ORIGINAL ARTICLE

In Vitro Antibacterial and Antibiofilm Activities of *Piper betle* L. Ethanolic Leaf Extract on *Staphylococcus aureus* ATCC 29213

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ABSTRACT

Background and Objective. *Staphylococcus aureus* is the leading cause of skin and soft tissue infections such as abscesses, furuncles, and cellulitis. Biofilm forming strains of *S. aureus* have higher incidence of antimicrobial resistance to at least three or more antibiotics and are considered as multidrug resistant. Since *S. aureus* biofilm-producing strains have higher rates of multidrug and methicillin resistance compared to non-biofilm-producing strains, the need for alternative therapeutic option is important. Furthermore, rates of methicillin-resistant *Staphylococcus aureus* (MRSA) in Asia remain high. Results of the study may provide support for the clinical uses of *P. betle* as a topical antibacterial and antiseptic in the treatment and prevention of infections involving the skin, mouth, throat, and indwelling medical devices. Thus, this study aimed to evaluate the in vitro antibacterial and antibiofilm activities of *Piper betle* L. ethanolic leaf extract (PBE) against a biofilm-forming methicillin-sensitive *Staphylococcus aureus* ATCC 29213 (MSSA).

Methods. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of PBE against MSSA were determined using the agar dilution assay. The biofilm inhibition and eradication assays using crystal violet were done to quantify the antibiofilm activities of PBE on MSSA biofilm.

Results. PBE showed activity against MSSA in agar dilution assay with MIC and MBC values of 2500 µg/mL and 5000 µg/mL, respectively. At subinhibitory concentrations, PBE showed biofilm inhibition activity at 1250 µg/mL but a lower percent eradication of biofilms as compared to oxacillin was noted.

Conclusion. PBE showed antibacterial activities including biofilm inhibition against methicillin-sensitive *Staphylococcus aureus* ATCC 29213 (MSSA).

Keywords: Piper betle, Staphylococcus aureus, anti-bacterial agents, biofilms

INTRODUCTION

S. aureus is one of main pathogens in community and hospital infections which can range from mild skin infections to severe life-threatening bacteremia.[1] Among the biofilm-forming pathogens, *S. aureus* is one of the most commonly reported bacteria found on the surfaces of medical devices, and that two-thirds of indwelling devices infections are linked to staphylococcal species, with majority being related to *S. aureus* and coagulase-negative staphylococci.[2–5] In addition, biofilm producing strains of *S. aureus* have higher rates of multidrug and methicillin resistance compared to non-biofilm producing strains.[6–10] Biofilm-forming pathogens are extremely difficult to treat with conventional antibiotics due to their high resistance characteristics.[11]

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The leaves of *Piper betle (P. betle)* has been used in common ailments by many Asian countries, where *P. betle* is widely cultivated particularly in India, Malaysia, Sri Lanka, and Thailand.[12] Locally available *P. betle* plant has shown antibacterial property using its various extracts against clinical isolates of *S. aureus* and MRSA.[13–17] *P. betle* extracts have shown 17.67 mm zone of inhibition in the disk diffusion assay and an MIC value ranging between 0.59 to 4.69 mg/mL against *S. aureus*.[13,14] Hydroxychavicol and eugenol are two primary compounds that have been linked to *P. betle*'s antibacterial activity. Both of these compounds have exhibited mechanisms resulting to bactericidal effects which include ATPase inhibition, ROS generation, membrane damage, DNA damage, and apoptosis-like death induction.[18–20] Local study suggested that the high concentrations of eugenol and eugenol isomers in the leaf extracts of *P. betle* make it a promising source of antimicrobial metabolites.[17]

Unfortunately, studies on biofilm inhibition and eradication effects of *P. betle* leaf extracts are lacking against *S. aureus*. With this, the study aimed to evaluate the in vitro antibacterial and antibiofilm activities of PBE against MSSA.

MATERIALS AND METHODS

Equipment, Reagents and Bacterial Inoculum

Standard laboratory equipment such as UV-visible spectrophotometer (Thermo Scientific™ GENESYS™ 180) for inoculum standardization and biofilm quantification, rotary evaporator (DLAB RE100-Pro) to concentrate the plant extract, and incubator (Lab Companion) for *S. aureus* cultures were used. A blender (Imarflex) was used to homogenize the dried *P. betle* leaves.

Absolute ethanol (Scharlau) and Mueller Hinton broth (MHB), Mueller Hinton agar (MHA), trypticase soy broth (TSB), and trypticase soy agar (TSA) from Hi-Media were used as growth media. Tween 80 (Sigma-Aldrich) and USP-grade oxacillin powder (Sigma-Aldrich) were also procured from a local supplier. Sodium chloride (APS) and glucose (MC SCIENTIFIC) were used as supplements for the agar and TSB, respectively. The *Staphylococcus aureus* ATCC 29213 was obtained from the Department of Medical Microbiology, College of Public Health, University of the Philippines (UP) Manila.

Extract Preparation

The fresh leaves of *P. betle* were collected from the mountaintops of Sitio Balagbag, San Jose del Monte, Bulacan, Philippines. Pesticides were not used during cultivation, and the farm was located far from highways and industrial areas as described by the local supplier. Collection was done in July 2021 and November 2021. The *P. betle* plant was authenticated by the Institute of Biology, College of Science in UP Diliman.

The preparation of PBE was based on previously reported method with minor modifications.[17] *P. betle* leaves were air dried and pulverized into small pieces until powder formed prior to ethanolic extraction. A total of 1500 g of powdered *P. betle* leaf was soaked in 6 L of absolute ethanol for 7 days with occasional shaking. After the maceration, the mixture was filtered through a Whatman filter paper No. 1. All the filtrates were concentrated using a rotary evaporator at 50°C at 600 mmHg while rotating at 90 rpm. The extract was then further concentrated under the same temperature using a water bath. This gave a 2.67% extract yield from the dried leaves. The crude extract was stored in a container at -4°C until further used.[12]

S. aureus Stock and Inoculum Preparation

The following procedures were based on the methods as described by Missiakas and colleagues and Clinical and Laboratory Standards Institute (CLSI).[21,22] Briefly, using a sterile inoculating loop, a small amount of MSSA from the stock was streaked onto a small section of a TSA plate using aseptic technique. A new sterile inoculating loop was passed through the initially streaked quadrant, repeating the process 1-2 more times. The plates were incubated for 16 hours at 37°C.

An overnight growth method was used to prepare the necessary broth suspensions containing 1 x 10^8 CFU/mL for the procedures in the in vitro assays. The initial overnight broth culture was standardized at optical density at 600 nm (OD600) to CFU/mL. The computed conversion factor of the OD600 to CFU/mL was the basis of the dilution of the overnight cultures for in vitro antibacterial and antibiofilm assays.[23]

Agar Dilution Assay

Following standard methods (i.e., CLSI M07-A9, CLSI M100-S25) with minor modifications, in vitro antibacterial assay was conducted via the agar dilution method.[17,21,23,24] Two-fold dilution concentrations of the PBE in Tween 80 ranging from 10000 µg/mL to 9.8 µg/mL were incorporated into MHA with 2% NaCl where the inocula were applied and observed for absence or presence of microbial growth. The plate with the lowest concentration that did not show growth, after overnight incubation at 37°C, was considered the MIC. The culture plates at MIC and the rest of plates without visible growth as compared to the untreated control were subcultured to another 2% NaCl MHA plates without any treatments to determine the MBC value. The plate with the lowest concentration without growth, after overnight incubation at 37°C, was considered the MBC. For the positive control, oxacillin was used at 2 µg/mL while an extract control was devoid of the bacterial inoculum. Preparations also consisted of a negative control which did not contain the plant extract but with the inoculum and diluent. Three replicates were carried out for each concentration and controls for the entire procedure.

Biofilm Inhibition and Eradication Assays using Crystal Violet

The biofilm inhibition assay was done primarily as described by Melo and colleagues, but with slight modifications for the study.[25–28] All procedures were done to make six wells for each concentration and controls. Briefly, from an initial 200 mg/mL of PBE concentration in 5% Tween 80, PBE was serially diluted. This was followed by the addition of a 100 µL of PBE to the inoculum making 200 µL of 0.25 x and 0.5 x MIC in 0.02% and 0.03% of Tween 80, respectively. The mixture containing 1 x 10^6 CFU/mL of MSSA and PBE were dispensed onto the 96-well microwell plate. This was then incubated at 37°C for 24 h. Afterwards, the supernatant was removed and the wells were washed with sterile normal saline. The wells were dried and stained using 100 µL of 0.01% crystal violet for five (5) minutes. Crystal violet was removed and the dried stains were released using 95% ethanol. This solution (125 µL) was transferred to a new microtiter plate for absorbance detection at 490 nm (A490).[28,29] A vehicle control containing 0.05% Tween 80 was used while 128 µg/mL oxacillin was used as the positive control.[29] Two (2) sets of blank control were added containing only the broth and PBE without the inoculum and another set which contained only the broth. The A490 from these wells were subtracted from the wells with and without PBE, respectively.

As for the biofilm eradication assay, the biofilms were allowed to form first for 24 h prior to the addition of PBE.[25–28,30] Succeeding steps were the same as in the biofilm inhibition assay except that oxacillin at a higher concentration (256 µg/mL) was used.[29] An untreated control was added containing only the broth to grow the biofilms for both the biofilm assays. Heavy growth accompanied by saturated stain after washing in the wells containing the untreated control were used to visually confirm biofilm formation.

After reading the A490 and subtracting the blank controls, the percentage inhibition or eradication was computed using the equation:

$$\% = \left[1 - \left(\frac{\text{A490 of the test}}{\text{A490 of untreated control}} \right) \right] \times 100$$

Statistical Analysis

The results of the assays were expressed as means for each group. Statistical differences in the biofilm inhibition and eradication assays were evaluated using paired-samples t-test. Results were considered to be statistically significant at $p<0.05$. The researcher utilized IBM SPSS Statistics 23 for the statistical analysis.

Ethical Statement

This research was exempted from ethical review by the UP Manila Review Ethics Board with code 2021-018-EX. It was also registered with the protocol code RGAO-2022-0296 and underwent review and approval process by the Institutional Biosafety and Biosecurity Committee with protocol number 2022-008.

RESULTS

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

PBE showed activity against MSSA in agar dilution assay with MIC and MBC values of 2500 µg/mL and 5000 µg/mL, respectively. The positive control (i.e., oxacillin at 2 µg/mL) inhibited the growth of MSSA while the negative control (i.e., 0.5% Tween 80) plates had visible growth similar to the untreated control. In contrast, the plant extract control did not show growth indicating prepared extract was not contaminated.

Percent Biofilm Inhibition

At 0.5 x MIC or at 1250 µg/mL, 71% of the biofilm was inhibited while at 0.25 x MIC or at 625 µg/mL, only 41% of the biofilm was inhibited. The % inhibition at 0.5 x MIC (71%) failed to show significant difference with oxacillin (99%). Although there was a higher inhibition of 0.5 x MIC (71%) than Tween 80 (54%), this failed to show significant difference ($p>0.05$). This is summarized in Table 1 while Figure 1 visually presents the intensity of crystal violet stains.

Percent Biofilm Eradication

At subinhibitory concentrations, 0.5 x MIC and 0.25 x MIC showed biofilm eradication of 22% and 26%, respectively, but were comparable to the Tween 80's effect at 0.05% (vehicle control). Oxacillin at 256 µg/mL showed 43% eradication which failed to show significant difference with the vehicle control and plant extracts. Table 1 summarizes the data on biofilm eradication assay while Figure 2 visually presents the intensity of crystal violet stains.

DISCUSSION

PBE has shown antibacterial activities against MSSA with MIC of 2500 µg/mL and MBC of 5000 µg/mL. This results to an MBC/MIC ratio of 2 which indicates that the extract is bactericidal.[31] However, based on a published

Table 1. Calculated % Inhibition and Eradication of MSSA Biofilms by Various Concentrations of PBE

Test Materials	Concentrations	% Inhibition	% Eradication
PBE in 0.02% Tween 80	0.25 x MIC µg/mL	41*	26
PBE in 0.03% Tween 80	0.5 x MIC µg/mL	71	22
Oxacillin	128 µg/mL	99	–
	256 µg/mL	–	43
Tween 80	0.05%	54*	34

**p<0.05 vs. positive control*

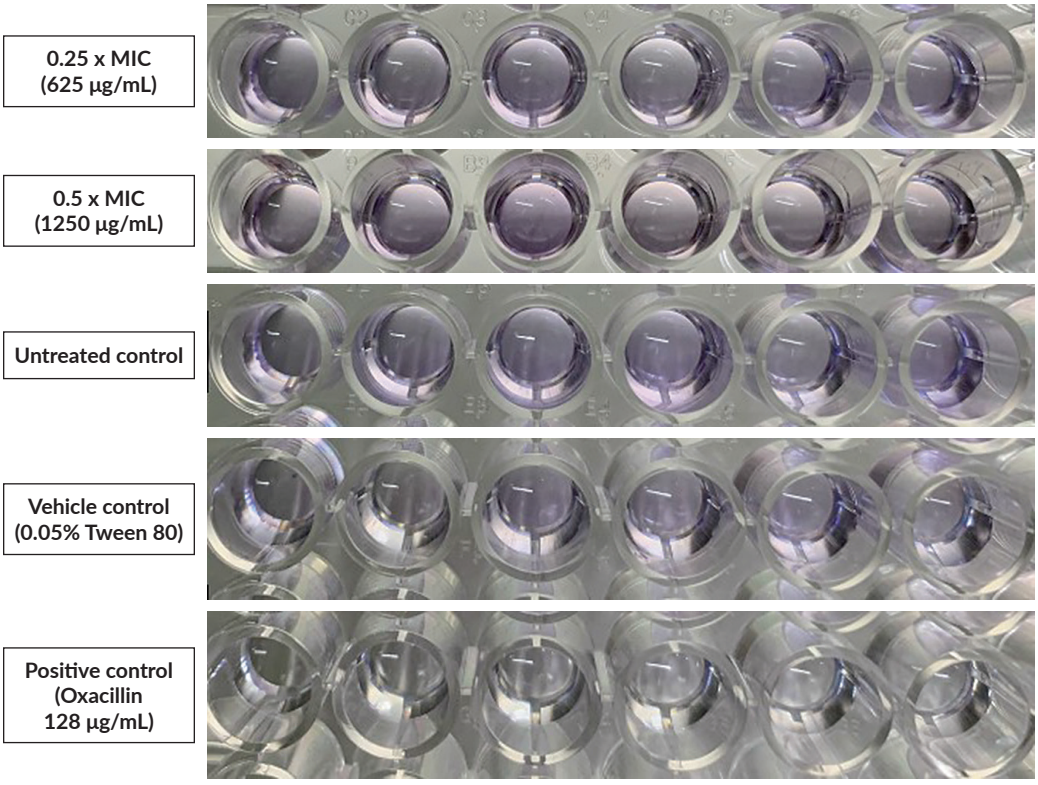

Figure 1. Dissolved crystal violet stain in ethanol of the remaining *S. aureus* biofilms after inhibition.

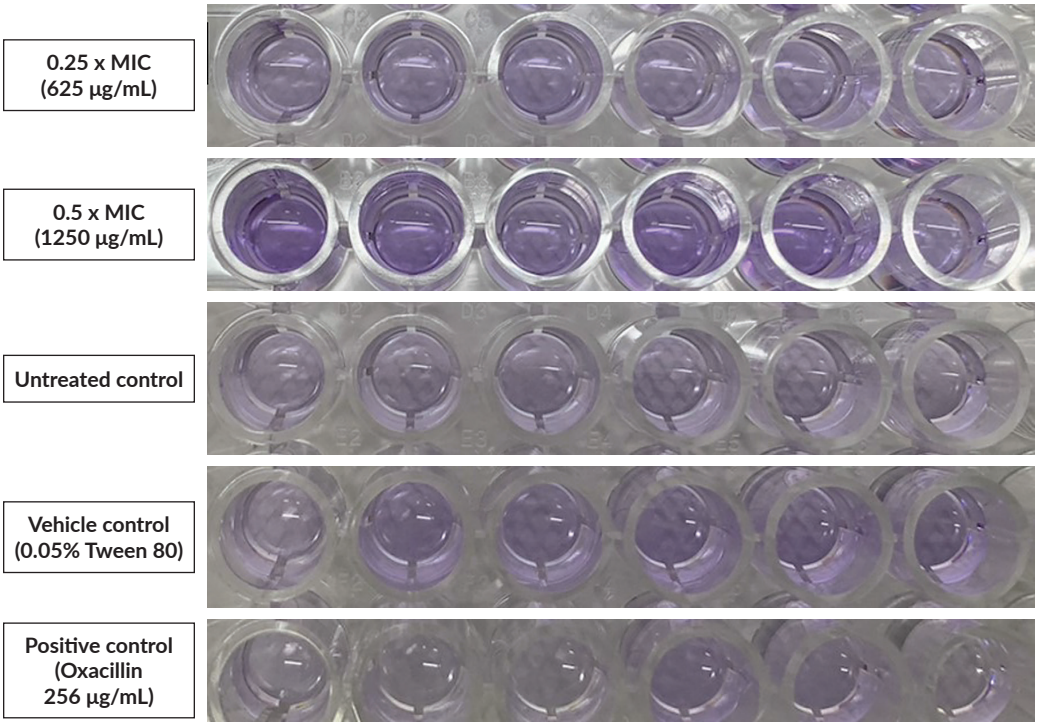

Figure 2. Dissolved crystal violet stain in ethanol of the remaining *S. aureus* biofilms after eradication.

categorization of antibacterial activity of plant extracts, the obtained MIC of PBE at 2500 µg/mL can be interpreted to have a weak antimicrobial effect.[32–34] The MIC in the study was higher than the MIC of the same species in the Philippines which were at 312-590 µg/mL, suggesting that the extract in the current study was less potent than those found in other locations.[14,16] Among these studies, the most potent PBE came from General Nakar, Quezon which was harvested from September to November.[16] Differences in their MIC and MBC may be explained by the various environmental factors involving the location and harvest time of *P. betle*.

Phytochemicals in the plant can differ significantly depending on the environmental factors such as temperature, humidity, and precipitation.[35–37] Of the phytochemicals, eugenol and hydroxychavicol are one of the most widely considered responsible for the antibacterial properties of *P. betle.* In the Philippines, eugenol was reported while hydroxychavicol has not been identified yet.[17] In the bioautographic assays, alkaloids, flavonoids, terpenoids, and saponins from *P. betle* have shown activities against MDR bacteria (e.g., MRSA). Ethyl diazoacetate, 3-fluoro-2-propynenitrite, tris(trifluoromethyl)phosphine, 4-(2-propenyl) phenol, and heptafluorobutyrate have also been reported.[17] If the same plant species was harvested under different environmental conditions, changes in phytochemical contents can affect their bioactivity including antimicrobial effects.[38,39]

Hydroxychavicol (HC) isolated from *P. betle* has been reported to possess antibacterial activity but its mechanism of actions has not yet been fully understood.[20,40] A study conducted on *E. coli* described a few possible mechanisms.[20] Reactive oxygen species (ROS) generation, DNA damage, and apoptosis-like death induction were some of the mechanisms noted in the study. HC treated *E. coli* cells resulted to growth inhibition and DNA condensation. ROS generation produces DNA damage which was also confirmed by using DNA damage repair deficient mutants versus wild type. In addition, antioxidants, thiourea, and GSH have protected *E. coli* against HC-induced death. Clinical isolates of *E. coli, Klebsiella, Pseudomonas*, and *Proteus* have shown susceptibility to HC with MIC ranging from 250 µg/mL to 500 µg/mL while MBC at 500 µg/mL to 750 µg/mL.[20] Another compound isolated from *P. betle* leaves was eugenol which when compared to hydroxychavicol is less abundant in the extract and may be less potent.[40] Eugenol's antibacterial activity is attributed to its ability to produce ROS generation and membrane damage.[18]

Differences in *P. betle* phytochemicals may have primarily contributed to the lower potency of the extract in the current study. Unfortunately, there is no known study of ideal conditions and cultivation methods of local *P. betle* for an optimal antibacterial effect. In addition, the current study did not perform chemical analysis of the the PBE's components. Other factors that could have contributed to the differences in the MIC values of *P. betle* are the differences in the assays conducted and the strains and diluents used. In particular, these other studies used *S. aureus* ATCC 25923 in broth microdilution assay and diluted PBE in dimethylsulfoxide at 0.2% and 5%.[14,16]

Biofilm formation was inhibited when exposed to sub-inhibitory concentration of PBE (0.5 x MIC). Contrastingly, percentage biofilm eradicated was lower than the oxacillin (positive control) and Tween 80 (vehicle control). In the current study, PBE showed biofilm inhibitory effects by up to 71% against MSSA at subinhibitory concentration of 0.5 x MIC or 1250 µg/mL (Table 1). This biofilm inhibitory effect of the extract was comparable or failed to show statistical difference at $p<0.05$ with the positive control oxacillin at 128 µg/mL and showed to be 17% higher than 0.05% Tween 80 (vehicle control).

Previous studies have shown that Tween 80 has biofilm inhibition at concentrations as low as 0.05% against MRSA and 0.1% against MSSA.[41,42] In the current study, although Tween 80 at 0.05% showed 54% biofilm inhibition, this was lower and significantly different from oxacillin (99%), and only when the PBE was added at 0.5 x MIC that the biofilm inhibition (71%) became comparable to oxacillin (Table 1). In vivo and in vitro studies against *S. aureus* have shown that a combination of an antibiofilm agent with an antibiotic produces greater inhibition of bacterial growth and lower MIC and MBC values.[43]

Staphylococcus aureus has different stages of biofilm development in the order of attachment, multiplication, exodus, maturation, and dispersal.[44,45] During the attachment stage, *S. aureus* utilizes hydrophobic and electrophilic interactions to attach on abiotic surfaces. The initial attachment stage happens approximately within two (2) hours before the multiplication starts. During the multiplication stage, in the presence of a sufficient nutrient source, the adherent cells starts dividing and accumulating. In this time, EPS and its components are also starting to stabilize the biofilm.[44,45] Interestingly, from a time-kill kinetics study, the exponential phase of the MSSA coincides with the multiplication stage of the adherent *S. aureus*.[46]

In literature, *P. betle* extract against biofilm formed by *S. marcescens* revealed that it can reduce exopolysaccharide production and hydrophobicity index, both of which are important in the formation and adherence of *S. aureus* biofilm.[47] Furthermore, at 0.5 x MIC or 1250 µg/mL, PBE significantly prolonged the lag time, slowed down the growth rate, and decreased the maximum optical density of MSSA.[46] These mechanisms together suggest that the effects exerted by *P. betle* at subinhibitory concentrations may be early during adherence and multiplication stages of the *S. aureus* biofilm formation. Therefore, the biofilm inhibition of PBE may suggest that early administration of the extract enhances its overall antibacterial effect against MSSA. However, more direct studies correlating these findings are needed. Phytol, 4-chromanol, and 1,8 cineole were few of

the bioactive compounds found in *P. betle* extract that may be responsible to its antibiofilm effects.[47–49]

In the biofilm eradication assay, PBE at subinhibitory concentrations (0.5 x MIC, 0.25 x MIC) has shown 22-26% eradication of the biofilm, respectively. Although oxacillin at 256 µg/mL showed higher eradication at 43%, this failed to show statistical difference with the PBE and Tween 80 (34%). In literature, it was determined that oxacillin at 256 µg/mL eradicated biofilm by up to 90%.[29] However, in another study, the minimum biofilm eradication concentration in preformed biofilms is at concentrations >1024 µg/mL.[50] Due to variability in the observed biofilm eradication activity of oxacillin, an alternative positive control may be preferred.

With the results of the study, *P. betle* can be an important source of a new antibacterial and antibiofilm agent against *S. aureus*. The results strengthen its potential for clinical uses as a topical antibacterial and antiseptic or disinfectant in the treatment and prevention of infections involving the skin, mouth, throat, and indwelling medical devices. A topical preparation of the *P. betle* leaf ethanolic extract has already been studied and shown activity against *S. aureus,* further supporting the possibility of an effective topical drug delivery system in treating or preventing bacterial infections.[51–53] Consistent antibacterial effect of local PBE is also encouraging for bioactive isolation as a potentially new systemic antibiotic. The results of the study may also help improve and guide in vivo designs of antibacterial assays using *P. betle* ethanolic leaf extracts.

CONCLUSION AND RECOMMENDATIONS

PBE obtained from a farm on mountaintops of San Jose del Monte, Bulacan showed activity against MSSA in agar dilution assay with MIC and MBC values of 2500 µg/mL and 5000 µg/mL, respectively. This MIC is considered to be of weak antibacterial activity for plant extracts.[32–34] PBE has also shown antibiofilm activity at 1250 µg/mL during the biofilm formation; however, a lower percent eradication of biofilms as compared to oxacillin and Tween 80 was noted.

It is recommended to further study favorable environmental conditions that can provide a consistent harvest of *P. betle* with maximum antibacterial effect. Isolation of the bioactives from the extract and conducting in vivo assays to further elucidate the antibacterial and antibiofilm activities of PBE against MSSA and MRSA are also highly recommended. Use of an alternative positive control for the biofilm eradication assay should be considered.

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Statement of Authorship

RCCL contributed in the conceptualization, laboratory work, formal analysis, writing the original draft, review and editing, project administration, and funding acquisition; AMY contributed in the conceptualization, formal analysis, writing the original draft, review and editing, project administration, funding acquisition, supervision, and final approval of the version to be published; MTA, LCRP and IRM contributed in the conceptualization, review and editing, and final approval of the version to be published.

Author Disclosure

All authors declare that there is no conflict of interest.

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