Evaluation of *Psidium guajava* (Guava) Leaf Extract Against Gram-Negative Bacteria in Planktonic and Biofilm Lifestyles

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Authors’ contributions

Authors CJOC, KJAC, and PMBM contributed equally to this work. Authors CJOC and KJAC designed the study, performed the experiment, and analyzed the data. Author PMBM designed the study, performed the experiment, analyzed the data, and wrote the manuscript. Authors TMCC and VAC performed the experiment and wrote the manuscript. Authors KMDGC and DAMDC designed the study and performed the experiment. Authors JSC, JEEC, AITC, KAC, BMPC, MKAC, FRAC, KMEC, and VACC performed the experiment. All authors read and approved the final manuscript.

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ABSTRACT

**Aims:** To test and compare relative efficiencies of Guava Leaf Ethanolic Extract (GLEEx) and antibiotic standards against two pathogenic gram-negative bacteria in planktonic and biofilm lifestyles.  
**Study Design:** *In vitro* antibacterial study.  
**Place and Duration of Study:** Department of Biochemistry & Molecular Biology in the University of the Philippines Manila, between January 2014 and April 2014.  
**Methodology:** The susceptibility of planktonic and biofilm forms of *Escherichia coli* and
Pseudomonas aeruginosa to GLEEx and to appropriate antibiotic standards were evaluated using the Kirby-Bauer disc diffusion assay (KB) and the BioTimer Assay (BTA), respectively.

**Results:** In KB, GLEEx exhibited comparable antibacterial strengths against both bacteria ($P > .05$), but was significantly less than the antibiotic standards ($P < .001$). However in BTA, there is no significant difference between the antibacterial properties of GLEEx and the antibiotic standards ($P > .05$).

**Conclusion:** The relative antibacterial potencies of GLEEx and standard antibiotics vary depending on bacterial lifestyle, thus suggesting the need to use BTA alongside KB when assessing the antibacterial properties of natural products.

**Keywords:** Guava; biofilm; planktonic; BioTimer assay; Kirby-Bauer assay.

1. **INTRODUCTION**

Psidium guajava Linn (guava) is a plant with antibacterial properties currently being explored by researchers from different parts of the world. The leaves were shown to be effective against *Bacillus subtilis* [1], *Escherichia coli* [1,2,3] *Pseudomonas aeruginosa* [1,3] multi-drug resistant *Vibrio cholera* [4] and *Staphylococcus aureus* [5]. It was shown to inhibit the growth of diarrhea-causing enteric bacteria [6]. Other guava parts, such as bark and stem, was shown to exhibit antibacterial properties [4,7].

Clinically, bacteria usually are difficult to eradicate because they predominantly live in a biofilm lifestyle [8,9]. Biofilms are formed by a wide variety of bacteria, most notably of those with medical implications and pathogenicity to humans, such as gram-negative *Escherichia coli*, and *Pseudomonas aeruginosa* and gram-positive bacteria *Staphylococcus aureus*, as well as *Bacillus subtilis* – a species commonly used to test antibiotic susceptibility [10]. These biofilms are formed when unicellular organisms of single or multiple species come together to form a community that is attached to a solid surface and encased in an exopolysaccharide matrix [9]. This protective configuration is formed as a response to several factors as observed in human infections [11]. In line with this, the biofilm lifestyle has been comparatively more common than the planktonic one in observation of bacterial behavior, particularly within living systems. This is attributed to the fact that microorganisms organized in biofilm exhibit higher levels of antibiotic resistance than in planktonic form. A great part of therapeutic regimens based on susceptibility of planktonic forms fail to eradicate most biofilm infections [11]. In fact, when cells exist in a biofilm, they can become $10–1000$ times more resistant to the effects of antimicrobial agents [12].

Research done to assess the antibacterial properties of guava typically used agar diffusion assays (e.g., Kirby-Bauer). The appeal of the methods lies in its simplicity, lack of expensive equipment, and reproducibility [13,14]. However, the bacteria tested in these assays are in planktonic forms, and not in biofilm, which is the more clinically relevant bacterial lifestyle. To address this issue, BioTimer Assay is one of the methods developed to provide a means to assess the efficiency of substances against bacteria in biofilm [15]. BTA uses pH-sensitive pigments that change color as an indicator for the presence of metabolizing bacteria.

We have sought to address the glaring disconnect between the way antibacterial agents have been traditionally evaluated, and the prevalent nature of bacteria present in infections, by testing GLEEx using BTA alongside KB. This would determine if testing with KB and BTA...
would result in the same valuation of a potential antibiotic agent and, consequently, verifying whether or not testing with KB alone is sufficient. We also limited our investigation to gram-negative bacteria to resolve the contradicting findings by Biswas et al. [16] and previous research [1,2,3]. Whereas past literature all show that guava leaf ethanolic extract is effective against gram-negative bacteria, the experimental results of Biswas, et al. indicated otherwise. We showed that GLEEx possessed antibacterial properties against gram-negative bacteria, confirming previous research. Moreover, we demonstrated that GLEEx in KB is not as effective as the antibiotic standards. Lastly, we discovered that GLEEx and the antibiotic standards performed similarly in BTA. The different relative strengths of GLEEx and the antibiotic standards against bacteria in planktonic and biofilm forms suggest that KB and BTA should be used together when evaluating the antibacterial properties of natural products.

2. MATERIALS AND METHODS

2.1 Sample Preparation and Extraction

2.1.1 Guava leaf collection and extraction

Guava leaves were obtained from San Pedro, Laguna, Philippines (14 degrees 22’19” N, 121 degrees 3’27” E). The leaves were washed with tap water, air-dried indoors for 3 days and blended. Fifty grams were placed in flasks with 100mL 95% ethanol, left in a mechanical shaker for 8 hours [17], and allowed to stand for 16 hours before filtering. The resulting filtrate was concentrated using a rotary evaporator (BÜCHI Rotavapor R-200) at 40°C, 120 rpm, air dried under a hood for 3 hours and further dried in a Pyrex®3080 desiccator (Corning, Tewksbury, MA) overnight to obtain the crude extract. One gram of the crude extract was resuspended in 10mL 95% ethanol to obtain the 1000 µg in 10 µL concentration and a serial dilution was performed to obtain the 500 µg, 250 µg and 100 µg in 10 µL concentrations. The resulting ethanolic extracts were kept in airtight containers and were refrigerated at 4°C until use.

2.1.2 Test organisms

*Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC27853) were obtained from the College of Public Health, University of the Philippines Manila, Philippines. The cultures were checked for purity and were maintained on nutrient agar slants.

2.2 Kirby-Bauer Disc Diffusion Assay (KB)

Twenty four-hour old cultures of *E. coli* and *P. aeruginosa* were each inoculated into nutrient broth (Merck, Whitehouse Station, NJ), adjusted to the turbidity of a 0.5 McFarland Standard and spread into nutrient agar plates (HiMedia Laboratories, Marg, Mumbai) [13]. A puncher was used to form 6-mm diameter discs from Whatman grade 3 filter papers (Whatman International Ltd., Maidstone, UK). These discs were autoclaved before they were infused with different concentrations of the guava extract (1000 µg, 500 µg, 250 µg, and 100 µg in 10 µL) and placed on each quadrant of the *E. coli* and *P. aeruginosa* plates. Appropriate antibiotic BD BBL™ Sensi-discs™ (Franklin Lakes, NJ) and ethanol discs were used as positive and negative controls, respectively. 30 µg chloramphenicol discs [1] and 95% ethanol discs were placed on *E. coli* plates, while 30 µg ceftazidime discs and ethanol discs
were placed on *P. aeruginosa* plates \[18\]. The plates were incubated at 37°C for 24 hours and the zones of inhibition (ZOIs) were measured after the incubation period.

### 2.3 BioTimer Assay (BTA)

#### 2.3.1 Media preparation

The BioTimer-Phenol Red (BT-PR) medium was prepared for the correlation line generation of *E. coli* while the BioTimer-Rezasurin (BT-RZ) medium was prepared for correlation line generation the BTA proper of *P. aeruginosa*. For BT-PR, 21 g Mueller Hinton (MH) broth (Merck, Whitehouse Station, NJ), 10 g glucose, and 25 mg phenol (Sigma, St. Louis, MO), were dissolved in 1000 mL distilled water.\[15\] The solution was sterilized at 121°C for 15 minutes, its pH was checked and adjusted to 7.2 ± 0.1 after sterilization.\[15\] For BT-RZ, 3.7 g brain-heart infusion (Merck, Whitehouse Station, NJ) was dissolved in 940 mL distilled water and sterilized at 121°C for 15 minutes. It was combined with 50 mL 10% sterile glucose solution, and 10 mL 0.1% sterile resazurin (Sigma, St. Louis, MO) solution only on the day of its use. The pH of the media was checked and adjusted to 7.0 ± 0.1 for BT-RZ. The final media appeared clear and red-orange for BT-PR, and clear and blue for BT-RZ.\[15\]

#### 2.3.2 Establishing the correlation line

In determining the correlation line for *E. coli*, 0.2 mL of MH-overnight broth culture was mixed with 1.8 mL of BT-PR medium. Serial two fold dilutions in 1mL of BT-PR medium were performed in 24 well plates and simultaneously counted using the colony forming unit (CFU) method \[15\]. Ten microliters were obtained from each well, plated in nutrient agar and the number of colony forming CFUs after a 24-hr incubation period at 37°C was determined. Each well was continuously monitored until color change from red-orange to yellow. The amount of time for color switch to occur was recorded and plotted against the log, base 10, of the number of CFUs of the corresponding well \[15\]. The same procedure was performed for *P. aeruginosa* using BT-RZ medium instead of BT-PR, where the color change is from blue to pink. Three trials were done in determining the correlation lines for the two bacteria.

#### 2.3.3 Biofilm formation and detection

To allow biofilm formation, each of the two bacteria was incubated overnight at 37°C in a 15 mL brain heart infusion broth without agitation in the presence of 35 sterile glass beads (5 mm diameter). After incubation, the colonized glass beads were washed thrice in sterile saline (0.9% NaCl) and divided into two groups: one group of fifteen beads for the crystal violet test to measure biofilm and the other twenty for BTA. Fifteen colonized beads and fifteen sterile beads were used in the crystal violet test. These were placed on a petri dish and soaked in 1% (w/v) crystal violet solution for 20 minutes at room temperature. The crystal violet solution was drained and sterile water was poured into the petri dish until the beads were submerged (approximately 20 mL). The dish was gently swirled for 30 seconds and drained. These steps were repeated as many times as necessary until the crystal violet is no longer visible on the water (around four to five times). The beads were then air-dried. Biofilm was quantified by eluting crystal violet with a mixture of ethanol and acetone (80:20 v/v) and was determined using the absorbance of the eluted dye at 570 nm \[15\]. Those used in BTA were placed in 24 well plates containing the appropriate medium (BT-PR or BT-RZ).
2.3.4 Measuring antibacterial activity of guava against bacteria in biofilm

1.0 mL of BioTimer medium (BT-PR for *E. coli*; BT-RZ for *P. aeruginosa*) was added to all wells of the 24 well plates. 10 µL of each of the four different GLEEx concentrations (same as those used in KB) was added to wells, in three replicates for every concentration. Each well with GLEEx was inoculated with one colonized glass bead. A well without an inoculated bead was prepared for each GLEEx concentration set-up to serve a reference point for color changes. For the controls, set-ups were created as previously described for the GLEEx set-ups, but instead of GLEEx, 10 µL of 95% ethanol was used in the negative control and antibiotic discs were used in the positive control. The standard antibiotics used were chloramphenicol for *E. coli* and ceftazidime for *P. aeruginosa*. Two wells contained only BioTimer medium and colonized beads, which served to provide the initial bacterial population count for BTA, to which population counts determined in experimental and control wells were compared.

2.4 Analysis of Data

The procedures were repeated thrice to obtain mean values and standard deviations. Correlation lines were obtained by linear regression analysis, and linear correlation coefficients were calculated using Microsoft Excel (Redmond, WA). The mean absorbance values of eluted crystal violet from sterile and the different colonized beads were analyzed using one-way ANOVA (95% confidence interval, α = .05). The antibacterial activity of the guava extract concentrations in the Kirby-Bauer disc diffusion assay and in the BioTimer Assay were also analyzed using one-way ANOVA (95% confidence interval, α = .05), with post hoc analysis using Tukey’s range test. Results between the Kirby-Bauer disc diffusion assay and BioTimer Assay were compared using t-test (95% confidence interval, α = .05).

3. RESULTS AND DISCUSSION

3.1 GLEEx Inhibits *E. coli* and *P. aeruginosa* Growth in Planktonic Form

All GLEEx concentrations used formed ZOIs in *E. coli* and *P. aeruginosa* (Fig. 1). In *E. coli*, the mean diameters of ZOIs increase as GLEEx concentration increases, with a range from 8.00 millimeters to 11.67 millimeters. However, the only statistically significant change, revealed by the post hoc test, was observed between 100 µg and 1000 µg in 10 µL of GLEEx (*P* = .03). A similar trend was observed in *P. aeruginosa*, wherein the ZOIs range from 7.33 millimeters to 11.33 millimeters, and 100 µg (*P* = .011) and 250 µg (*P* = .02) were both significantly different from 1000 µg in 10 µL. Comparing GLEEx against *E. coli* and *P. aeruginosa* showed that there was no significant difference between them across all four concentrations of GLEEx (*P* > .05).

All ZOIs formed by GLEEx in *E. coli* and *P. aeruginosa* were significantly greater than the negative control (*P* < .001), which had no detectable ZOI. In contrast, the ZOIs formed by GLEEx were significantly less than the ZOIs formed by the antibiotic standards (*P* < .001).

3.2 GLEEx and Antibiotic Standards Exhibit Comparable Antibacterial Activity against Gram-negative Bacteria Biofilm

BTA counts bacteria based on bacterial metabolism of the surrounding medium, which causes color change of the pH indicator in the medium (red-orange to yellow for BT-PR; blue
to pink for BT-RZ). The amount of time required for color change to occur is correlated to the initial bacterial concentration. Hence, given an amount of time for color switch to occur, the initial population of bacteria (at time 0) can be determined based on the specific correlation line for the bacteria. Fig. 2 illustrates the correlation lines that were generated for *E. coli* and *P. aeruginosa* and used in interpreting the BTA results.

![Image](image_url)

**Fig. 1.** Guava leaf ethanolic extracts (GLEEx) exhibits antibacterial activity against planktonic forms but significantly less than the antibiotic standards

The mean diameters of the zones of inhibition from three replicates show no significant difference among adjacent GLEEx concentrations. Ethanol (negative control) did not produce any zones of inhibition.

The percentages of CFUs, i.e., viable bacteria, listed in Table 1 were subjected to statistical analysis. In *E. coli* and *P. aeruginosa*, the post hoc test reveals that all GLEEx concentrations caused a decrease in viable bacterial population that was significantly greater from the negative control (*P* < .001). However, in *E. coli*, there was no significant difference among the four GLEEx concentrations (*P* > .05). Additionally, no significant difference was observed between all GLEEx concentrations and chloramphenicol (*P* > .05). In *P. aeruginosa*, there was also no significant difference between the four concentrations of GLEEx (*P* > .05), even though an apparent decreasing trend was observed as GLEEx concentration increases. Furthermore, there was also no difference between all GLEEx concentrations and ceftazidime performance (*P* > .05).

Previous studies on BTA typically include minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC). Determination of MBIC and MBEC require wells that did not change color after a certain length of incubation time, typically 16 to 24 hours. In our hands, all of the wells changed color at the 24-hour time point, thus MBIC and MBEC cannot be determined. However, we were still able to directly compare the relative efficiencies of GLEEx and the antibiotic standards by noting the actual times of color change for each treatment. The time it took for color to change translated to the number of surviving viable bacteria by referring to the previously determined correlation line (Fig. 2).
Fig. 2. The correlation lines of both *E. coli* and *P. aeruginosa* both cap at approximately 300 minutes

The well plates were continuously monitored to determine the precise time of color change. BioTimer Assay correlation lines for each bacterium were determined through linear regression analysis by plotting the time of color change against the logarithm of CFU (viable bacteria) in the well.

Table 1. Surviving viable bacteria in biofilm after treatment

| Test Organism | Viable Bacteria (%) | Guava Extract Concentration (µg/10 µL) | Antibiotic (µg) | 95% Ethanol* (µL) |
|---------------|---------------------|----------------------------------------|----------------|------------------|
|               |                     | 100                                    | 250            | 500              | 1000 | 30  | 10  |
| *E. coli*     |                     | 3.84±1.35                              | 1.08±0.48      | 2.19±1.85        | 0.05±0.00 | 0.00±0.00 | 51.19±11.67 |
| *P. aeruginosa*| 0.44±0.30            | 0.07±0.09                              | 0.03±0.03      | 2.08±0.79        | 2.08±0.79 | 33.90±5.91 |

The BTA set-ups for *E. coli* and *P. aeruginosa* were observed continuously to note the precise time when color change occurred in each well. The recorded times for color change for both BT-PR and BT-RZ, which transpired in less than 24 hours, were translated to CFUs and normalized against the initial CFU to calculate the percentage of viable bacteria.

(*the percentages of viable bacteria in ethanol are the only significantly different (i.e., higher) values in the data set)

3.3 GLEEx is More Effective Against Gram-negative Bacteria in Biofilm than in Planktonic Forms

As determined using KB, the ZOIs produced by the highest GLEEx concentration were significantly lower than those of the antibiotic standards against planktonic *E. coli* and
P. aeruginosa (Fig. 1). This suggests that GLEEEx is less effective than the antibiotics used in the study when treating gram-negative planktonic bacteria. In contrast, the BTA revealed that the reduction in viable bacteria in all four GLEEEx concentrations were not significantly different from the reduction of viable bacteria in the antibiotics used (Table 1). The apparent parity between GLEEEx and the antibiotic standards indicates that they have equivalent efficacies against gram-negative bacteria in biofilm. A comparison of the antibacterial performances of GLEEEx relative to the antibiotic standards in both planktonic and biofilm bacteria is illustrated in Fig. 3.

Fig. 3. Gram-negative bacteria in biofilm are equally susceptible to both GLEEEx and the antibiotic standards

Relative antibacterial activity of decreasing concentrations of ethanolic guava extract to antibiotic standards against E. coli (A) and P. aeruginosa (B) for the Kirby-Bauer (KB) and the BioTimer Assay (BTA). All values were normalized against the antibiotic standard (chloramphenicol for E. coli and ceftazidime for P. aeruginosa)

The remarkable difference in KB and the unexpected similarities in BTA that have been observed between GLEEEx and the antibiotics may be due to a variety of reasons. In KB, where the bacteria are in planktonic form, we propose two possibilities. It may be that the active component found in GLEEEx is innately less potent than the antibiotic standards used. However, it can also be argued that the active component in GLEEEx occurs in a much lower concentration than the antibiotics, thus limiting its antibiotic activity. In BTA, where the bacteria are in biofilm, we offer two scenarios. First, the active component in GLEEEx may diffuse more rapidly in biofilm, having an increased interaction with the bacteria than the antibiotics. This could explain the similarity of the results between the two, even though the active component in GLEEEx may be innately weaker or less concentrated than the antibiotics. Secondly, further biofilm in GLEEEx treatment is prevented from forming, thus enhancing the interaction of its active component with the bacteria. Previous studies have revealed that guava leaves contain quercetin, which inhibits biofilm formation [19,20,21]. In the antibiotic treatment, more tolerant bacteria may be continuously producing biofilm, which may further impede the antibiotic activity.

The evaluation of GLEEEx as an antibacterial agent using KB and BTA, uncovers the limitations of the former to accurately assess the efficacy of an extract. This highlights the need for a secondary form of assessment, such as BTA, that will complement KB, which is
typically the only method used in screening for antibacterial activity. Since our research tested only gram-negative bacteria, we also need to test gram-positive bacteria to determine if they would behave differently in planktonic and in biofilm lifestyles in an antibacterial screen.

Our study was able to demonstrate that GLEEx is effective against gram-negative bacteria in biofilm. However, the research was limited to an *in vitro* design. We feel that it is warranted that an *in vivo* experiment be done to ascertain the consistency of the antibacterial properties of GLEEx in an animal model. We also suggest isolating, purifying, and identifying the active component in GLEEx to further expound on its mechanism of action.

4. CONCLUSION

Several previous research have already validated the antibacterial properties of guava leaves but have only tested them with planktonic forms of bacteria. Our research evaluated the efficacy of guava leaf ethanolic extract with bacteria in both planktonic and biofilm forms. In doing so, we have revealed that the potency of GLEEx, relative to antibiotic standards, was less in planktonic than in biofilm bacteria. These points highlight the ability of the BioTimer Assay to detect antibacterial activities of plant extracts that may not be adequately represented in the Kirby-Bauer disc diffusion assay alone; thereby stressing the importance of simultaneously testing the potencies of certain substances against both lifestyles of bacteria when evaluating antibacterial properties of natural products.

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CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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