ORIGINAL ARTICLE

Antibacterial and Biofilm-inhibiting Activity of the Crude *Psidium guajava* Ethanolic Leaf Extracts against Biofilm-forming *Staphylococcus epidermidis* (ATCC 12228)

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ABSTRACT

Background and Objective. The emergence of antimicrobial resistance (AMR) poses a significant global health threat, with developing countries such as the Philippines facing particularly severe impacts due to resource limitations. The most affected by AMR is Healthcare Acquired Infections (HAIs), including Catheter-Related Bloodstream Infections (CRBSIs). These are commonly associated with biofilm-forming bacteria like *Staphylococcus epidermidis*, which complicates treatment due to antibiotic resistance. The Philippine variety of *Psidium guajava*, a folklorically used medicinal plant, has shown potential antimicrobial properties that could offer a new avenue for combating resistant pathogens.

Methods. This study evaluated the antibacterial and antibiofilm efficacy of crude *Psidium guajava* ethanolic leaf extracts (PGELE) against biofilm-forming *S. epidermidis* (ATCC 12228). PGELE was tested at five concentrations (ranging from 312.5 µg/mL to 10,000 µg/mL) using two-fold serial dilution to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) using agar dilution count method. For the Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC), crude PGELE was tested at 0.25 x MIC, 0.5 x MIC, MIC, 2 x MIC and 4 x MIC.

Results. The MIC for PGELE against *S. epidermidis* was determined to be 2500 µg/mL, and the MBC was 5000 µg/mL, indicating that PGELE exhibits bactericidal activity. In biofilm assays, PGELE demonstrated strong antibiofilm activity at concentrations as low as 625 µg/mL, inhibiting biofilm formation by more than 50%. However, PGELE did not eradicate preformed biofilms, as indicated by the MBEC results at concentrations ranging from 625 µg/mL to 10,000 µg/mL.

Conclusion. *Psidium guajava* ethanolic leaf extracts exhibit antibacterial and antibiofilm activities against *S. epidermidis*, particularly in preventing biofilm formation. These findings suggest that PGELE could be developed as an effective natural antimicrobial agent for use in healthcare settings to prevent CRBSIs and other infections caused by biofilm-forming pathogens. Further research and development are warranted to explore the potential of PGELE for antimicrobial drug development.

Keywords: Psidium guajava, catheter-related infections, Staphylococcus epidermidis, agar dilution

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INTRODUCTION

Over the past decades, antimicrobial resistance (AMR) has emerged as a major public health concern, leading to health crises and the persistence of diseases globally. In low to middle-income countries like the Philippines, patients often face resource constraints, causing them to self-medicate and misuse antibiotics, exacerbating the significant health burden caused by AMR.[1,2] Healthcare facilities, particularly hospitals, are among the most affected by AMR.[2] Healthcare Acquired Infections (HAIs), such as those associated with medical devices like catheters or ventilators, are prevalent. Catheter-Related Bloodstream Infections (CRBSIs) are notably common and significant.[3] Approximately 60% of CRBSI cases are caused by *S. epidermidis* from the patient's skin transferring to the catheter.[4-6] Evidence indicates that nosocomial genotypes of *Staphylococcus epidermidis* can colonize medical devices and cause a substantial proportion of healthcare-associated infections.[7-9] In the Philippines, *S. epidermidis* is the most frequently reported resistant isolate found in blood samples.[10]

Managing *S. epidermidis* is particularly challenging due to its ability to adhere to and form biofilms on indwelling medical devices, rendering it resistant to many antibiotics.[11] Despite the threat *S. epidermidis* poses, its epidemiology and transmission in healthcare settings are less understood, primarily due to the lack of focused studies.[12] Moreover, addressing AMR associated with *S. epidermidis* requires a multifaceted, interdisciplinary approach due to its complex nature.[13] Effective cutaneous antisepsis and preventive strategies that inhibit microorganisms from the skin or catheter hub from adhering to the catheter are among the best measures to prevent CRBSIs.[14] Discovering and developing new antibacterial agents that can be used to prevent CRBSIs is one avenue to help address this threat.

Plants have always been used as a source of medicine, and many parts of the world continue to rely on herbal medicines. Notably, 70-95% of people in underserved communities depend on plants for medicine.[15,16] Traditional healers have long used plants to treat various conditions, and many plants have multiple secondary metabolites (i.e., tannins, terpenoids, alkaloids, phenolics, essential oils, lectins, polyacetylenes and flavonoids) that have been found in vitro to have antimicrobial properties.

Psidium guajava (commonly known as "bayabas") has demonstrated antimicrobial properties, wherein the leaves of *Psidium guajava* are traditionally used as oral or skin antiseptics and have been proven effective against various pathogens. Its activity is high against Gram-positive bacteria and moderate against Gram-negative bacteria. Plant leaves and bark of *Psidium guajava* have shown to possess antimicrobial activity against species of *Bacillus* and *Salmonella*. The leaves have shown greater antimicrobial activity against *Staphylococcus aureus* and *Bacillus cereus* but showed moderate activity against *Escherichia coli* and *Salmonella enteritidis*.[17-24] Bioactive compounds such as terpenoids, tannins, flavonoids, glycosides, saponins and alkaloids found in *Psidium guajava* leaves, all of which targeting multiple bacterial pathways, contribute to its antimicrobial properties.[19,20,25] These compounds can act synergistically, contributing to the overall antibacterial effect of guava leaves. Not only that, the presence of multiple bioactive compounds in the guava leaves may help in the prevention of AMR emergence.[26] Yet despite the numerous medicinal benefits of *Psidium guajava*, there are limited number of products currently available in the Philippine market that capitalizes on the antibacterial use of *Psidium guajava*.

Majority of the antibacterial researches done on *Psidium guajava* was against Gram-positive *Staphylococcus aureus* and it showed great promise, but there are limited studies on the antibiofilm activity of *Psidium guajava*, and more so on its antibacterial effects against *S. epidermidis*. *Psidium guajava* holds potential as a source for a new antibacterial agent that can be used to prevent CRBSIs and combat the growing threat of AMR due to its multiple bioactive compounds can target multiple bacterial pathways simultaneously. Thus, this study aimed to determine the antibacterial activity (using Minimum Inhibitory Concentration and Minimum Bactericidal Concentration) and antibiofilm activity (using Minimum Biofilm Inhibitory Concentration and Minimum Biofilm Eradication Concentration) of the crude *Psidium guajava* ethanolic leaf extracts (PGELE) against biofilm-forming *Staphylococcus epidermidis* (ATCC 12228). The findings of this research could provide foundational data for developing a drug product from *Psidium guajava*, aimed at addressing biofilm-forming *S. epidermidis*.

MATERIALS AND METHODS

Study Design

This study conducted an in vitro evaluation of the antibacterial effect of crude *Psidium guajava* ethanolic leaf extracts (PGELE) against biofilm-forming *Staphylococcus epidermidis* (ATCC 12228). Five different dose levels of crude PGELE were used to determine the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration.

Chemicals and Reagents

The following chemicals and reagents were used in the study: Ethanol Undenatured 100% AR (64-17-5, Chem-Supply, Port Adelaide, Australia), Acetic Acid, Glacial (64-19-7, Chem-Supply, Port Adelaide, Australia), Mueller Hintor Agar (70191, Merck KGaA, Darmstadt, Germany), Mueller Hintor Broth (M391, HiMedia Laboratories LLC, Pennsylvania, USA), Crystal Violet (548-62-9, Techno PharmChem, India), and Vancomycin HCl 500 mg USP Reference Standard (1709007, USP, Maryland, USA, Material mfd in Denmark).

Equipment and Materials

Staphylococcus epidermidis ATCC 12228 was sourced from CBC Link of Manila, Inc., arriving in KWIK-STIK formats (lot number 371-459-4) containing lyophilized bacterial pellets, hydrating fluid ampoules, and inoculating swabs. It was purchased in the month of August 2023, and was stored in a refrigerator at 2-8 ˚C, as specified in its package insert prior to use. Laboratory equipment used were as follows: UV-Vis spectrophotometer (GENESYS 180, Thermo Fisher Scientific Inc., Waltham, MA, USA), Absorbance Microplate Reader (BioTek 800 TS, Agilent, CA, USA), Rotary Evaporator (DLAB RE100-Pro, HCS Scientific & Chemical Pte Ltd, Singapore), Biological Safety Cabinet (BSC-2000IIA2-X, JINAN BIOBASE BIOTECH CO., LTD., Shandong, China), Incubator (IB-21E, Jeio Tech Lab Companion, Korea), Autoclave (ST-65G, Jeio Tech Lab Companion, Korea), IR Moisture Analyzer (FD-720, Kett Electric Laboratory Co. Ltd., Tokyo, Japan), 96 Well, Flat Bottom with Lid Cell Culture Plate (Costar 3596, Corning Incorporated, Wujiang, Jiangsu Province, China) and standard laboratory ware.

Plant Collection

Fresh leaves of *Psidium guajava*, with lot number 231026-LB-BaC was sourced in the month of November 2023 from the National Integrated Research Program on Medicinal Plants-Institute of Herbal Medicine (NIRPROMP-IHM) farm at the Organic University of the Philippines-Los Baños. Authentication of the samples was carried out at the Institute of Biology, College of Science, University of the Philippines Diliman.

Upon collection, the leaves were stored at the Department of Pharmacology and Toxicology, University of the Philippines Manila. Post-harvest, the leaves were thoroughly washed with tap water and air-dried for three days, protected from direct sunlight. The dried leaves were further oven dried for six hours at a temperature of 60˚C, then ground into a fine powder and was subsequently stored in airtight plastic bags until required for extraction. To ensure the absence of any residual microbial contaminants, the powdered leaves were placed in an oven at 50˚C for two days prior to extraction/maceration.

Plant Quality Control

The *Psidium guajava* leaves exhibited a mild and slightly aromatic scent, with faint fruity undertones. The leaves were simple, opposite, and ranged from ovate to elliptic. They featured a smooth texture with slightly wavy margins and a glossy surface. Typically, the leaves measured between 5 and 15 cm in length and 3 to 7 cm in width. The dried leaves showed a moisture content of less than 10%. A sample of the crude PGELE was sent to the Terrestrial Natural Products Laboratory at the Institute of Chemistry, College of Science, UP Diliman, Quezon City for phytochemical analysis. It showed the presence of tannins, terpenoids, flavonoids, cardiac glycosides, and phenolic compounds.

Percent Moisture Analysis

To analyze the moisture content of the *Psidium guajava* leaves using an IR Moisture Analyzer, 5g of the dried, powdered *Psidium guajava* leaf sample was placed into the moisture analyzer tray and was evenly distributed to ensure consistent drying. The analyzer was set to a drying temperature of 105˚C, with automatic stop functionality based on stabilization of moisture loss. Upon completion of the drying process, the analyzer displayed the final moisture content and the result was recorded and was replicated three times. The average of three moisture analysis trial of the leaves must not be more than 12%.

Phytochemical Analysis

A sample of the crude PGELE, amounting to 10 g was sent to the Terrestrial Natural Products Laboratory at the Institute of Chemistry, College of Science, UP Diliman, Quezon City for phytochemical analysis. Phytochemical screening procedures were based on Harborne (1984), Edeoga et al. (2005), and Onwukaeme et al. (2007) with slight modifications.[27-29]

Tannins

The plant extract was dissolved in 1 mL distilled water. The formation of blue-black precipitate or a brownish-green precipitate with the dropwise addition of 15% FeCl3 solution, is indicative of the presence of tannins.

Saponins

The plant extract was dissolved in 5 mL distilled water. The solution was brought to boiling, cooled down, then vigorously shaken for 5 mins. The occurrence of frothing after 30 mins indicates presence of saponins.

Terpenoids

Two (2) mL CHCl3 was added to the dried extract and then layered with concentrated H_2SO_4. The presence of a reddish-brown interface indicates the presence of terpenoids.

Flavonoids

The plant extract was dissolved in 2 mL ethanol and acidified with 3 drops conc. HCl. Formation of pinkish to red-orange solution with the addition of magnesium ribbon indicates the presence of flavonoids.

Cardiac Glycosides (killiani-keller test):

The extract was dissolved with 2 mL glacial acetic acid. To the solution, 1% $FeCl_3$ was added dropwise. One (1) mL concentrated H2SO4, was added without disturbing the solution. The observance of a brown ring is indicative of the presence of cardiac glycosides.

Phenolic Compounds

The plant extract was dissolved in 2 mL distilled water. The formation of blue-black precipitate with dropwise

addition of 1% FeCl3 solution indicates the presence of phenolic compounds.

Alkaloids

The plant extract was dissolved in 3 mL distilled water. Formation of brown-black precipitate with the dropwise addition of Wagner's reagent indicates the presence of alkaloids.

Quality Control of Test Microorganism

In this study, the *S. epidermidis* strain (ATCC 12228) underwent a series of examinations to verify the characteristics and purity of the obtained *S. epidermidis*. It was carried out using the specifications and recommended tests issued by CLSI M100 – Performance Standards for Antimicrobial Susceptibility Testing, 34th Edition, 2024.[30] The tests used were as follows:

Gram Staining

Bacterial cultures were first smeared onto a glass slide and allowed to air dry. The smear was then heat-fixed by passing the slide through a flame. Crystal violet stain was applied to the smear and left for one minute before rinsing with distilled water. Gram's iodine solution was added next for one minute to form a complex with the crystal violet. The slide was then decolorized with alcohol for 15-20 seconds and rinsed immediately with water. Finally, the smear was counterstained with safranin for 30 seconds, rinsed again, and gently blotted dry before microscopic examination. The bacteria were classified as Gram-positive or Gram-negative based on their color retention, with Gram-positive appearing purple and Gram-negative appearing pink.

Catalase Test

A small amount of bacterial culture was placed on a clean glass slide. A few drops of hydrogen peroxide were added directly to the culture. The presence of bubbles was observed as an indicator of catalase activity, which confirmed the production of oxygen gas from the breakdown of hydrogen peroxide. A positive catalase test was indicated by the rapid formation of bubbles, while no bubble formation indicated a negative result.

Coagulase Test

Bacterial colonies were mixed with a few drops of rabbit plasma on a clean slide. The mixture was observed for clumping or coagulation, which indicated the presence of the coagulase enzyme. A positive coagulase test was confirmed by the visible clumping of cells, indicating the bacteria's ability to convert fibrinogen to fibrin. In contrast, a lack of clumping indicated a negative result.

Preparation of Crude Extract

Five hundred (500) grams of powdered *Psidium guajava* leaves were accurately weighed and then soaked in 2L of 100% ethanol for 24 hours at room temperature, ensuring full submersion of the powder, maintaining a solvent-to-sample ratio of 4:1. Following soaking, the mixture was filtered through Whatman filter paper using a vacuum flask where the filter paper was changed three times during the course of the filtration. The filtrate was subjected to rotary evaporation at 50˚C to remove the ethanol. The resultant crude extract was then further dried in a water bath at 50˚C until only an incipient dryness (where the PGELE begins to show the initial signs of drying but has not yet become completely dry) was achieved. To ensure complete drying, the extract was placed in a desiccator until a powdered form was obtained. This crude ethanolic extract, referred to as *Psidium guajava* ethanolic leaf extract (PGELE), was stored at -18˚C for subsequent analysis.

Determination of MIC and MBC

Preparation of Stock Solutions and Working Concentrations of Crude PGELE

Based on previously reported MIC, five concentrations of crude *Psidium guajava* ethanolic leaf extract (PGELE) were prepared through serial two-fold dilutions: 5000 µg/mL, 2500 µg/mL, 1250 µg/mL, 625 µg/mL, and 312.5 µg/mL. In this study, Vancomycin at 2 µg/mL was used as the positive control, while sterile distilled water served as the negative or growth control.[31,32] To ensure the absence of microbial contamination in the crude PGELE, a plant control was employed by incorporating 5000 µg/mL of the extract into the agar medium and incubating it without any inoculum.

Preparation of the Inoculum

The *S. epidermidis* (ATCC 12228) used in the study was supplied in Kwik-Stik formats, which included a lyophilized bacterial pellet, a hydrating fluid reservoir, and a swab. The internal ampoule was activated to release the hydrating fluid, saturating the lyophilized *S. epidermidis* pellet. The Kwik-Stik was gently shaken to ensure thorough mixing and then left at room temperature for 15 to 30 minutes to fully rehydrate the bacterial pellet. Following rehydration, the moistened swab was used to streak a prepared sterile nutrient agar plate to promote the formation of isolated colonies, which were then incubated for 24 hours. After incubation, a single colony was selected and transferred to a prepared Mueller-Hinton broth (MHB), which was subsequently incubated for another 24 hours. This culture served as the stock for preparing fresh, actively growing inoculums for the microbiological assays.

The initial turbidity of the freshly made inoculum was determined using a UV-Vis spectrophotometer set at 600 nm absorbance (OD600), and adjusted to 0.1 OD600 (corresponding to approximately 1×10^8 CFU/mL) using the formula C1V1 = C2V2, where C1 is the starting (initial) OD600, C2 is the target OD600 (which is 0.1), V1 is the unknown volume in µL, and V2 is the final volume (1000 µL). The turbidity of the inoculum was adjusted by adding

more *S. epidermidis* (ATCC 12228) to the MHB to increase turbidity, or further diluting the MHB by adding more MHB to reduce the turbidity.[30]

MIC and MBC Assay

To determine the Minimum Inhibitory Concentration (MIC), varying concentrations of the crude *Psidium guajava* ethanolic leaf extract (PGELE) were incorporated into 20 mL of molten Mueller Hinton agar at approximately 50˚C and poured into Petri dishes to solidify. Following the cooling of the plates, a prepared inoculum was streaked onto each plate and was incubated using IB-21E, Jeio Tech Lab Companion incubator, set at aerobic conditions, with a temperature of 37˚C for 24 hours. The MIC endpoint was identified as the lowest concentration of crude PGELE, which completely inhibited bacterial colony growth on the agar plates. Each concentration was tested in triplicate to ensure the reliability of the results.

After the MIC determination, the plates with the MIC for the crude PGELE, as well as the plates with concentrations higher than the MIC, were swabbed using a sterile single-use inoculating loop and streaked onto fresh Mueller Hinton agar plates. The plates were then incubated for 24 hours at 37 ˚C. The MBC endpoint was recorded as the lowest concentration of the crude PGELE where no colonies of the bacteria grew on the agar plates.

The MIC of vancomycin against *S. epidermidis* was determined prior to use for the assays for verification purposes and a negative control was included to validate the test. Sterility controls were implemented to check for contamination (if any) in the plant samples used in the assay. The tests were performed in triplicate to confirm reproducibility. The procedure used in determining the MIC was based on the CLSI guideline.

Determination of MBIC and MBEC

MBIC and MBEC Assays

The Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC) were evaluated using the broth microdilution method in a 96-well flat-bottom microplate, following the Clinical & Laboratory Standards Institute (CLSI) guidelines using Crystal Violet (CV) assay for quantification.[30]

Preparation of Stock Solutions and Working Concentrations of PGELE

For the MBIC and MBEC determinations, serial two-fold dilutions were used to prepare five concentrations of the crude *Psidium guajava* ethanolic leaf extract (PGELE) at 0.25× MIC, 0.5× MIC, MIC, 2× MIC, and 4× MIC. Vancomycin served as the positive control (10 µg/mL) and Mueller Hinton Broth (MHB) was used as the negative/vehicle control.[33] All treatments were performed in triplicate.

Determination of the MBIC

The Minimum Biofilm Inhibitory Concentration (MBIC) was determined using a BioTek 800 TS Microplate Reader under the following conditions: temperature set at 37˚C and absorbance measurements taken at a wavelength of 600 nm.

The preparation of the bacterial inoculum was similar to the procedure used during the MIC/MBC assay. A 100 µL aliquot of the prepared bacterial inoculum, adjusted to an optical density of 0.1 at 600 nm (OD600), was dispensed into each designated well in a Costar (3596) 96 Well, flat bottom with lid, Cell Culture Plate. To each well, 100 µL of the respective treatment concentrations were added, thoroughly mixed and incubated for 24 hours at 37˚C to allow biofilm formation within the wells. Post-incubation, the bacterial cultures, together with the PGELE extracts were removed by inverting the plate and shaking out the contents. The wells were washed three times with phosphate-buffered saline (PBS, pH 7.4) to remove residual planktonic cells and remaining PGELE extracts.

To stain the biofilms formed in the wells, 200 µL of a 1% (w/v) crystal violet solution was added, and the plate was left at room temperature for 20 minutes. Excess stain was removed by washing the wells three to four times with PBS, and the plate was inverted and left to air dry. Once dried, 125 µL of a 30% acetic acid solution was added to each well to solubilize the crystal violet stain, and the plate was incubated for 10 minutes at room temperature to elute the stain. The absorbance of the de-staining solution in each well was measured at 600 nm using a microplate reader, with a 30% acetic acid solution serving as the blank.

The optical density readings reflected the biofilm mass. The mean absorbance (OD600) for each sample was calculated, and the percent inhibition was determined using the formula:

$$\text{Percent (\%)Inhibition} = \frac{\text{OD negative control} - \text{OD sample}}{\text{OD negative control}} \times 100$$

The crude PGELE concentration demonstrating percent inhibition greater than 50% was classified as having strong antibiofilm (ABF) activity (++). In contrast, those with percent inhibition between 0% and 50% were designated as having moderate ABF activity (+). Percent inhibition less than 0 (-) was considered as indicating no inhibition. The MBIC was identified as the minimum concentration, resulting in 0% inhibition.[22]

A similar procedure described for MBIC determination was done for the MBEC determination, with the respective treatment concentrations added to the designated wells containing the formed biofilms. Then, plates were incubated for an additional 24 hours at 37˚C. The staining procedure used for the MBIC was also employed for MBEC determination. The MBEC was defined as the lowest concentration of the treatment required to achieve 0% inhibition, representing the

minimum concentration necessary to eradicate preformed biofilms effectively.

Ethical Considerations

This research was registered under the University of the Philippines – Manila (UPM) Research Grants Administration Office (RGAO) with the code RGAO-2022-0954 and received approval from the UPM Institutional Biosafety and Biosecurity Committee (IBBC) under protocol number 2023-013. An exemption from ethical review was granted by the UP-Manila Review Ethics Board (UPMREB 2023-0089-EX).

RESULTS

Plant Quality Control

Moisture Analysis

The average of the three moisture analysis trial was 2.79% ± 0.03, which is all below 12% and falls within standards.

Phytochemical Analysis

Phytochemical analysis showed that the *Psidium guajava* ethanolic leaf extracts showed presence of tannins, terpenoids, flavonoids, cardiac glycosides, and phenolic compounds, and absence of saponins and alkaloids.

Percent Yield

From the 500 g of powdered *Psidium guajava* leaves, 24.8 g of powdered PGELE was obtained, thus resulting to a 4.96% yield.

Quality Control of Test Microorganism

Gram Staining

The microorganism exhibited characteristics consistent with a Gram-positive bacterium, displaying a purple hue when observed under the microscope. Moreover, it manifested in clusters, adopting configurations reminiscent of grape-like arrangements (See Appendix A). These characteristics are consistent with *S. epidermidis*.

Catalase Test

When hydrogen peroxide was introduced to a colony, the microorganism generated bubbles, indicative of the presence of catalase enzyme (See Appendix A). This observation aligns with the characteristic property of *S. epidermidis*.

Coagulase Test

No clumping or agglutination was evident within 30 seconds following the introduction of a colony of the microorganism to a slide containing plasma (See Appendix A). This absence of coagulation implies the lack of coagulase activity, consistent with the identification of *S. epidermidis*.

MIC and MBC

Figure 1 shows the results of the MIC of the crude PGELE against *S. epidermidis* while Figure 2 shows the MBC.

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the crude *Psidium guajava* ethanolic leaf extract (PGELE) against *Staphylococcus epidermidis* were 2500 µg/mL and 5000 µg/mL, respectively. The ratio of MBC to MIC showed a ratio of 2, indicating bactericidal activity.[30,34,35] This suggests that the crude PGELE is capable of inhibiting the growth of *S. epidermidis* at a concentration of 2500 µg/mL and achieving a ≥99.9% reduction in the bacterial population of *S. epidermidis* at a concentration of 5000 µg/mL after 24-hours incubation.

Minimum Biofilm Inhibitory Concentration (MBIC)

Table 1 presents the percentage of biofilm inhibition by the crude *Psidium guajava* ethanolic leaf extract (PGELE) and the positive control, vancomycin (See Appendix B for the OD600 reading of the MBIC).

As shown in Table 1, the crude PGELE moderately inhibited biofilm formation even at concentrations four-fold below the MIC (625 µg/mL). This means that the PGELE is effective, requiring only a small concentration to inhibit biofilm formation, which is advantageous for therapeutic applications. It could also indicate that the PGELE acts on specific pathways or mechanisms that are crucial for the initial stages of biofilm formation, such as quorum sensing or surface attachment.[36]

Minimum Biofilm Eradication Concentration (MBEC)

Table 2 displays the percentage of biofilm eradication by the crude and semi-purified PGELE fractions, alongside the positive control, vancomycin (See Appendix C for the OD600 reading of the MBEC).

Results showed that only the positive control, vancomycin, exhibited biofilm eradication capabilities among the treatments. This indicates that the PGELE is not effective at breaking down or removing biofilms that have already matured.

DISCUSSION

Guava leaf extracts have shown to possess antimicrobial properties, and its activity is high against Gram-positive bacteria, such as *S. aureus*, and moderate against Gram-negative bacteria. Plant leaves and bark of *Psidium guajava* have shown to possess antimicrobial activity against species of *Bacillus* and *Salmonella*, but the leaves have shown greater antimicrobial activity against *Staphylococcus aureus* and *Bacillus cereus*. It showed moderate activity against *Escherichia coli* and *Salmonella enteritidis*.[17-21] Both the methanol and ethanolic extracts of guava leaves showed inhibitory properties against Gram-positive bacteria (Staphylococcus

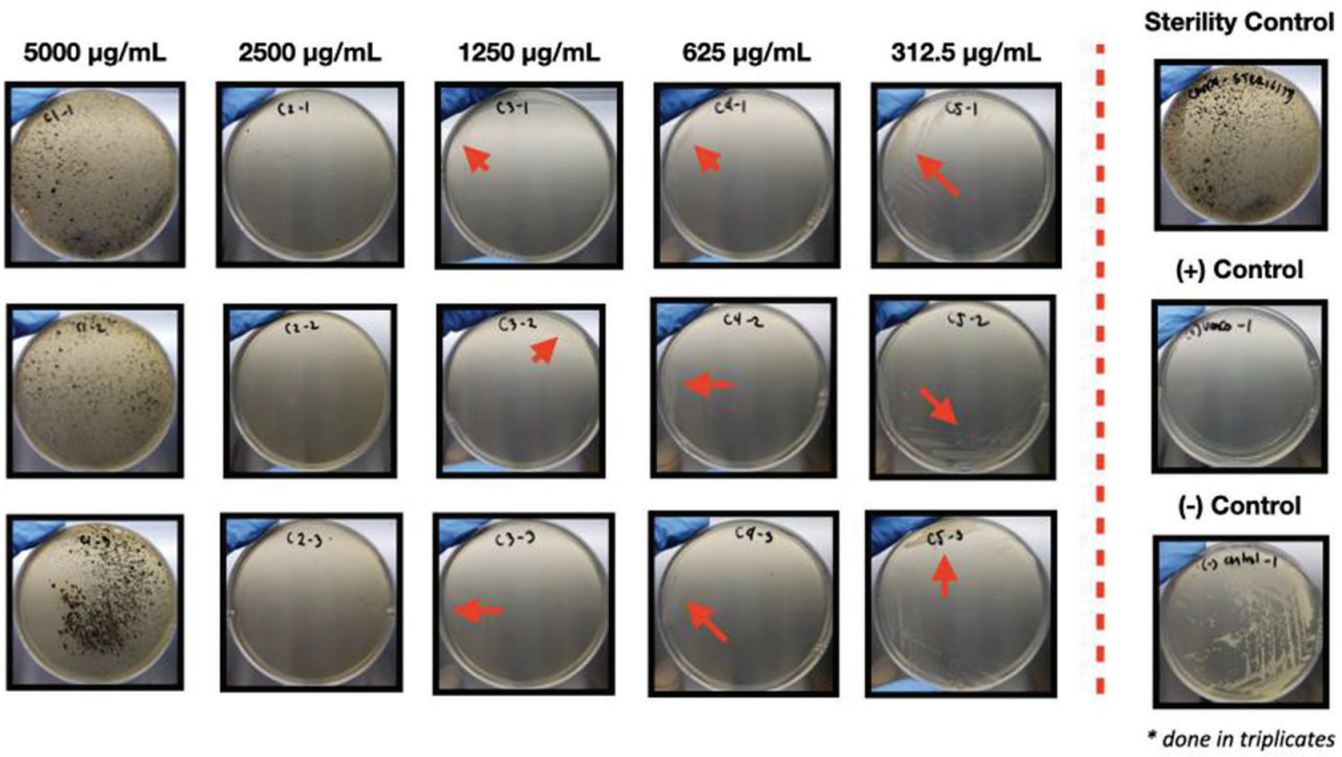

Figure 1. MIC of crude PGELE against *S. epidermidis*.

aureus and Bacillus cereus) but not on Gram-negative bacteria (Escherichia coli and Salmonella enteritidis).[18]

The flavonoids (i.e., quercetin and catechin), polyphenols (i.e., tannins), and terpenoids (i.e., caryophyllene) in guava leaves are compounds that can disrupt bacterial cell walls and membranes, leading to cell lysis and death.[37] They may inhibit bacterial enzymes that are critical for cell wall synthesis and energy production, and may also inhibit bacterial replication and protein synthesis by binding to nucleic acids or interfering with enzyme function.[38] Guava leaves contain antioxidants that can generate reactive oxygen species (ROS) when in contact with bacteria. The ROS can damage bacterial DNA, proteins, and lipids, leading to cell death.[39] The diverse bioactive compounds in guava leaves can target multiple bacterial pathways simultaneously. This

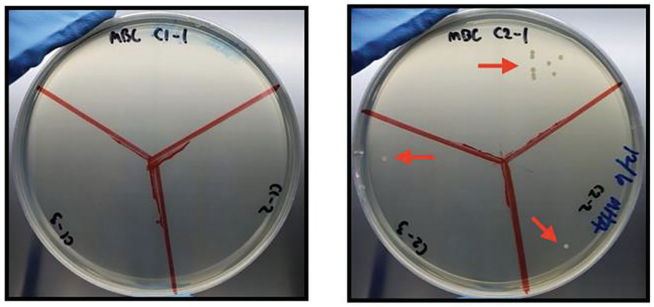

Figure 2. MBC of crude PGELE against *S. epidermidis*.

Table 1. Percent Biofilm Inhibition of the Crude PGELE against *S. epidermidis* after 24-hour Exposure

Concentration	Percent Biofilm Inhibition		
	Crude PGELE	(-) Control	(+) Control
0.25 x MIC	35.03% ± 11.5 (+)	0%	39.31% ± 11.4 (+)
0.5 x MIC	45.20% ± 8.8 (+)		
MIC	34.23% ± 4.4 (+)		
2 x MIC	36.97% ± 4.7 (+)		
4 x MIC	34.00% ± 2.8 (+)		

Note: MIC of crude PGELE is 2500 µg/mL. MIC for vancomycin in inhibiting biofilm formation is 10 µg/mL.

Table 2. Percent Biofilm Eradication of the Crude and Semi-purified PGELE Fractions against *S. epidermidis* after 24-hour Exposure

Concentration	Percent Biofilm Eradication		
	Crude PGELE	(-) Control	(+) Control
0.25 x MIC	-42.80% ± 7.5 (-)	0%	13.75% ± 6.3 (+)
0.5 x MIC	-17.89% ± 9.1 (-)		
MIC	-35.41% ± 7.6 (-)		
2 x MIC	-21.27% ± 13.7 (-)		
4 x MIC	-54.35% ± 14.1 (-)		

Note: MIC of PGELE is 2500 µg/mL. MIC for vancomycin in eradicating biofilm formation is 10 µg/mL.

multifaceted approach makes it more difficult for bacteria to develop resistance because they would need to undergo multiple mutations or adaptations at the same time to overcome the various mechanisms. These compounds can act synergistically, contributing to the overall antibacterial effect of guava leaves.[26] The specific mechanisms may vary depending on the type of bacteria and the concentration of active compounds in the guava leaf extract.

The antibacterial activity of *Psidium guajava* is confirmed in this with MIC of the crude *Psidium guajava* ethanolic leaf extract (PGELE) at 2500 µg/mL. This is within the reported MIC values for *Psidium guajava* against *Staphylococcus* species ranging from 625 µg/mL to 7500 µg/mL.[23,24,35]

The determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for antimicrobial agents holds theoretical significance and has proven to be a valuable tool in research settings.[26] However, this process is strongly influenced by numerous biological, methodological, and technical variables, which can introduce complexities and potential biases into the obtained results, thereby complicating their interpretation. Consequently, evaluating the clinical applicability of MBC determination has posed considerable challenges. Relying solely on MIC and MBC assessments may insufficiently characterize the antibacterial effectiveness of an antimicrobial agent and its potential relevance in clinical contexts.

To determine the bactericidal potential of the crude PGELE relative to its inhibitory effects, the ratio between the Minimum Bactericidal Concentration (MBC) and Minimum Inhibitory Concentration (MIC) of PGELE against *S. epidermidis* was calculated. It is postulated that an MBC/MIC ratio ≤4 signifies bactericidal activity, whereas an MBC/MIC ratio >4 indicates bacteriostatic effects.[30,34] Based on the MBC/MIC ratio determined in this study, the crude PGELE can be interpreted to exhibit bactericidal properties. However, based on the findings of other researches, it was identified that *Psidium guajava* leaves possess bacteriostatic properties against most Gram-positive bacteria. The bacteriostatic property of *Psidium guajava* is consistent with other published literatures stating that *Psidium guajava* leaves possess bacteriostatic properties based on time-kill assays.[18,23,24] An antimicrobial might have a low MIC/MBC ratio indicating bactericidal activity, but the time-kill assay could reveal that it kills bacteria very slowly. If the 3-log reduction (99.9% kill) criterion is not met within 24 hours, TKA will categorize it as bacteriostatic despite MIC/MBC results suggesting it is bactericidal. While MIC/MBC and time-kill assays are generally complementary, they can occasionally yield different results due to their differing methodologies and what they measure. MIC/MBC tests provide essential information about inhibitory and bactericidal concentrations, but TKA provides a more detailed picture of the antimicrobial's activity over time, including the rate of kill and potential for regrowth. Thus, it is ideal that future researches on the Philippine variety of *Psidium guajava* exploring its antimicrobial property should perform time-kill assays as well.

The crude PGELE inhibited the formation of *S. epidermidis* biofilms even at the lowest concentrations tested (625 µg/mL). Similar to other reported studies, *Psidium guajava* leaves have been shown to have antibiofilm and anti-quorum sensing activity even at sub-MIC levels.[36] This finding shows the potential of crude PGELE for various clinical applications. Chronic wounds, such as diabetic ulcers and pressure sores, are frequently complicated by biofilm formation from local skin bacteria like *S. epidermidis*, which impedes healing and increases the risk of secondary infections.[40] The topical application of crude PGELE can help manage chronic wounds by preventing biofilm formation and promoting healing. Additionally, using these extracts for central catheter site hygiene may reduce the risk of catheter-related bloodstream infections (CRBSIs) by preventing microbial colonization and biofilm formation at the catheter site.

However, crude PGELE does not exhibit biofilm-eradicating properties. This may be limited only to the Philippine variety of *Psidium guajava* since there are other reports that *Psidium guajava* in other countries (i.e., Brazil, Portugal, Turkey) possess biofilm eradicating properties.[36,41,42] Several factors might explain why PGELE inhibits biofilm formation but does not eradicate established biofilms. One reason could be that PGELE targets specific pathways involved in the initial stages of biofilm development, such as microbial adhesion and biofilm matrix production, thereby preventing biofilm formation without affecting mature biofilms with distinct metabolic and physiological characteristics.[43] Another reason may be the limited ability of PGELE to penetrate the dense extracellular matrix of mature biofilms, making it challenging to reach and effectively target the microbial cells within.[41] Future research should focus on elucidating the specific mechanisms of PGELE's antibiofilm activity.

Our study showed that the Philippine variety of *Psidium guajava* possesses antimicrobial activity against *S. epidermidis* (ATCC 12228) and inhibits biofilm formation. This plant offers a promising, affordable, and culturally accepted source of antimicrobial agents in the Philippines, where traditional herbal remedies are widely used. Additionally, guava extract could be developed into an effective topical antiseptic to prevent CRBSIs by inhibiting microbial adhesion to catheters, which is crucial in reducing the risk of catheter-related bloodstream infections.[14]

In summary, future researches exploring the antimicrobial and antibiofilm activity of the Philippine variety of *Psidium guajava* should also use Time-Kill Assays to further expand on the antimicrobial property of PGELE, and perform anti-quorum sensing assay for the antibiofilm activity. Future endeavors should also explore on compounding topical creams or ointments out of *Psidium guajava* leaves and assess them for their efficacy and stability. An oral preparation (i.e., syrups or

solutions) of *Psidium guajava* as an oral antibiotic may not be advisable since its MIC against *S. epidermidis* is 2500 µg/mL, which would mean that large amounts of *Psidium guajava* leaves must be ingested to elicit a desired antimicrobial effect. Future researches should also consider the possible synergistic effect of combining *Psidium guajava* extracts with established antimicrobials to prevent development of microbial resistance or the possibility of reducing the dose of antimicrobials to prevent toxicity.

CONCLUSION

The study revealed that the crude PGELE effectively inhibits *Staphylococcus epidermidis* (ATCC 12228) with an MIC of 2500 µg/mL and an MBC of 5000 µg/mL, showing a bactericidal nature given the MBC/MIC ratio of 2. Notably, PGELE prevented biofilm formation at a concentration as low as 625 µg/mL, indicating its potential as a preventive agent against biofilm-related infections. However, it did not demonstrate the ability to eradicate established biofilms. This suggests that while PGELE is promising for preventing initial biofilm formation and bacterial growth, further research is needed to enhance its biofilm-eradicating potential.

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Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

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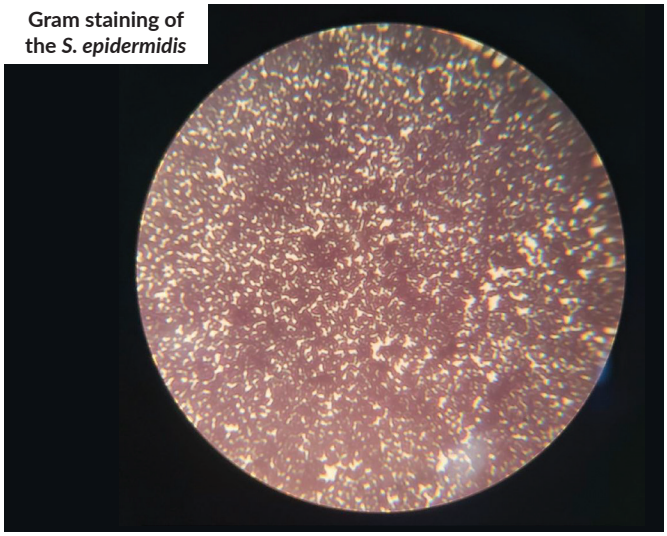
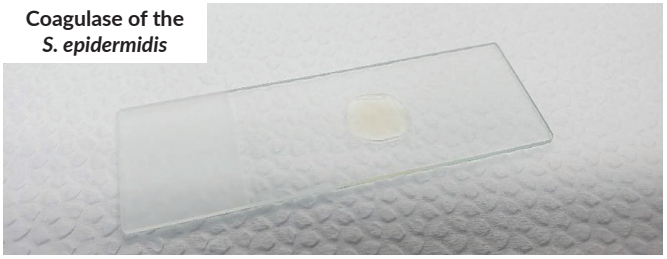
APPENDICES





Appendix A. Quality Control of Test Microorganism.

Appendix B. MBIC Results

OD600 Readings

	Concentration	R1	R2	R3	Mean
Crude	625 ug/mL	0.439	0.306	0.392	0.379
	1250 ug/mL	0.333	0.363	0.263	0.319667
	2500 ug/mL	0.401	0.396	0.354	0.383667
	5000 ug/mL	0.396	0.366	0.341	0.367667
	10000 ug/mL	0.403	0.371	0.381	0.385
Vancomycin	10 ug/mL	0.301	0.429	0.332	0.354
Negative Control (30% Acetic acid)	0 ug/mL	0.525	0.644	0.581	0.583333

MBIC – % Inhibition

	Concentration	R1	R2	R3	Mean	SD	Antibiofilm Activity (Inhibition)
Crude	625 ug/mL	24.74286	47.54286	32.8	35.02857	11.56222	Moderate
	1250 ug/mL	42.91429	37.77143	54.91429	45.2	8.797031	Moderate
	2500 ug/mL	31.25714	32.11429	39.31429	34.22857	4.42516	Moderate
	5000 ug/mL	32.11429	37.25714	41.54286	36.97143	4.720775	Moderate
	10000 ug/mL	30.91429	36.4	34.68571	34	2.806407	Moderate
Vancomycin	10 ug/mL	48.4	26.45714	43.08571	39.31429	11.44727	Moderate

Appendix C. MBEC Results

OD600 Readings

	Concentration	R1	R2	R3	Mean
Crude	625 ug/mL	0.389	0.359	0.353	0.367
	1250 ug/mL	0.31	0.277	0.322	0.303
	2500 ug/mL	0.333	0.37	0.341	0.348
	5000 ug/mL	0.32	0.273	0.342	0.311666667
	10000 ug/mL	0.435	0.392	0.363	0.396666667
Vancomycin	10 ug/mL	0.215	0.21	0.24	0.221666667
Negative Control (30% Acetic acid)	0 ug/mL	0.288	0.24	0.243	0.257

MBEC – % Inhibition

	Concentration	R1	R2	R3	Mean	SD	Antibiofilm Activity (Eradication)
Crude	625 ug/mL	-51.36	-39.68	-37.35	-42.80	7.504786584	None
	1250 ug/mL	-20.62	-7.78	-25.29	-17.89	9.067066302	None
	2500 ug/mL	-29.57	-43.96	-32.68	-35.40	7.575067056	None
	5000 ug/mL	-24.51	-6.22	-33.07	-21.27	13.71468843	None
	10000 ug/mL	-69.26	-52.52	-41.24	-54.34	14.09577493	None
Vancomycin	10 ug/mL	16.34	18.28	6.61	13.74	6.25398882	Moderate