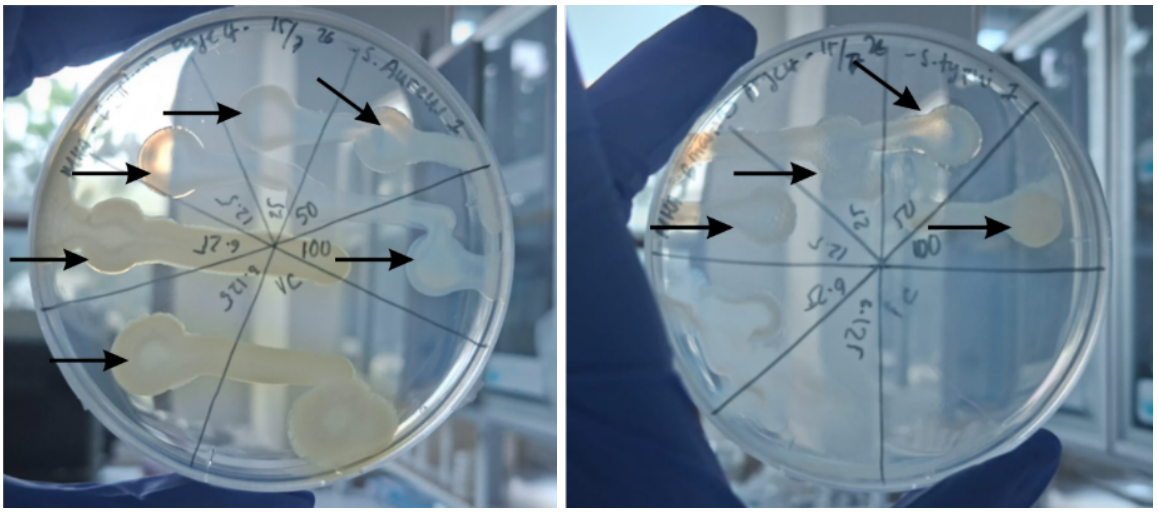

Figure 4. Minimum Bactericidal Concentration Assay

Figure 4 shows representative Mueller–Hinton agar plates used for the Minimum Bactericidal Concentration (MBC) assay. Bacterial growth was observed at all tested Binahong (*Anredera cordifolia*) extract concentrations for both *Staphylococcus aureus* and *Salmonella typhimurium*. As no concentration completely eliminated bacterial growth, the MBC could not be determined under the experimental conditions.

4.4. Antibiofilm Activity Assay

The present study investigated the antibiofilm activity of Binahong extract against *Staphylococcus aureus* and *Salmonella enterica serovar Typhimurium* using the crystal violet microtiter plate assay. After 24 hours of incubation in the presence of varying concentrations of Binahong crude extract, biofilm formation was evaluated by measuring biofilm biomass through crystal violet staining. In the biofilm disruption assay, preformed mature biofilms were first established for 24 hours before treatment with different concentrations of the extract for an additional 24 hours to evaluate its effectiveness in reducing established biofilm biomass. As shown in **Figure 5**., biofilm inhibition was quantified and compared across groups treated with different extract concentrations (3.125%, 6.25%, 12.5%, 25%, 50%, 100%) and in **Figure 6,** it is shown that biofilm disruption was qualitatively observed and compared across groups treated with different extract

concentrations (3.125%, 6.25%, 12.5%, 25%, 50%, 100%) to assess the concentration-dependent antibiofilm effects of Binahong extract on bacterial biofilm formation and disruption.

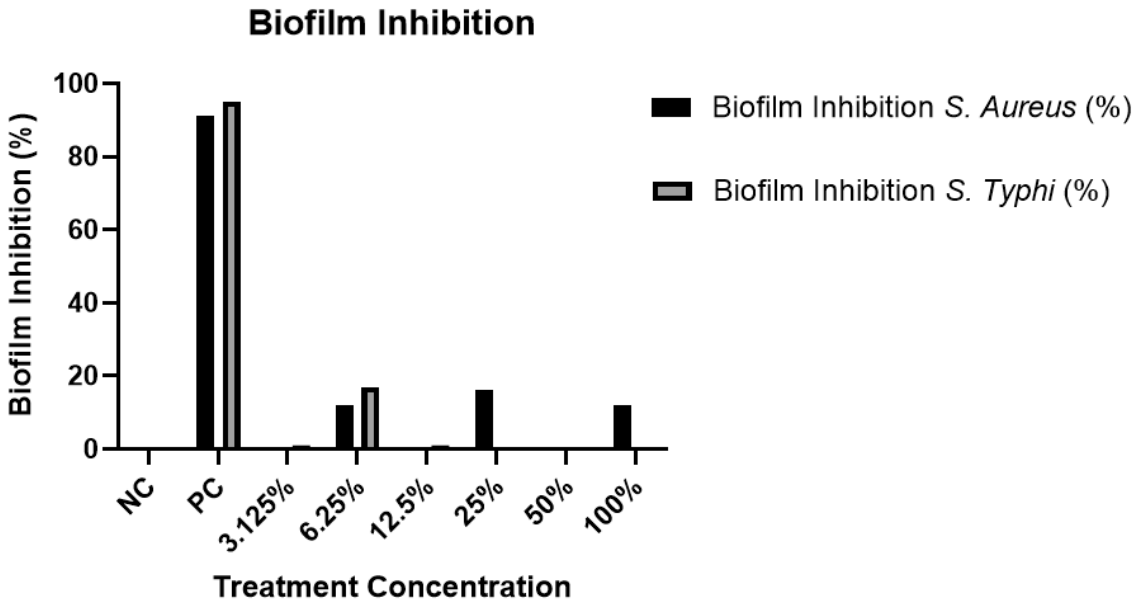

Figure 5. Biofilm Inhibition (%) of *Staphylococcus aureus* and *Salmonella typhimurium*

Figure 5 shows the biofilm inhibition of *Staphylococcus aureus* and *Salmonella typhimurium* following treatment with Binahong water extract. The positive control exhibited the highest inhibition for both bacteria (approximately 91% and 94%, respectively). The extract produced only low levels of biofilm inhibition, with the highest inhibition against *Staphylococcus aureus* observed at 25% (approximately 16%) and against *Salmonella typhimurium* at 6.25% (approximately 16%). Little or no inhibition was detected at the remaining concentrations, indicating limited antibiofilm activity under the tested conditions.

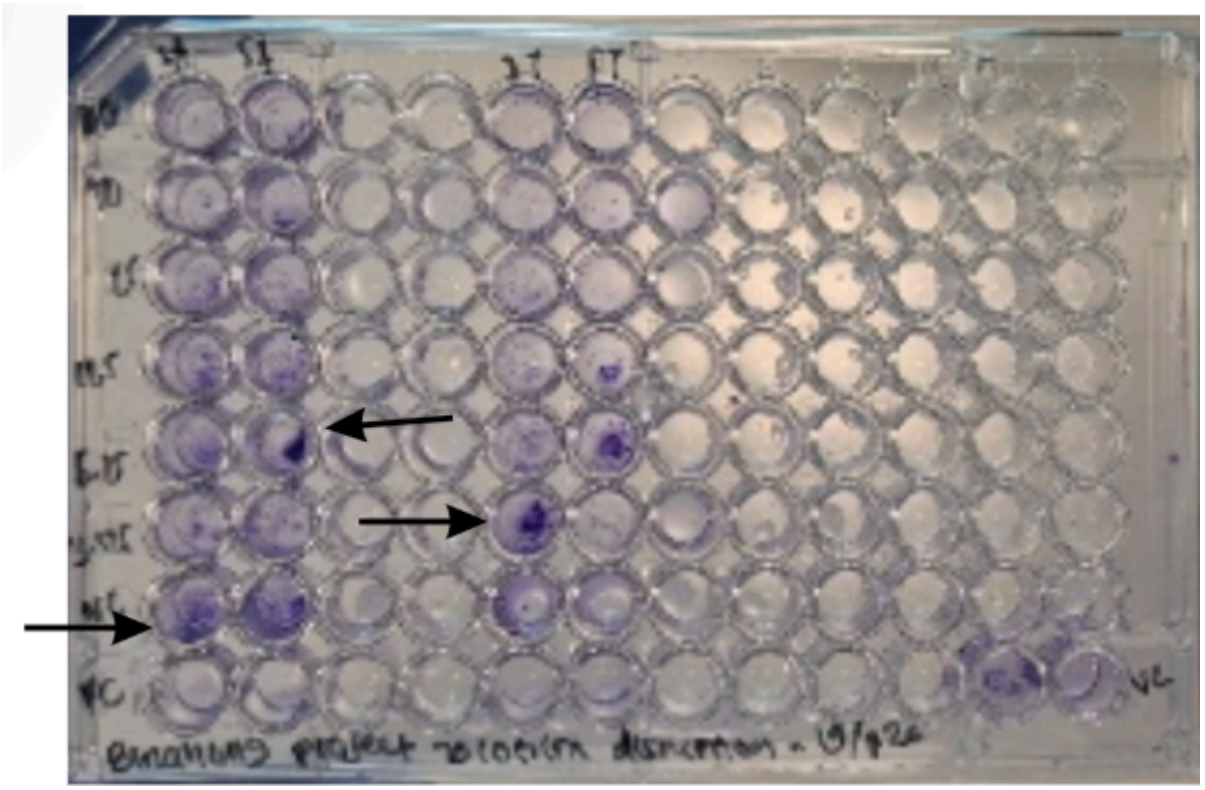

Figure 6. Qualitative Biofilm Disruption Assay

Figure 6 presents a representative image of the crystal violet-stained microplate used for the biofilm disruption assay. Purple staining indicates the presence of residual biofilm following treatment with different concentrations of Binahong (*Anredera cordifolia*) water extract. Variation in staining intensity was observed among the treatment groups; however, no clear concentration-dependent reduction in biofilm biomass was visually apparent.

Chapter 5

Discussion

5.1. Extraction of Binahong Powder

In the present study, a water extract of Binahong (*Anredera cordifolia*) was prepared using the aqueous maceration method. Water was selected as the extraction solvent because it is non-toxic, inexpensive, environmentally friendly, and suitable for developing extracts intended for food-contact applications. Unlike organic solvents such as ethanol or methanol, water does not leave harmful solvent residues, making it a more appropriate extraction medium for applications where food safety is a primary consideration (Plaskova & Mlcek, 2023). The extraction of 10 g of Binahong powder yielded 35 mL of filtered aqueous extract, corresponding to an estimated concentration of 285.7 mg raw material equivalent/mL. Since the extract was used directly after filtration without evaporation or drying, the concentration was expressed as milligrams of raw material equivalent per milliliter rather than milligrams of dried extract per milliliter (Meryem Tourabi et al., 2025). This value represents the amount of original Binahong powder corresponding to each milliliter of the final filtrate and does not indicate the actual quantity of phytochemicals dissolved in the extract.

The aqueous maceration method was selected because it is a simple extraction technique that minimizes thermal degradation of heat-sensitive phytochemicals (Meryem Tourabi et al., 2025; Plaskova & Mlcek, 2023). By allowing the plant material to remain in contact with water for an extended period, water-soluble bioactive compounds such as phenolics, flavonoids, saponins, and polysaccharides can diffuse into the solvent. However, the extraction efficiency depends on the polarity of the target compounds (Lefebvre et al., 2021). While water effectively extracts polar constituents, less polar or non-polar compounds with potential antimicrobial activity may remain in the plant matrix (Abubakar & Haque, 2020). Consequently, the phytochemical composition of the aqueous extract may differ from that of extracts prepared using organic solvents. Furthermore, the final extract volume (35 mL) was lower than the initial extraction volume because a portion of the solvent was retained by the plant material during filtration. Such volume loss is common during

aqueous maceration and does not necessarily indicate poor extraction performance (Neis et al., 2025). The filtered extract was subsequently sterilized using a 0.22 µm syringe filter before being used in the antibacterial, antibiofilm, and cytotoxicity assays to ensure sterility without altering the chemical composition of the extract. Overall, the aqueous maceration method successfully produced a sterile Binahong water extract suitable for subsequent biological evaluation while maintaining compatibility with the intended food-contact application.

5.2. In Vitro Cytotoxicity Assay

The cytotoxicity of the Binahong (*Anredera cordifolia*) water extract was evaluated using the MTT assay on HaCaT cells to determine its biocompatibility for potential food-contact applications. HaCaT cells, an immortalized human keratinocyte cell line, were selected because they provide a stable and reproducible in vitro model for evaluating the cytotoxic effects (Colombo et al., 2017). Keratinocytes constitute the major cell type of the epidermis and are among the first human cells that may come into contact with food-contact materials through skin exposure during food handling or manufacturing. Compared with primary human keratinocytes, HaCaT cells exhibit consistent growth characteristics, unlimited proliferation, and reduced donor-to-donor variability, making them widely used for cytotoxicity screening and biocompatibility assessment (Liao et al., 2024).

The MTT assay is a colorimetric method that measures cell metabolic activity as an indicator of cell viability. Viable cells possess active mitochondrial dehydrogenase enzymes that reduce the yellow tetrazolium salt (MTT) into insoluble purple formazan crystals (van Meerloo et al., 2011). Following solubilization with dimethyl sulfoxide (DMSO), the amount of formazan produced is quantified spectrophotometrically by measuring absorbance at 570 nm. Since the quantity of formazan is directly proportional to the number of metabolically active cells, higher absorbance values indicate greater cell viability (Colombo et al., 2017). Treatment with the Binahong water extract at concentrations ranging from 3.13% to 50% resulted in HaCaT cell viability values above 70%

across all tested concentrations. The highest cell viability was observed at the 12.5% concentration, while the remaining concentrations showed only minor variations without an apparent dose-dependent trend. Compared with the untreated control, the extract-treated groups maintained relatively high cell viability, whereas the positive and vehicle controls exhibited lower viability. These findings suggest that the Binahong water extract has low cytotoxic potential toward HaCaT cells under the experimental conditions and supports its potential biocompatibility for applications requiring contact with human tissues, such as food-contact surfaces.

The high cell viability observed across all extract concentrations suggests that the aqueous Binahong extract is well tolerated by HaCat cells. This low cytotoxicity may be attributed to the relatively mild nature of the aqueous extraction process, which primarily extracts hydrophilic phytochemicals while avoiding potentially cytotoxic solvent residues associated with organic extraction methods (Bitwell et al., 2023). Furthermore, water extraction preserves the suitability of the extract for applications where human safety is an important consideration, such as food-contact materials. Overall, the MTT assay demonstrated that the Binahong water extract maintained high HaCaT cell viability and exhibited low cytotoxicity, supporting its potential safety for further investigation in food-contact applications.

5.3. Antibacterial Activity Assay

The antibacterial activity of the Binahong (*Anredera cordifolia*) water extract was evaluated using the broth microdilution method to determine its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *Staphylococcus aureus* and *Salmonella typhimurium*. The MIC is defined as the lowest concentration of an antimicrobial agent that completely inhibits visible bacterial growth, whereas the MBC is the lowest concentration capable of killing at least 99.9% of the initial bacterial population (Gregory & Langland, 2025).

The present study demonstrated that the aqueous Binahong extract exhibited limited antibacterial activity against both bacterial species. Against *Staphylococcus aureus*, the Binahong water extract exhibited limited antibacterial activity. No growth inhibition was observed at concentrations of 3.125%, 6.25%, 12.5%, and 100%, whereas slight inhibition was detected at 25% (approximately 14%) and increased to approximately 32% at 50%. Despite this increase, the highest inhibition achieved was insufficient to completely suppress bacterial growth. Therefore, the MIC could not be determined within the tested concentration range. In contrast, the positive control (5 µg/mL ciprofloxacin) exhibited almost complete inhibition of bacterial growth, confirming that the assay conditions were appropriate for detecting antibacterial activity. Against *Salmonella typhimurium,* the Binahong water extract demonstrated no measurable antibacterial activity across all tested concentrations, with growth inhibition remaining close to 0%. The positive control again showed substantial inhibition, confirming the validity of the assay. Overall, these findings indicate that the aqueous Binahong extract exhibited weak antibacterial activity against *Staphylococcus aureus* and no detectable antibacterial activity against *Salmonella typhimurium* under the experimental conditions.

The greater susceptibility of *Staphylococcus aureus* may be explained by differences in bacterial cell envelope structure. As a Gram-positive bacterium, *Staphylococcus aureus* lacks the outer membrane present in Gram-negative bacteria, allowing antimicrobial compounds to interact more readily with the cytoplasmic membrane and cell wall (Martínez de Tejada et al., 2012). In contrast, the outer membrane of *Salmonella typhimurium*, which contains lipopolysaccharides, acts as an additional permeability barrier that limits the penetration of many plant-derived antimicrobial compounds, thereby reducing their antibacterial effectiveness (Zhang, 2022). The limited antibacterial activity observed in this study may also be related to the extraction method. Water preferentially extracts polar compounds, including certain phenolics, flavonoids, and saponins (Ng et al., 2020). However, several antimicrobial constituents reported in Binahong have moderate or low polarity and are extracted more efficiently using organic solvents such as ethanol or methanol (Cheng

et al., 2021). Consequently, the aqueous extract may have contained insufficient concentrations of antimicrobial phytochemicals to completely inhibit bacterial growth.

The MBC assay further supported the MIC findings. After subculturing aliquots from the MIC assay onto agar plates, bacterial colonies were observed at every tested extract concentration for both bacterial species. Since viable bacteria remained after treatment, no concentration achieved the required 99.9% reduction in bacterial viability. Therefore, the MBC could not be determined within the tested concentration range, indicating that the aqueous Binahong extract did not exhibit bactericidal activity under the experimental conditions. Overall, the results indicate that the Binahong water extract possessed only weak antibacterial activity against *Staphylococcus aureus* and negligible activity against *Salmonella typhimurium*. Although partial growth inhibition was observed for *Staphylococcus aureus* at the highest tested concentration, neither the MIC nor the MBC could be established. These findings suggest that higher extract concentrations, extract concentration by evaporation or drying, fractionation of bioactive compounds, or alternative extraction solvents may be required to improve the antibacterial efficacy of Binahong while preserving its favorable safety profile

5.4. Antibiofilm Activity Assay

The antibiofilm activity of the Binahong (*Anredera cordifolia*) water extract was evaluated through two complementary assays: biofilm inhibition and biofilm disruption. The biofilm inhibition assay assessed the ability of the extract to prevent bacterial attachment and biofilm formation, whereas the biofilm disruption assay evaluated its ability to remove or degrade pre-established biofilms. Since biofilm-associated bacteria exhibit greater resistance to antimicrobial agents than planktonic cells, both assays are important for assessing the potential of natural products as antibiofilm agents (Azeem et al., 2025).

The biofilm inhibition assay demonstrated that the Binahong water extract exhibited limited ability to prevent biofilm formation by both *Staphylococcus aureus* and *Salmonella typhimurium*. The aqueous Binahong extract demonstrated limited biofilm inhibitory activity against both *Staphylococcus aureus* and *Salmonella typhimurium* under the tested conditions. Although minor variations in biofilm inhibition were observed among the extract concentrations, inhibition remained low overall. The highest biofilm inhibition was observed at 25% against *Staphylococcus aureus* and at 6.25% against *Salmonella typhimurium*, while negligible or no inhibition was detected at the other concentrations. These findings suggest that the aqueous extract had limited capacity to prevent biofilm formation. Furthermore, *Staphylococcus aureus* appeared to be slightly more susceptible to the extract than *Salmonella typhimurium*, consistent with the trend observed in the antibacterial assay. The thicker but more permeable peptidoglycan layer of Gram-positive bacteria allows antimicrobial compounds to reach their cellular targets more readily, whereas the outer membrane of Gram-negative bacteria serves as an additional barrier that limits the penetration of many plant-derived compounds (Saxena et al., 2023). Nevertheless, the observed inhibition remained insufficient to produce statistically significant antibiofilm activity.

The limited biofilm inhibition observed in this study may also be associated with the aqueous extraction method. Water primarily extracts hydrophilic phytochemicals, whereas certain compounds reported to interfere with bacterial adhesion, quorum sensing, or extracellular polymeric substance (EPS) production may be extracted more efficiently using organic solvents (Royani et al., 2024). As a result, the concentration of antibiofilm-active constituents in the aqueous extract may have been too low to significantly inhibit biofilm formation.

The biofilm disruption assay produced similar findings. Visual examination of the crystal violet-stained microplate revealed persistent purple staining in most treatment wells, indicating that established biofilms remained attached to the well surfaces after treatment. Furthermore, no clear concentration-dependent reduction in staining intensity was observed. These observations suggest

that the Binahong water extract was unable to effectively disrupt mature biofilms under the tested conditions. The resistance of mature biofilms may explain the limited disruption observed. Once a biofilm has formed, bacterial cells become embedded within an extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, extracellular DNA, and other macromolecules (Flemming et al., 2022). This matrix acts as a physical and chemical barrier that restricts the penetration of antimicrobial agents and protects bacterial cells from environmental stress. Consequently, disrupting mature biofilms generally requires higher concentrations of antimicrobial compounds or agents capable of degrading the EPS matrix (Azeem et al., 2025).

The limited antibiofilm activity observed is consistent with the MIC and MBC results obtained in this study. Since the aqueous Binahong extract demonstrated only weak antibacterial activity against planktonic bacteria, its ability to inhibit or disrupt the more resistant biofilm form was also limited. This relationship is expected because bacterial survival within biofilms depends not only on antimicrobial susceptibility but also on the protective properties of the EPS matrix (Singh et al., 2025). Overall, the findings indicate that the Binahong water extract exhibited minimal antibiofilm activity against both *Staphylococcus aureus* and *Salmonella typhimurium*. Neither biofilm inhibition nor biofilm disruption was significantly affected by the tested extract concentrations. Future studies should investigate higher extract concentrations, concentrated or fractionated extracts, alternative extraction solvents, or combinations with established antimicrobial agents to improve the antibiofilm efficacy of Binahong while maintaining its low cytotoxicity.

5.5. Limitations

This study has several limitations that should be considered when interpreting the findings. First, the Binahong (*Anredera cordifolia*) sample used in this study was obtained as a commercially prepared powder purchased from an online supplier. This decision was made after repeated unsuccessful attempts to prepare Binahong powder from fresh leaves due to technical difficulties. To ensure product quality and safety, the purchased powder was certified with a Halal MUI certification

and a Home Industry Food Production Certificate (PIRT) issued by the local health authority. Nevertheless, differences in processing, drying conditions, and storage prior to purchase may have influenced the phytochemical composition of the plant material.

Second, the study employed aqueous extraction as a safer and more environmentally friendly method suitable for food-contact applications. However, water primarily extracts polar compounds and may not efficiently recover less polar phytochemicals that have been reported to possess antibacterial and antibiofilm activities (Wahab & Chua, 2022). Consequently, the biological activity observed in this study may underestimate the full antimicrobial potential of Binahong. Third, the aqueous extract was used directly after filtration without concentration or purification. Therefore, the concentration of bioactive constituents in the final extract was likely lower than that of concentrated or dried extracts, which may have contributed to the limited antibacterial and antibiofilm activities observed.

Fourth, phytochemical screening was not performed. As a result, the presence and relative abundance of specific bioactive compounds responsible for the observed biological effects could not be confirmed. Future studies should include qualitative and quantitative phytochemical analyses to better correlate the chemical composition of the extract with its biological activities. Fifth, the minimum bactericidal concentration (MBC) could not be determined because bacterial growth was observed on all agar plates following subculture from the MIC assay. In addition, colony overlap on several plates made interpretation difficult and may have been caused by movement of the culture plates during incubation, resulting in possible cross-contamination between adjacent samples. Consequently, bactericidal activity could not be accurately evaluated under the experimental conditions.

Sixth, the biofilm disruption assay was evaluated qualitatively through visual observation because the microplate reader required for quantitative absorbance measurement was unavailable due to equipment malfunction. Combined with the limited experimental time, quantitative