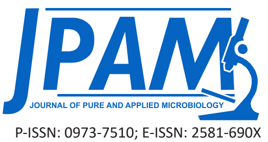
Salcedo-Catulong & Hinay Jr | Article 11380
J Pure Appl Microbiol. 2026;20(2):1810-1818. doi: 10.22207/JPAM.20.2.70
Received: 28 January 2026 | Accepted: 07 May 2026
Published Online: 05 June 2026



P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Molecular Characterization of Biofilm-associated Genes in Methicillin-resistant *Staphylococcus aureus* and their Influence on the Antibiofilm Activity of Oregano (*Plectranthus amboinicus*) Ethanolic Extract

Keziah Amor D. Salcedo-Catulong and Alfredo A. Hinay Jr*

Graduate School Department, University of the Immaculate Conception, Davao City, Philippines.

Abstract

Methicillin-resistance in *Staphylococcus aureus* is frequently associated with enhanced biofilm formation and the presence of biofilm-associated genes that contribute to treatment failure and persistence. This study characterized the phenotypic biofilm formation and genotypic profiles of 10 clinical methicillin-resistant *Staphylococcus aureus* (MRSA) isolates and evaluated the dose-dependent antibiofilm activity of the ethanolic extract of oregano (*Plectranthus amboinicus*). All isolates carried *mecA,* confirming methicillin resistance in all isolates. The biofilm-associated genes *icaA, icaD,* and *sarA* were detected, whereas *bap* was absent. Despite the absence of *bap*, all isolates exhibited moderate to strong biofilm formation, consistent with *ica*-dependent polysaccharide-mediated adhesion. Based on PCR-based detection of the *agrII* gene (presence/absence), the isolates were classified into *agrII*-positive (n = 5) and *agrII*-negative (n = 5) groups. Biofilm formation was predominantly moderate (0.140 < OD < 0.280), with two isolates showing strong biofilm formation (OD > 0.280). Extract dilutions (100%-3.125% v/v) were tested using a modified crystal violet assay. The *agrII*-positive isolates exhibited significantly lower biofilm inhibition (12.9% ± 7.4%) than *agrII*-negative isolates (33.0% ± 1.2%) at 100% v/v (P = 0.003). Nonlinear regression using a four-parameter log-logistic model generated distinct dose-response curves, with 95% confidence bands exhibiting minimal overlap (12.5%-100%). These findings suggest a potential association between *agrII* status and differential biofilm susceptibility to *P. amboinicus* extract. However, given the limited sample size and in vitro design, these results should be interpreted with caution. Overall, this study highlights the potential of *P. amboinicus* as an antibiofilm agent and supports further investigation of genotype-informed strategies against MRSA.

Keywords: Methicillin-resistant *Staphylococcus aureus,* Biofilm-associated Genes, Oregano Plant Extract, Antibiofilm Activity

***Correspondence:** ahinay@uic.edu.ph

Citation: Salcedo-Catulong KAD, Hinay Jr AA. Molecular Characterization of Biofilm-associated Genes in Methicillin-resistant *Staphylococcus aureus* and their Influence on the Antibiofilm Activity of Oregano (*Plectranthus amboinicus*) Ethanolic Extract. *J Pure Appl Microbiol.* 2026;20(2):1810-1818. doi: 10.22207/JPAM.20.2.70

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INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains a clinically significant pathogen associated with persistent and difficult-to-treat infections. While its global burden is well recognized, treatment failure is increasingly attributed not only to antimicrobial resistance but also to the ability of MRSA to form biofilms, which enhance survival under hostile conditions and reduce the efficacy of antibiotics.[1,2]

Biofilm formation plays a central role in MRSA persistence by protecting bacterial cells from host immune responses and limiting antibiotic penetration.[3,4] These structured communities contribute to chronic and device-associated infections, for which conventional therapeutic strategies are often insufficient. Consequently, increasing attention has been directed toward understanding the molecular mechanisms underlying biofilm development, particularly the regulatory systems governing adhesion and maturation. Disruption of quorum sensing (QS)-regulated genes, such as the accessory gene regulator (agr) system, which governs intercellular signaling and biofilm maturation, may reduce bacterial adherence and virulence.[5,6]

Biofilm-associated genes, including *icaA, icaD, sarA*, and *bap,* are traditionally implicated in biofilm matrix formation; however, MRSA can also form biofilms through alternative *ica*-independent mechanisms, such as protein-mediated adhesion and quorum-sensing regulation. Among these, the *agr* system is a key modulator of virulence and biofilm dynamics, influencing both structural integrity and dispersal. Plant-based QS inhibitors show promise for attenuating biofilm formation; however, studies on readily available medicinal species remain limited.[7] In particular, there is limited evidence on whether antibiofilm activity varies according to MRSA genotypic differences, especially in relation to quorum-sensing systems.

Oregano (*Plectranthus amboinicus*), although not among the ten medicinal plants officially endorsed by the Philippine Department of Health, is widely used in folk medicine and exhibits antibacterial properties.[8] Its ethanolic extract contains phenolic compounds, such as carvacrol and thymol, which disrupt bacterial membranes, alter signaling pathways, and degrade biofilm matrix components.[9-11]

Despite these known properties, the relationship between MRSA genotype and the antibiofilm activity of *P. amboinicus* remains unclear. In this study, the phenotypic biofilm characteristics of clinically isolated MRSA were evaluated, along with genotypic profiling of biofilm-associated genes. The ethanolic extract of *P. amboinicus* was assessed for dose-dependent antibiofilm activity. We aimed to determine whether *agrII* status is associated with differential antibiofilm susceptibility, thereby addressing a gap in genotype-informed antibiofilm strategies.

MATERIALS AND METHODS

Bacterial strains

Ten clinically isolated MRSA strains were obtained from a tertiary hospital Microbiology laboratory in Davao City. This study was conducted as a preliminary pilot investigation, and the sample size (n = 10) was selected to explore initial genotype–phenotype associations.

Isolates were collected from different patients, and duplicate isolates from the same patient were excluded. Inclusion criteria included confirmed *Staphylococcus aureus* isolates with methicillin resistance, while contaminated or mixed cultures were excluded. Clinical specimens were derived from diverse sources, including tracheal aspirates, wound and tissue samples, blood, urine, catheter tips, and abscesses.

Isolates were phenotypically identified by standard biochemical tests and confirmed using the automated VITEK system (bioMérieux, Marcy-l'Etoile, France). Identification confidence values (probability scores ≥95%) were used for species-level confirmation.

S. aureus ATCC 25923 was used as a positive control for the biofilm formation assays. Isolates were transported under cold-chain conditions in nutrient agar slants and subcultured on blood agar at 37 °C for 24 hrs. Colonies with round, convex morphology (1-4 mm diameter) and weak to clear β-hemolysis were confirmed on Mueller–Hinton agar supplemented with 4% NaCl and 6 µg/mL of oxacillin.

Assessment of the biofilm formation capability of the clinically isolated MRSA

Biofilm formation was quantified using a crystal violet microtiter plate assay.[3,12] All experiments were performed using three biological replicates (independent cultures) and three technical replicates per condition. MRSA strains were cultured on blood agar at 37 °C for 24 hrs, and three colonies were inoculated into 5 mL tryptic soy broth (TSB) and incubated for 18 hrs. Bacterial suspensions were standardized to OD_{580} = 0.50-0.63 McFarland Standard using VITEK® DensiCHEK (bioMérieux, Marcy-l'Etoile, France) in phosphate-buffered saline (PBS; pH 7.4). A 100 µL bacterial suspension was added to 100 µL of TSB in 96-well plates and incubated for 24 hrs at 37 °C. Wells were washed twice with sterile PBS, fixed with 96% ethanol (15 min), stained with 2% crystal violet (5 min), and destained with 33% acetic acid. Absorbance was measured at 492 nm. Biofilm production was categorized as none, weak ($0.070 < OD < 0.140$), moderate ($0.140 < OD < 0.280$), or strong ($0.280 < OD$) based on the cut-off optical density (ODc = mean OD of blank + 3 × SD) as previously described.[13]

Screening the clinically isolated MRSA for biofilm-associated genes

Isolates demonstrating biofilm formation (weak to strong) were screened for biofilm-associated genes (*icaA, icaD, bap, sarA,* and *agrII*) using polymerase chain reaction (PCR). Genomic DNA was extracted using a GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies, Malaysia; Cat. No. GF-BA-050). A 25 µL PCR mixture contained 2 µL template DNA, 1 µL of each primer, 12.5 µL 2× Taq Master Mix (Vivantis Technologies, Malaysia; Cat. No. PLMM01), and 8.5 µL of nuclease-free water. Amplification was performed in a miniPCR mini16X thermocycler (miniPCR Bio, Cambridge, MA, USA) under the following conditions: 30 cycles at 94 °C for 30 sec (denaturation), primer-specific annealing temperatures (40-50 °C depending on the primer set; exact temperatures are provided in Table 1) for 30 sec, and extension at 72 °C for 17-59 sec. Each PCR run included a negative control (no template control) and a reference strain (*Staphylococcus aureus* ATCC 25923) used as a control based on documented gene characteristics to verify amplification. PCR products were visualized by 1.5% agarose gel electrophoresis using the GELATO™ system (miniPCR Bio, Cambridge, MA, USA) at a running voltage of 135 V for 20-25 min. Primers were selected from a previously published study[14] and validated for specificity, with the sequences and corresponding details presented in Table 1.

Plant extract preparation

Fresh *P. amboinicus* plants cultivated in Calinan, Davao city were authenticated by a botanist prior to experimentation. Mature leaves (2.5-3 cm long) were harvested before flowering, washed, and dehydrated at 30 °C to preserve phenolic compounds.[15,16] Dried leaves were ground, and 5.5 g of powder was macerated in 100 mL of 60% ethanol for 7 days at ambient temperature with daily agitation. This process was repeated three times, and the extracts were pooled, filtered through Whatman No. 1 paper, and concentrated by rotary evaporation.[17] The resulting extract exhibited an oily consistency and low recovery following lyophilization; therefore, the crude extract was used directly without determining the percentage yield. The crude extract was diluted in 5% dimethyl sulfoxide to prepare two-fold serial dilutions.[18]

Phytochemical Analysis

Qualitative phytochemical screening of the *P. amboinicus* ethanolic extract was performed using standard colorimetric assays. Phenols were detected using the ferric chloride test and characterized by the formation of a deep bluish-black coloration. Flavonoids were identified using the sodium hydroxide test, which produced an intense yellow color that turned colorless upon acidification. The presence of tannins was confirmed using the lead acetate test (yellowish precipitate), indicating their presence in the extract.

This analysis was limited to qualitative detection, and the quantitative determination of phytochemical constituents using chromatographic techniques such as high-performance liquid chromatography (HPLC) or gas chromatography–mass spectrometry (GC-MS) was not performed. As this study was designed as a preliminary pilot investigation, the phytochemical assessment aimed to provide an initial characterization of the

Table 1. Primer sequences and PCR conditions for the screening of biofilm-associated genes in clinically isolated MRSA

Genes	Primer Sequence	Product size (bp)	Annealing time (s)
mecA	5'-ATCGATGGTAAAGGTTGG-3' 5'-AGTTCTGCAGTACCGGATTTG-3'	533	32
icaA	5'-TGGCTGTATTAAGCGAAGTC-3' 5'-CCTCTGTCTGGGCTTGACC-3'	669	41
icaD	5'-AAACGTAAGAGAGGTGG-3' 5'-GGCAATATGATCAAGATAC-3'	381	23
bap	5'-CCCTATATCGAAGGTGTAGAATTG-3' 5'-GCTGTTGAAGTTAATACTGTACCTGC-3'	971	59
sarA	5'-TTAGCTTTGAAGAATTCGCTGT-3' 5'-TTCAATTTCGTTGTTTGCTTC-3'	275	17
agrII	5'-GTAGAGCCGTATTGATTCC-3'' 5'-GTATTTTCATCTCTTTAAGG-3	463	28

extract. Therefore, the relative concentrations of the active compounds could not be determined.

Dose-response analysis of *P. amboinicus* ethanolic extract

The dose-dependent antibiofilm activity was evaluated using a modified crystal violet assay. Bacterial suspensions standardized to OD_{580} = 0.50-0.63 McFarland Standard using VITEK® DensiCHEK (bioMérieux, Marcy-l'Etoile, France) were co-incubated with equal volumes (50 µL) of serially diluted *P. amboinicus* ethanolic extract and 100 µL TSB in 96-well plates for 24 hrs at 37 °C. The experiments were conducted in triplicate. Following incubation, the wells were washed, fixed, stained, and analyzed as described above. Cultures without extract served as growth controls, and TSB alone served as a blank.

The percentage of biofilm inhibition was calculated as follows:

$$\text{OD Inhibition (\%)} = (1 - OD_{GC} / OD_x) \times 100$$

where OD_{GC} is the mean optical density of the growth control, and OD_x is the mean optical density at the tested concentration as previously described.[19]

Statistical analysis

Biofilm-inhibitory activity (%) was compared between *agrII*-positive and *agrII*-negative MRSA isolates at each concentration

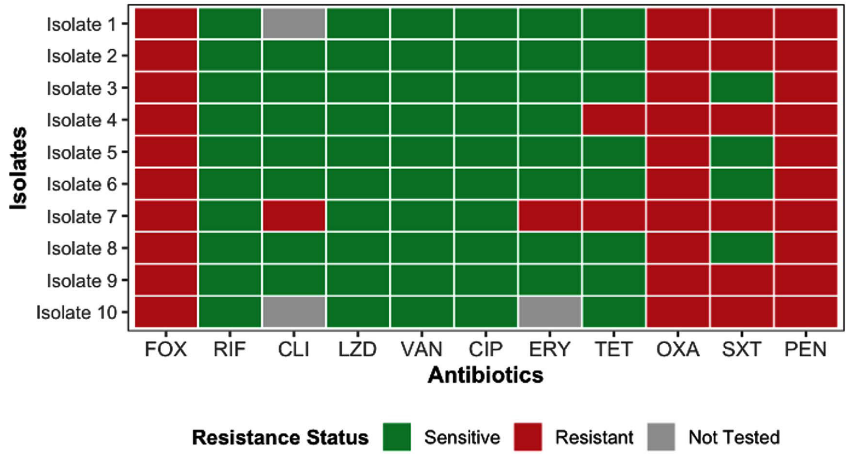

Figure 1. Antimicrobial Resistance Profile of the Clinically Isolated MRSA

using Welch's two-sample t-test (GraphPad Prism v10.2.3), with significance set at P < 0.05. Normality within groups was evaluated using the Shapiro-Wilk test, and homogeneity of variance was assessed using the F-test. Nonlinear regression using the four-parameter log-logistic (LL.4) model (DRC package, R v4.4.1) was applied to generate dose-response curves characterizing genotype-specific biofilm inhibition profiles.

RESULTS

Antimicrobial resistance profile of clinically isolated MRSA

The antimicrobial susceptibility profiles of the 10 *Staphylococcus aureus* isolates confirmed their methicillin-resistant phenotype (Figure 1). All isolates were resistant to cefoxitin (FOX), penicillin (PEN), and oxacillin (OXA), and most were resistant to trimethoprim-sulfamethoxazole (SXT). Variable resistance was observed for erythromycin (ERY) and clindamycin (CLI), whereas vancomycin (VAN), linezolid (LZD), tetracycline (TET), rifampicin (RIF), and ciprofloxacin (CIP) remained effective. These resistance patterns are consistent with the phenotypic characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) and were used as an initial screening step, with molecular confirmation (*mecA* detection) presented in subsequent analyses.

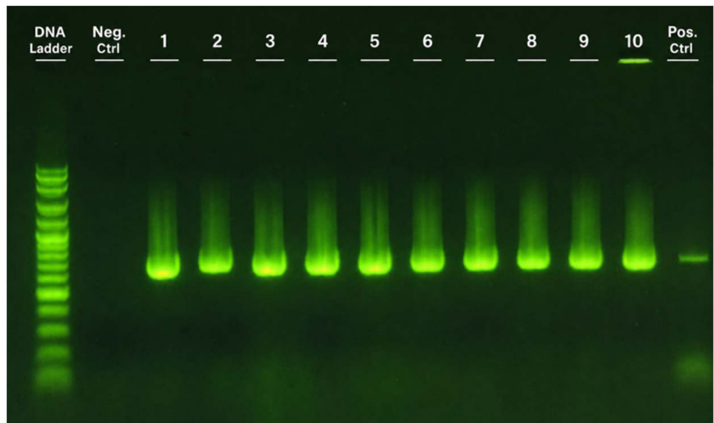

Figure 2. Agarose gel electrophoresis (1.5%) showing PCR amplification of the *sarA* gene across samples 1-10, including DNA ladder, negative control, and *Staphylococcus aureus* ATCC 25923 as the positive control, visualized using the GELATOTM system (miniPCR Bio, Cambridge, MA, USA)

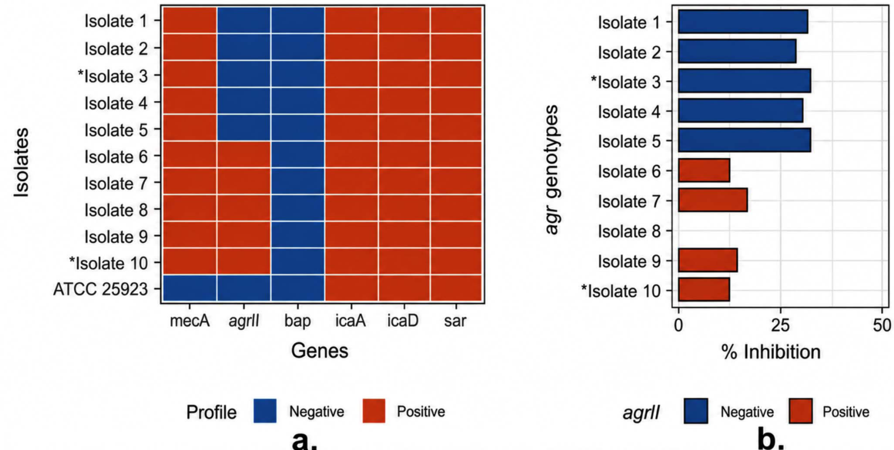

Figure 3. Biofilm-associated gene profile (a) and biofilm-inhibitory activity of *P. amboinicus* ethanolic extract against clinically isolated MRSA (b). Asterisks (*) mark phenotypically strong biofilm-forming isolates

Although antimicrobial resistance in *S. aureus* has been associated with biofilm formation in previous studies, no direct association was evaluated in the present study, and the data are presented as descriptive findings. These findings served as the basis for assessing the biofilm-forming capability and genotypic profiles of the isolates in subsequent analyses.

Biofilm-associated gene profiles of MRSA isolates

The phenotypic biofilm formation capability of the ten MRSA isolates was predominantly moderate (0.140 < OD < 0.280), with two isolates (3 and 10) classified as strong (OD > 0.280). However, marked differences were observed in the biofilm-associated gene profiles. Representative agarose gel electrophoresis confirming PCR amplification of selected biofilm-associated genes (*sarA*) is shown in Figure 2. The expected amplicon size (~275 bp) was observed in the positive control, whereas no amplification was detected in the negative control, confirming assay specificity. All isolates possessed *mecA*, confirming phenotypic methicillin resistance. The biofilm-associated genes *icaA, icaD,* and *sarA* were detected in all isolates, whereas *bap* was not detected. Despite the absence of *bap,* moderate to strong biofilm formation was observed, consistent with *ica*-dependent polysaccharide-mediated biofilm formation. Notably, five isolates carried *agrII*, whereas the remaining five were negative (Figure 3a), indicating variability in the quorum-sensing profiles that may influence biofilm regulation rather than initial formation.

Antibiofilm activity of *P. amboinicus* extract

To determine whether this genotypic variation influenced antibiofilm susceptibility, the activity of the *P. amboinicus* ethanolic extract was assessed against these isolates (Figure 3b). Isolates carrying *agrII* exhibited lower susceptibility to biofilm inhibition (12.92% ± 7.45%) compared to *agrII*-negative isolates, which showed higher inhibition (32.98% ± 1.18%) at 100% concentration. These trends are supported by the numerical values presented in Table 2. The observed antibiofilm activity is likely attributable to the phenolic, flavonoid, and tannin contents of the extract. Moreover, the differential response suggests that *agrII* may modulate biofilm responsiveness to the extract, consistent with its role in quorum sensing and matrix formation stability.

Dose-dependent antibiofilm activity of *P. amboinicus* extract

P. amboinicus ethanolic extract demonstrated dose-dependent biofilm inhibition across serial dilutions (100%-3.125% v/v), with *agrII*-positive strains consistently exhibiting

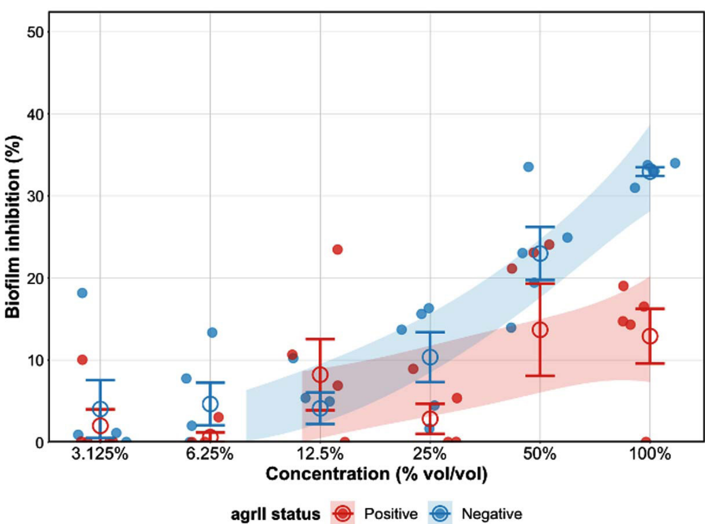

Figure 4. Dose-dependent Biofilm Inhibition of Oregano Ethanolic Extract Against *agrII*-positive and *agrII*-negative Biofilm-forming MRSA Genotypes

Table 2. Biofilm Inhibition by Plectranthus amboinicus Extract across *agr*II genotypes

Concentration (% vol/vol)	*Agr*II positive genotypes (Mean ± SD), n = 5	*Agr*II negative genotypes (Mean ± SD), n = 5	t-statistic	P-value
100	12.9 ± 7.4	33.0 ± 1.2	-5.947	0.0034
50	13.7 ± 12.5	23.0 ± 7.2	-1.435	0.1984
25	2.8 ± 4.1	10.4 ± 6.8	-2.122	0.074
12.5	8.2 ± 9.7	4.1 ± 4.3	0.862	0.4244
6.25	0.6 ± 1.3	4.6 ± 5.8	-1.516	0.1974
3.125	2.0 ± 4.5	4.0 ± 7.9	-0.502	0.6325

lower levels of inhibition than *agrII*-negative strains (Figure 4). Nonlinear regression using the four-parameter log-logistic (LL.4) model further illustrated distinct dose-response curves for each genotypic group, with associated 95% confidence bands (shaded blue = *agrII*-negative; red = *agrII*-positive) showing minimal overlap across concentrations, where model predictions were reliable (12.5%-100%).

These trends are supported by the numerical values presented in Table 2. Statistical analysis confirmed that *agrII* status significantly affected inhibition at 100% concentration (P = 0.003), whereas the differences were non-significant at lower concentrations (50%: 13.7% ± 12.5% vs. 23.0% ± 7.2%, P = 0.198; 25%: 2.8% ± 4.1% vs. 10.4% ± 6.8%, P = 0.074; 12.5%: 8.2% ± 9.7% vs. 4.1% ± 4.3%, P = 0.424). However, relatively high standard deviation values at certain concentrations (e.g., 50%) indicate substantial variability among isolates, and these results should be interpreted cautiously.

Nevertheless, given the observed variability and limited sample size, these differences should be considered indicative of trends rather than definitive genotype-specific effects.

DISCUSSION

Emerging evidence from recent studies have highlighted the central role of biofilm-associated genes and quorum-sensing systems in the persistence and treatment failure of MRSA infections.[12,13,18] In particular, the accessory gene regulator (agr) system is a key modulator of biofilm architecture, virulence factor expression, and antimicrobial tolerance.[20,21] In this context, the present study contributes to the growing body of work on biofilm-targeted strategies by demonstrating that the ethanolic extract of *P. amboinicus* exhibits genotype-associated antibiofilm activity in vitro, with *agrII*-positive MRSA isolates showing reduced susceptibility compared to *agrII*-negative genotypes at the same concentration of extract.

All clinical isolates carried *mecA*, confirming their methicillin resistance. The biofilm-associated genes *icaA* and *icaD*, which are core components of the intercellular adhesion (ica) operon responsible for polysaccharide intercellular adhesin (PIA) biosynthesis, were detected in all isolates.[22] In addition, *sarA* was present, whereas *bap* was not detected. These findings indicate that the classical *ica*-dependent biofilm pathways remain intact in these isolates. While the presence of *agrII* may be associated with differences in biofilm behavior, its role in modulating biofilm susceptibility cannot be definitively established without gene expression analyses.

The phytochemical profile of *P. amboinicus* ethanolic extract, characterized by the presence of phenolic compounds, flavonoids, and tannins, provides a plausible basis for the observed antibiofilm activity of the extract. Phenolics, such as carvacrol and thymol, have been reported to interfere with quorum-sensing pathways and disrupt biofilm structure.[23] However, quorum-sensing inhibition and gene expression changes were not directly assessed in this study; therefore, mechanistic interpretations should be considered hypothetical.

Comparable antibiofilm effects of phenolic-rich plant extracts have been reported in MRSA and other bacterial systems, supporting the broader potential of natural products as biofilm-

disrupting agents in various bacterial systems. The observed genotype-associated differences in inhibition are consistent with previous finding, suggesting that bacterial regulatory systems may influence the responsiveness to antimicrobial compounds.[24]

The application of a four-parameter log-logistic model to dose-response data provides a quantitative framework for comparing inhibition profiles. However, given the limited sample size and number of data points, these results should be interpreted as indicative of trends rather than definitive genotype-specific effects.

The findings of this study should be interpreted in light of its limitations. The relatively small sample size and in vitro design limit the statistical robustness and generalizability of the findings, and the absence of gene expression analysis and quantitative chemical characterization restricts mechanistic interpretation. In addition, detailed antimicrobial efficacy parameters and safety-related assessments were not performed. These constraints highlight the preliminary nature of the findings and the need for further research.

From a clinical perspective, the potential application of *P. amboinicus* extract as an antibiofilm agent has yet to be established. Future studies should address the optimal dosing, delivery strategies, safety, and in vivo efficacy of these compounds to determine their therapeutic feasibility.

Overall, while this study provides preliminary evidence of genotype-associated differences in antibiofilm activity, further investigations incorporating molecular, chemical, and translational approaches are required to confirm these findings and elucidate their underlying mechanisms.

CONCLUSION

This in vitro study demonstrated that the ethanolic extract of *P. amboinicus* exhibited dose-dependent antibiofilm activity against MRSA isolates, with differences observed between the *agrII*-positive and *agrII*-negative genotypes. These findings suggest a potential association between *agrII* status and variability in antibiofilm response, although this relationship requires further validation. However, these results should be interpreted in light of several limitations, including the small sample size, absence of gene expression analysis, and lack of quantitative chemical and efficacy assessments.

Overall, this study provides preliminary evidence supporting the potential of *P. amboinicus* extract as an antibiofilm agent, warranting further investigation through mechanistic studies and in vivo validation to assess its clinical applicability.

ACKNOWLEDGMENTS

None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Both authors listed have made substantial, direct, and intellectual contributions to the work and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the University of the Immaculate Conception (protocol code GS-EX-07-25-0058).

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