounds maintained their functional integrity throughout the experimental window. This sustained effect may be attributed to the complementary physicochemical properties of *binahong* extract and honey derived terpenoids, which collectively enhance compound persistence and penetration within the biofilm matrix. Moreover, the dominance of concentration as a determining factor underscores the importance of precise formulation optimization in achieving maximal antibiofilm efficacy. From a practical perspective, this stability over time is advantageous for therapeutic or topical applications, as it suggests prolonged antibiofilm action without rapid loss of effectiveness, thereby supporting the potential translational value of the K4 formulation for managing *Staphylococcus aureus* biofilm-associated infections.

CONCLUSION

The combination of stingless bee honey (*Homotrigona apicalis*) and *binahong* leaves extract exhibits strong synergistic anti-biofilm activity against *Staphylococcus aureus*, which is concentration-dependent. The 1:1 combination (K4) was the most effective, achieving over 90% inhibition at 8% concentration after 48 hours of incubation. The anti-biofilm effect remained relatively stable over the 24-72 hours observation period, highlighting its potential as an alternative agent to control *S. aureus* biofilms.

SUGGESTIONS

Future research should focus on identifying the specific bioactive compounds responsible for the synergistic effect and elucidating their molecular mechanisms. Further testing against other clinically relevant pathogens, including MRSA, is recommended. In vivo toxicity and efficacy studies, formulation optimization and steps toward developing this natural combination into a practical alternative or adjunctive agent for treating biofilm-associated infections.

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