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*Research article*

# Bactericidal Properties, Biofilm Formation Inhibition, and Chemical Profiling of *Piper argyrites* and *Piper betel* L.

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**Abstract:** Betel leaf has been an important medicinal plant for therapeutic purposes since ancient times. This study aims to investigate the ethanolic leaf extracts of *Piper betel* (PB) and *Piper argyrites* (PA), native to Satun Province, Thailand. The antibacterial properties of the extracts were evaluated using a disc diffusion assay on four bacterial isolates, including *Escherichia coli* PT005, *Pseudomonas aeruginosa* PT00A2, *Klebsiella pneumoniae* PT00A1, and *Staphylococcus aureus* RTV01. PB leaf extracts demonstrated a substantial effect on *K. pneumoniae* PT00A1 with an inhibition zone of 25.77 ± 0.04 mm, while PA leaf extract showed potent activity against *E. coli* PT005 with an inhibition zone of 17.23±0.44 mm. In addition, both extracts exhibited bactericidal effects, with low MIC and MBC values ranging from 6.3 to 50 μg/mL, as determined by a broth dilution assay. Moreover, biofilm formation inhibition was evaluated using the crystal violet method. The results showed that both extracts effectively inhibited biofilm formation in bacterial strains. PA and PB extracts demonstrated high efficiency in reducing the biofilm formation of *S. aureus* RTV01, achieving reductions of 94.94% and 88.43%, respectively. The extracts showed a reduction in biofilm formation of 36.18% to 94.94% in the tested strains. The chemical profiles of the plants were analyzed using the GC-MS technique, which revealed that hydroxychavicol was a major constituent of both plant species, with varying amounts and distinct profiles. *P. argyrites* exhibited a more diverse range of compounds than *P. betel*. The findings suggest that these betel plant extracts need further investigation for potential therapeutic applications.

**Keywords:** Antibacterial agents; antibiotic resistance; biofilm; *Piper* spp.

## 1. Introduction

Bacterial infections pose a serious global health challenge, especially with the rise of multidrug resistance (MDR) in both Gram-negative strains, such as *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, as well as Gram-positive strains like *S. aureus, which* can lead to various infections. *S. aureus* is particularly concerning as it can cause a wide range of illnesses, from minor skin infections to severe conditions like sepsis [1]. Similarly, Gram-negative bacteria often produce extended-spectrum beta-lactamases (ESBLs), which can inactivate extended-spectrum cephalosporins. Clavulanic acid has been proven effective in treating certain infections [2-3]. However, the presence of biofilm-forming pathogenic bacteria significantly contributes to their virulence and their connection to multidrug resistance (MDR). The biofilm structure provides several mechanisms that allow bacteria to survive and resist both antibiotic treatments and the host's

immune responses. These mechanisms include creating a physical barrier, reducing metabolic activity, evading the immune system, facilitating gene transfer, and increasing mutagenesis. Infections caused by biofilm-forming bacteria are notoriously difficult to treat, often resulting in chronic, recurring infections that do not respond to conventional therapies. This resistance to treatment underscores the critical link between biofilm formation and the challenges of managing MDR, making it a crucial aspect of bacterial pathogenicity [4-6]. These infections contribute to extended hospital stays, increased disease complications, higher treatment costs, and higher mortality rates [2]. As a result, the search for natural compounds with antimicrobial properties has become a key focus of current research. The complex composition of natural antimicrobial agents makes it harder for microorganisms to develop resistance. The *Piper* genus, including *Piper betel* (cultivated betel, also known as Phlu-Ban) and *Piper argyrites* (wild betel, also known as Phlu-Pha), has been used as traditional medicine in Satun Province, Thailand, since ancient times to the present. Despite their traditional use, further research is needed to explore their pharmacological potential. Previous studies on *Piper* species have identified numerous bioactive compounds, including eugenol, hydroxychavicol, β-caryophyllene, methyl eugenol, chavibetol, chavicol, safrole, estragole, anethole, and iso-eugenol. The presence and properties of these compounds depend on extraction methods, cultivation regions, environmental conditions, and harvesting times [7]. Bioactive substances from *Piper* species exhibit significant antimicrobial, anti-inflammatory, antioxidant, anticancer, and antidiabetic properties. However, their composition varies depending on extraction methods and environmental factors [8]. This study aims to characterize the properties of two *Piper* species, *P. betel* and *P. argyrites*, that are native to Satun Province, Thailand. While *P. betel* has been extensively studied in other regions, data specific to Satun Province are limited. Additionally, this study is the first to investigate the chemical composition of *P. argyrites*, a wild betel species traditionally used by the Maniq people in Satun and surrounding regions for treating wounds, bruises, and skin conditions [9]. The antimicrobial activity and biofilm inhibition formation properties of both species were also evaluated.

## 2. Materials and Methods

### 2.1 Plant Materials

Whole plants of *P. argyrites* and *P. betel* were purchased from the local market in Satun, Thailand, from January to March 2024. The plants were identified and authenticated by an expert taxonomist, Assoc. Prof. Jarearnsak Sae Wai from the Division of Biological Science, Faculty of Science, Prince of Songkla University. The voucher specimens have been deposited at the PSU Herbarium with the collector numbers S. Watcharakul 7 and S. Watcharakul 9, corresponding to herbarium numbers 20538 and 20954. No specific permits were required, as the location is not protected.

### 2.2 Bacterial pathogens

Bacterial pathogens (*E. coli* PT005-ESBL, *P. aeruginosa* PT00A2-ESBL, *K. pneumoniae* PT00A1-ESBL, and *S. aureus* RTV01) were collected at the microbiology laboratory (PSU_Vet2, PSU, Thailand), briefly confirmed by the double-disk synergy method applied from Livermore and Brown (2001) [10]. All isolates were maintained on Mueller-Hinton Agar (MHA; pH 6.8-7.2) and stored in Cryostock mixed (MedEx™, EU) at -80 °C for further use.

### 2.3 Plant Extraction

The whole leaves of *P. argyrites* (PA) and *P. betel* (PB) were rinsed with water and dried in a hot air oven (Binder, Germany) at 60 °C for 3-5 days. They were then milled into a powder and extracted with ethanol ($CH_3CH_2OH$). Each ethanolic extract sample was evaporated using a Heidolph rotary evaporator (Hei-VAP HL, EU, Germany) with a Cooling Water Circulator (CF800, Yamato Scientific Co., Ltd., Japan) and stored at room temperature [11].

### 2.4 Antibacterial assays

***2.4.1 Disc Diffusion Assay:*** The antibacterial activity was evaluated using the disc diffusion assay according to CLSI [12]. Briefly, the crude extract of the sample was dissolved in dimethyl sulfoxide (DMSO)

(VWR Chemicals BDH®, France) to a final concentration of 50 mg/mL and then applied to a sterile paper disc. All bacterial strains were activated at 35 °C for 18-24 hours and then inoculated on MHA. The crude extract paper disc was placed on the inoculum plate and incubated at 35 °C for 18-24 hours. The antimicrobial activity was evaluated based on measurements of the inhibition zones' appearance around the discs, with some modifications from Padumanonda and Phontree [13]. Gentamicin CN10 (10 μg, Oxoid, UK) antimicrobial susceptibility discs were used as a standard antibiotic, belonging to the aminoglycoside class, with a broad spectrum of activity against severe Gram-negative bacterial infections.

***2.4.2 MIC/MBC:*** The antimicrobial activity was assessed by determining the Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The assay tested eight different concentrations for each extract (0.390, 0.781, 1.563, 3.125, 6.25, 12.5, 25, 50 μg/mL). The tests were conducted in a 96-well plate using varying concentrations of sample extract and sterile Mueller-Hinton Broth (MHB) inoculated with bacterial concentrations of $10^5$ cfu/mL, in accordance with CLSI guidelines (CLSI M07-A9, CLSI M100-S25). The plates were then incubated at 35 °C for 18-24 hours, as applied by Chanprapai et al. [11]. The MIC was determined as the lowest concentration of the extract that showed no visible bacterial growth. Subsequently, each well of MIC broth was spread over MHA plates and further incubated at 35 °C for 24 hours to determine the MBC, indicated by the absence of visible bacterial growth at the lowest concentration of each sample. The MIC values of each sample were analyzed using a one-way analysis of variance (ANOVA) and compared with those of the standard antibiotic gentamicin (Oxoid, UK).

**2.5 Effect of Extracts on Biofilm Formation**

The biofilm production was evaluated using the classical microbiological method described by Ngoc et al. (2024) [14] with some modifications. Briefly, the bacterial strains were activated in Tryptic Soy Broth (TSB) (Oxoid, UK) supplemented with 1% sucrose (Loba Chemie Pvt Ltd, India) at 37 °C for 18-24 hours. The extracts were prepared in TSB to obtain a final concentration of 50 mg/mL in sterile 96-well polystyrene flat-bottom microtiter plates (SPL Life Science Co., Ltd., Korea) after adding 100 μL of freshly prepared bacterial cell culture at $10^6$ cells/mL corresponding to a density of 0.1 (OD600 nm) (Thermo Scientific™, Evolution™ 201/220 UV-Visible Spectrophotometers, USA). The inoculated 96-well microtiter plate was incubated at 37 °C without shaking. After 4 hours of incubation, 100 μL aliquots of diluted extracts were added to the wells of the 96-well microtiter plates. Gentamicin (Oxoid, UK) was used as a positive control, while DMSO was used as a solvent. After incubation at 37 °C for 24 hours, non-adherent cells were gently removed, and the microtiter plate was washed with sterile phosphate-buffered saline (PBS) (Oxoid, UK). The samples were then fixed at 60 °C for 1 hour. Biofilms and attached cells were stained using a 0.1% crystal violet solution (Clinag Co., Ltd., TH). After 15 minutes of the staining procedure, the samples were rinsed with sterile distilled water and dried. For the quantitative evaluation of biofilm production, a 30% acetic acid (125 μL) solution was added to destain the biofilm-associated crystal violet, and the solution's absorbance was measured. at OD 492 nm. The percentage of biofilm inhibition was determined using the following formula:

$$\%\text{Biofilm reduction} = \frac{\text{ODcontrol-ODsample}}{\text{ODcontrol}} \text{x } 100 \quad (1)$$

Where ODcontrol is the absorbance of the bacteria without the extract solution, and ODsample is the absorbance of the extract solution with bacteria.

**2.6 GC-MS analysis**

The chemical composition of the ethanolic extracts from the samples was analyzed using a Gas Chromatograph Triple Quadrupole Spectrometer (GC-MS/MS) model Agilent 7000D Triple Quadrupole GC/MS. The separation was performed on a capillary column HP-5ms Ultra Inert (Agilent 19091S-433UI) with dimensions of 30 m × 250 μm × 0.25 μm. The carrier gas was helium, with a constant flow rate of 1 mL/min. The oven temperature was programmed to range from 60°C to 320°C at a rate of 5°C/min, then held for 10 minutes. at 300°C. A sample volume of 1 μL was injected using a splitless mode with a flow rate of 3 mL/min. The mass spectrometer was set to scan in the m/z 35–500 range with electron impact (EI) ionization mode. The relative percentage of the components was represented as the percentage using peak area normalization. Identifying

compounds using a spectral library in MassHunter Qualitative Analysis version 10.0. The relative proportions were calculated by dividing the individual peak area by the total area of all peaks. Only compounds over 1% were included.

### 2.7 Statistical Analysis

The data were obtained in triplicate and expressed as means ± SD. IBM SPSS Statistics version 29.0.1.0 (179) for Windows was used for data analysis. The significance of the means was compared at $p < 0.05$ using a one-way analysis of variance (ANOVA).

## 3. Results and Discussion

### 3.1 Plant Extracts

Ethanol was used as a solvent for extracting compounds from betel leaves due to its various advantages over other solvents. As a medium-polarity solvent, it is capable of extracting a diverse range of betel leaf compounds, including both polar compounds such as flavonoids and non-polar compounds like chavicol and eugenol, which are recognized as the major bioactive components (see Table 4). Moreover, ethanol is considered a safe solvent for producing extracts intended for the food, pharmaceutical, and cosmetic industries, as it is classified as "Generally Recognized As Safe (GRAS)" by the U.S. Food and Drug Administration (FDA). This safety profile makes it particularly suitable for commercial applications.

The average percentage yield of the ethanolic leaf extracts from *P. betel* and *P. argyrites* was 0.77 and 0.84, respectively. The crude extracts exhibited distinct characteristics, as shown in Figure 1. The ethanolic leaf extracts of *P. betel* were semisolid and dark green, while those of *P. argyrites* were reddish-brown with a liquid texture. This finding is consistent with a previous study on the extraction of leaves from the *Piperaceae* family [7], which reported that members of the *Piperaceae* family contain various substances due to species differences.

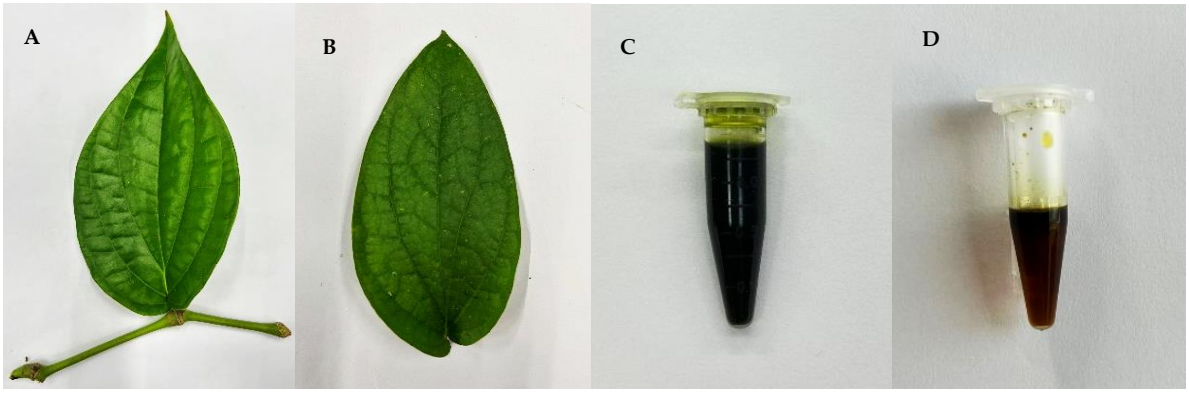


**Figure 1**. Characteristics of *P. betel* leaf (A), *P. argyrites* (B), the ethanolic leaf extract of *P. betel* (C), and *P. argyrites* (D)

### 3.2 Antibacterial Properties

#### *3.2.1 Disc Diffusion Assay*

The disc diffusion method was used to evaluate the antibacterial activity of all extracts. The results demonstrated their inhibitory effects on all tested strains, as shown in Table 1 and Figure 2. Notably, PB exhibited potent activity against *K. pneumoniae* PT00A1 and *E. coli* PT005, with inhibition zones of 25.77 ± 0.04 mm and 22.09 ± 0.23 mm, respectively. These results were very close to or even better than the gentamicin CN10 standard antibiotic (10 μg, Oxoid, UK), which produced inhibition zones of 24.41 ± 0.60 mm and 20.11 ± 0.32 mm against the same bacteria. However, its activity against *P. aeruginosa* PT00A2 and *S. aureus* RTV01 was minimal, with inhibition zones of 7.78 ± 0.18 mm and 12.15 ± 0.42 mm, as shown in Table 1. Moreover, PA showed significant antibacterial activity against *E. coli* PT005, with an inhibition zone of 17.23 ± 0.44 mm. The inhibition zones for *P. aeruginosa* PT00A2, *K. pneumoniae* PT00A1, and *S. aureus* RTV01 were 7.78±0.18 mm, 10.84±0.48 mm, and 12.15±0.42 mm, respectively. The *P. argyrites* extract was most effective against *E. coli*, but its overall activity was less potent than that of the *P. betel* extract and gentamicin.

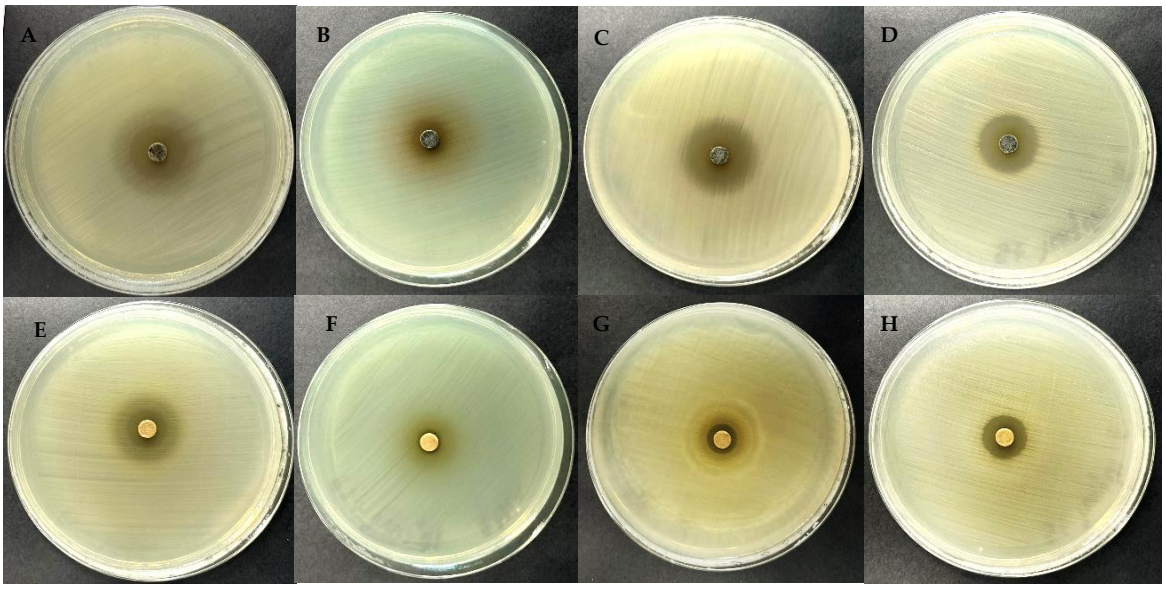


**Figure 2.** Disc diffusion assay of *P. betel* (A-D) and *P. argyrites* (E-H) ethanolic leaf extract against bacterial growth on MHA. (A, E) *E. coli* PT005, (B, F) *P. aeruginosa* PT00A2, (C, G) *K. pneumoniae* PT00A1, (D, H) *S. aureus* RTV01

**Table 1.** Antimicrobial activity of *P. betel* and *P. argyrites* ethanolic leaf extract.

| Bacterial Strains | Inhibition zone diameter (mm) | | |
|---|---|---|---|
| | ***P. betel*** | ***P. argyrites*** | **Gentamicin CN10** |
| *E. coli* PT005 | 22.09 ± 0.23 [a] | 17.23 ± 0.44 [c] | 20.11 ± 0.32 [b] |
| *P. aeruginosa* PT00A2 | 10.94 ± 0.31 [b] | 7.78 ± 0.18 [c] | 12.18 ± 0.29 [a] |
| *K. pneumoniae* PT00A1 | 25.77 ± 0.04 [a] | 10.84 ± 0.48 [c] | 24.41 ± 0.60 [b] |
| *S. aureus* RT0V1 | 15.42 ± 0.21 [a] | 12.15 ± 0.42 [b] | 11.66 ± 0.16 [c] |

Note: Values, an average of the mean inhibition zones (mm) ± standard deviation (SD) of duplicates of the extracts at 50 mg/disc of each isolate. Mean values within rows followed by a different letter differ significantly ($p<0.05$; DMRT).

### *3.2.2 MIC/MBC*

The ethanolic leaf extracts of PB and PA demonstrated significant antibacterial activities against all tested strains in the broth microdilution assay. MIC and MBC values ranged from 6.25 to 50 µg/mL, indicating potent antibacterial effects compared with the standard antibiotic gentamicin, as shown in Table 2. PB extracts displayed intense antibacterial activity against *E. coli* PT005 and *K. pneumoniae* PT00A1, with MIC values of 6.25 µg/mL, while the MIC values for *P. aeruginosa* PT00A2 and *S. aureus* RTV01 were 25 µg/mL and 12.5 µg/mL, respectively. Moreover, PA extracts demonstrated MIC results with lower antibacterial activity than PB extracts for all tested strains. MIC values for *E. coli* PT005 and *S. aureus* RTV01 were 12.5 µg/mL, whereas the MIC values for *P. aeruginosa* PT00A2 and *K. pneumoniae* PT00A1 were 50 µg/mL and 25 µg/mL, respectively (Table 2). These results represented a MIC index of ≤ 2 (MBC/MIC ratio) for both PB and PA extracts, suggesting a bactericidal mode of action. The MIC index has been used to determine whether a substance possesses bactericidal properties; for bacteriostatic properties, the MIC index is ≤ 4 [11]. The results clearly indicate that both extracts possess strong antibacterial properties, with low MIC and MBC values, suggesting that these extracts can effectively combat bacterial strains according to the MIC index. This confirms their bactericidal nature, as the extracts inhibit bacterial growth and kill bacterial strains directly. Additionally, the extracts were effective against both Gram-positive (*S. aureus* RTV01) and Gram-negative (*E. coli* PT005, *K. pneumoniae* PT00A1, *P. aeruginosa* PT00A2) bacteria, suggesting broad-spectrum antibacterial activity. In view of these findings, both extracts demonstrated a bactericidal mode of action with MIC and MBC values ranging from 6.25 to 50 µg/mL. The PB extract was particularly effective, as evidenced by its MIC

values of 6.25 μg/mL for *E. coli* PT005 and *K. pneumoniae* PT00A1. The MIC value is comparable to that of gentamicin against *K. pneumoniae*, indicating that the PB extract has a similar bactericidal effect to gentamicin on these specific bacterial strains. However, based on previous studies on the antibacterial activity of the *Piperaceae* family, the antimicrobial activity of mature leaves is effective against *E. coli*, *Streptococcus pyogenes*, *P. aeruginosa*, *S. aureus*, *Proteus vulgaris*, and *Salmonella* spp., with low MIC and MBC values, as presented in the bactericidal activity. [15-17]. The efficiency of extracting substances varies in terms of antibacterial activity and main chemical components due to various environmental factors, including the location and harvest time of the plant.

**Table 2.** The MIC and MBC of the plant's ethanolic leaf extract

| Strains | *P. betel* | | | *P. argyrites* | | | Gentamicin | | |
|---|---|---|---|---|---|---|---|---|---|
| | MIC | MBC | MBC/MIC ratio | MIC | MBC | MBC/MIC ratio | MIC | MBC | MBC/MIC ratio |
| *E. coli* PT005 | 6.25 | 6.25 | 1 | 12.5 | 12.5 | 1 | 12.5 | 12.5 | 1 |
| *P. aeruginosa* PT00A2 | 25 | 25 | 1 | 50 | 50 | 1 | 12.5 | 25 | 2 |
| *K. pneumoniae* PT00A1 | 6.25 | 12.5 | 2 | 12.5 | 25 | 2 | 6.25 | 12.5 | 2 |
| *S. aureus* RTV01 | 12.5 | 25 | 2 | 12.5 | 25 | 2 | 12.5 | 25 | 2 |

Note: The MBC/MIC ratio, where MBC is the minimum bactericidal concentration and MIC is the minimum inhibitory concentration, with an MBC/MIC ratio ≤ 4, indicates that a drug is considered bacteriostatic.

**3.3 Biofilm Formation Reduction**

The biofilm formation inhibition was carried out in microtiter plates using the classical crystal violet method [14] with some modifications. The ethanolic leaf extracts of PB and PA effectively inhibited biofilm formation in all tested bacterial strains (Table 3). At the same concentrations, PB and PA extracts demonstrated strong inhibitory effects on the biofilm formation of *S. aureus* RTV01, with reductions of 88.43% and 94.94%, respectively. Additionally, PA extracts reduced the biofilm formation of *K. pneumoniae* PT00A1, *E. coli* PT005, and *P. aeruginosa* PT00A2 by 88.35%, 88.05%, and 36.54%, respectively. In comparison, PB extracts exhibited lower inhibitory effects on the biofilm formation of *K. pneumoniae* PT00A1 and *E. coli* PT005 than PA extracts, with reductions of 36.54% and 79.88%, respectively. In contrast, the effect of PB extracts on reducing biofilm formation was higher in *P. aeruginosa* PT00A2, at 46.44%. Gentamicin was used as a positive control, as represented in Table 3. Based on the findings of this study, the percentage of biofilm formation reduction ranged from 36.18% to 94.94% for all tested strains, highlighting the significant antibiofilm potential of both plant extracts. Notably, the PA extract showed a slightly higher efficiency in reducing the formation of *S. aureus* RTV01 biofilm compared to the PB extract and standard antibiotic gentamicin.

However, the *in vitro* study revealed varying inhibitory effects of extracts on biofilm formation reduction for each strain. These variations could be due to differences in the mechanisms of biofilm formation among the different strains. The biofilm formation process involves several stages: attachment, multiplication, exodus, maturation, and dispersion. Moreover, the type of biofilm also varies according to bacterial functions, such as adhesion, metabolic pathways, and the synthesis of extracellular polymeric substances (EPS) [18]. However, the chemical composition of plants is responsible for their antibacterial properties; even if the same plant genus were harvested under the same environmental conditions, differences in phytochemical contents could affect their antimicrobial effects. Based on publications on the antimicrobial activity of plant extracts, *P. betel* has been shown to affect the biofilm formation of *Serratia marcescens* and *S. aureus* by reducing exopolysaccharide production and the hydrophobicity index at the early stages of biofilm formation, thereby postponing the lag time and slowing down the growth rate [19]. This information suggests that *P. betel* and *P. argyrites* ethanolic leaf extracts likely inhibit biofilm formation at the early adherence and multiplication stages of *S. aureus* and other tested strains due to their enhanced antibacterial properties. These findings indicate the significant antibiofilm formation activity of the examined extracts, making them a promising alternative therapy and highlighting the potential of both *P. argyrites* and *P. betel* as sources of new

antimicrobial drugs. *P. betel* is a powerful antibacterial agent, while the *P. argyrites* extract may be more valuable due to its antibiofilm properties. Their most significant potential lies in their dual-action ability to both kill bacteria and inhibit biofilm formation, a crucial advantage in the face of growing antibiotic resistance. Furthermore, extensive traditional and emerging scientific evidence suggests their value extends beyond antimicrobial use, indicating they could be developed into treatments for various conditions, including cancer, inflammation, and digestive ailments [7]. These findings warrant further research and development to explore their full therapeutic potential in modern medicine.

**Table 3.** Antibiofilm formation of plant ethanolic leaf extract at a concentration of 50 mg/mL

| Bacterial Strains | Biofilm reduction (%) | | |
|---|---|---|---|
| | ***P. betel*** | ***P. argyrites*** | ***Gentamicin*** |
| *E. coli* PT005 | 79.88 ± 0.41[b] | 88.05 ± 0.55 [a] | 69.97 ± 0.52 [c] |
| *P. aeruginosa* PT00A2 | 46.44 ± 0.35 [a] | 36.18 ± 0.61[c] | 42.17 ± 0.41 [b] |
| *K. pneumoniae* PT00A1 | 36.54 ± 0.54 [c] | 88.35 ± 0.74 [a] | 75.33 ± 0.69 [b] |
| *S. aureus* RT0V1 | 88.43 ± 0.84 [c] | 94.94 ± 0.58 [a] | 92.89 ± 0.63 [b] |

Note: Data shown as mean ± SD values from triplicate analysis
Mean values within rows followed by a different letter differ significantly ($p<0.05$; DMRT).

**3.4 Chemical Compositions of Extracts**

The chemical composition analysis using GC-MS of PA and PB ethanolic leaf extracts revealed distinct compositional profiles. PA exhibited a more diverse chemical composition than PB extracts, containing hydroxychavicol (24.8%), elemenes (5.45%), caryophyllene (5.02%), and many other constituents (Table 4). Under the same conditions, PB extracts revealed dominant compounds, including hydroxychavicol (64.01%), eugenol (22.40%), and phytol (1.43%), with minor components comprising less than 0.01% of the total, as shown in Table 4. Notably, hydroxychavicol was a main component in both plant species, aligning with their classification within the *Piper* genus. While both plant species shared these common compounds, the quantitative distribution differed significantly, with PB exhibiting approximately three times higher levels of hydroxychavicol than PA extract; conversely, PA extracts demonstrated a more varied chemical composition.

As stated above, it was revealed that the specific antibacterial and antibiofilm activities of the two extracts are influenced by their unique chemical profiles. Although *P. betel* contains a significantly higher concentration of hydroxychavicol, the more diverse composition of *P. argyrites* appears to give it a greater efficacy in certain areas, such as biofilm reduction in *S. aureus* RTV01. This finding suggests that the synergistic effects of various compounds, rather than the concentration of a single component, play a crucial role in the plant's biological activity.

According to previous studies on bioactive substances in the *Piper* genus, particularly in betel leaves, the presence of phytol, hydroxychavicol, eugenol, and caryophyllene is a significant constituent, although with varying concentrations across different cultivation regions and even within the same species. These variations in the chemical composition of betel leaves depend on environmental factors such as soil type, nutrient content, and climatic conditions, which influence the chemical composition profiles and, consequently, the plants' biological activities [20]. This report reveals the chemical composition profiling of wild betel (PA) ethanolic leaf extract for the first time. Remarkably, PA and PB leaf extracts contain natural antioxidant properties due to their polyphenol compounds, such as hydroxychavicol [21]. Additionally, the presence of various bioactive components, such as eugenol, phytol, caryophyllene, and terpenes [22], exhibits antimicrobial properties against *P. aeruginosa, S. aureus, E. coli, Streptococcus pyogenes*, and *Proteus vulgaris* [15, 17, 23]. The important bioactive compound in the plant extracts (PA and PB), hydroxychavicol, has been reported in previous studies to possess potent antibacterial properties, with a mechanism of action in *E. coli* involving the stripping of magnesium ions, inducing oxidative stress followed by membrane and DNA damage, and inducing cell permeabilization, which inhibits and damages the outer membrane that is responsible for resistance to several antibiotics [24]. Moreover, bioactive compounds in the *Piper* genus have been reported to possess anticancer properties responsible for the antiproliferation of cancer cells [25-26],

as well as anti-inflammatory activities and antidiabetic properties [7], highlighting their significant pharmacological potential. However, more research is needed to evaluate their efficacy and safety for therapeutic purposes.

**Table 4.** Chemical composition of the ethanolic leaf extract of plants

| Plants | Retention time | Compounds Name | Percent of the total* | Chemical formula |
|---|---|---|---|---|
| *P. argyrites* | 14.5866 | L-α-Terpineol | 2.19 | $C_{10}H_{18}O$ |
| | 20.2880 | Elemene | 5.45 | $C_{15}H_{24}$ |
| | 20.9998 | Caryophyllene | 5.02 | $C_{15}H_{24}$ |
| | 21.8594 | 1,5,9,9-tetramethyl-1,4,7-Cycloundecatriene | 2.5 | $C_{15}H_{24}$ |
| | 22.4159 | Hydroxychavicol | 24.8 | $C_9H_{10}O_2$ |
| | 22.9071 | γ-Selinene | 3.88 | $C_{15}H_{24}$ |
| | 30.6094 | Hexahydro-3H-benzofuran-2-one | 4.39 | $C_{15}H_{20}O_2$ |
| | 36.2518 | α-Linolenic acid | 4.12 | $C_{18}H_{30}O_2$ |
| | 54.7662 | γ-Tocopherol | 2.95 | $C_{29}H_{50}O_2$ |
| *P. betel* | 19.6819 | Eugenol | 22.41 | $C_{10}H_{12}O_2$ |
| | 20.9941 | Caryophyllene | 1.65 | $C_{15}H_{24}$ |
| | 22.4163 | Hydroxychavicol | 64.01 | $C_9H_{20}O_2$ |
| | 35.7476 | Phytol | 1.43 | $C_{20}H_{40}O$ |

* % of the total is implied to be the percent of the total in terms of the peak area relative to the total peak area.

## 4. Conclusion

The ethanolic leaf extracts of *P. argyrites* and *P. betel* showed potent antimicrobial properties and a substantial effect on the reduction of biofilm formation in MDR isolates and common pathogens, including *E. coli* PT005, *P. aeruginosa* PT00A2, *K. pneumoniae* PT00A1, and *S. aureus* RTV01. In addition, the extracts exhibited hydroxychavicol as a major constituent in significantly different amounts, followed by minor compounds, including eugenol, elemenes, caryophyllene, and various other compounds. This report presents the chemical composition of *P. argyrites* leaf extract, as determined by the GC-MS technique, and highlights its potent antimicrobial properties. Overall, this study suggests that *P. argyrites* and *P. betel* are rich sources of potent bioactive substances, which could potentially revolutionize the fields of alternative therapy and pharmacology.

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