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Antivirulence Potential of Betel Leaves *(Piper betle L.)* in Combating Antimicrobial Resistance

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Abstract: ***The global rise in antimicrobial resistance (AMR) has necessitated the exploration of alternative therapeutic strategies. Betel leaves (Piper betle L.), a traditional medicinal plant widely used in Asian cultures, have demonstrated promising antimicrobial and antivirulence properties. This paper reviews the phytochemical composition of betel leaves and examines their potential role in inhibiting virulence factors such as biofilm formation, quorum sensing, and toxin production in pathogenic microorganisms. Drawing from existing in vitro and in vivo studies, this research highlights the therapeutic relevance of natural antivirulence agents in addressing AMR while minimizing selective pressure for resistance development.***

Keywords: *Piper betle* L.; antimicrobial resistance; antivirulence; biofilm inhibition; quorum sensing; phytochemicals

1. Introduction

Antimicrobial resistance (AMR) has emerged as one of the most pressing global health challenges of the 21st century. The rapid and widespread misuse of antibiotics in clinical, agricultural, and veterinary settings has accelerated the evolution of resistant microbial strains, rendering many conventional antibiotics ineffective. The World Health Organization has repeatedly warned that the world is approaching a "post-antibiotic era" in which common infections may once again become life-threatening. Resistant pathogens such as *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* have demonstrated an alarming ability to evade multiple classes of antibiotics, complicating treatment outcomes and increasing morbidity, mortality, and healthcare costs.

In response to this growing crisis, contemporary research has shifted toward innovative therapeutic strategies that move beyond traditional bactericidal or bacteriostatic approaches. One such promising strategy is antivirulence therapy, which aims to neutralize pathogenic mechanisms rather than directly kill microorganisms. By targeting virulence factors such as toxin production, adhesion molecules, quorum sensing systems, and biofilm formation, antivirulence agents reduce the pathogenicity of microorganisms without imposing strong selective pressure for resistance development. This strategy is considered advantageous because it potentially minimizes the rapid emergence of resistant strains that commonly arises from conventional antibiotic use.

Natural medicinal plants have gained renewed attention as valuable sources of novel bioactive compounds. Among these, *Piper betle* L., commonly known as betel leaf, holds a prominent place in traditional medicine systems across South and Southeast Asia. Historically, betel leaves have been used for their antiseptic, anti-inflammatory, wound-healing, and digestive properties. Ethno pharmacological evidence supports their long-standing use in treating oral infections, respiratory disorders, and gastrointestinal ailments. Scientific investigations have validated many of these traditional claims, demonstrating significant antimicrobial, antioxidant, and anti-inflammatory activities (Arambewela et al., 2005).

Recent studies suggest that beyond its direct antimicrobial effects, *Piper betle* L. may also exert substantial antivirulence properties. The plant's rich phytochemical composition provides a biochemical basis for its therapeutic potential. Therefore, understanding the antivirulence mechanisms of betel leaf extracts could contribute to the development of alternative or adjunct therapies aimed at combating AMR. This study explores the phytochemical profile of betel leaves and critically examines their potential role in inhibiting key virulence factors in pathogenic microorganisms.

Figure 1: Betel Leaves

Phytochemical Composition of Betel Leaves

The therapeutic potential of *Piper betle* L. is largely attributed to its diverse array of bioactive phytochemicals. Betel leaves contain essential oils, phenolic compounds, alkaloids, tannins, flavonoids, and terpenoids. Among these, eugenol, chavicol, hydroxychavicol, chavibetol, and various phenolic derivatives are considered the primary active constituents responsible for antimicrobial and antivirulence effects.

Eugenol, a phenolic compound also found in clove oil, exhibits strong antimicrobial and antioxidant properties. It disrupts microbial cell membranes, increases permeability, and interferes with enzymatic activity. Chavicol and chavibetol similarly contribute to antimicrobial activity through membrane disruption and interference with metabolic processes. However, one of the most extensively studied compounds in betel leaves is hydroxychavicol**,** which has demonstrated potent antibacterial, antifungal, and antibiofilm activities.

Hydroxychavicol acts by inhibiting microbial adhesion, a critical first step in biofilm formation. Biofilms are structured microbial communities embedded within a self-produced extracellular matrix that protects pathogens from antibiotics and host immune responses. By preventing initial attachment and interfering with extracellular polymeric substance (EPS) production, hydroxychavicol significantly reduces biofilm establishment. This property is particularly important because biofilm-associated infections are notoriously resistant to conventional antibiotic therapy.

In addition to phenolic compounds, flavonoids and tannins present in betel leaves contribute antioxidant properties that reduce oxidative stress and inflammation. These compounds may indirectly support host defense mechanisms while impairing microbial virulence. The synergistic interaction among these phytochemicals enhances the overall therapeutic efficacy of betel leaf extracts, making them promising candidates for further pharmacological investigation (Prakash et al., 2014).

Antivirulence Mechanisms

The antivirulence properties of *Piper betle* L. have been systematically investigated through specific experimental models targeting critical virulence determinants, including coagulase activity, extracellular polymeric substance (EPS) production associated with capsule formation, and biofilm development assessed by the crystal violet assay. Unlike conventional antibiotics that aim to eradicate bacterial cells, these approaches evaluate the ability of betel leaf extracts to attenuate pathogenic traits without exerting direct bactericidal pressure.

One important virulence factor studied is coagulase, an enzyme predominantly produced by *Staphylococcus aureus*. Coagulase plays a crucial role in pathogenicity by converting fibrinogen to fibrin, leading to clot formation around bacterial cells. This clot acts as a protective barrier, shielding the pathogen from host immune defenses such as phagocytosis. Inhibition of the coagulase mechanism significantly reduces bacterial survival within host tissues. Experimental investigations have demonstrated that treatment with *Piper betle* leaf extracts leads to a marked reduction in coagulase activity, thereby impairing clot-mediated immune evasion. The suppression of this enzyme suggests that betel leaf phytochemicals interfere with virulence expression rather than bacterial growth, supporting their potential role as antivirulence agents.

Another key mechanism involves the inhibition of extracellular polymeric substance (EPS) production, which is essential for capsule formation. The bacterial capsule is a protective polysaccharide layer that enhances resistance to phagocytosis, antimicrobial agents, and environmental stress. EPS also serves as the structural foundation of biofilms, facilitating microbial adhesion and persistence on host tissues or medical devices. Studies evaluating *Piper betle* extracts have shown significant reductions in EPS synthesis, indicating disruption of capsule development. By inhibiting EPS production, betel leaf phytochemicals weaken bacterial structural defenses, making pathogens more susceptible to immune clearance and antimicrobial therapy. This effect is particularly relevant in encapsulated organisms where capsule formation directly correlates with virulence severity.

Biofilm inhibition represents another major antivirulence property evaluated using the crystal violet assay, a standard quantitative method for assessing biofilm biomass. Biofilms are structured microbial communities embedded in an EPS matrix that protects bacteria from antibiotics and host immune responses. Chronic infections, including urinary tract infections, wound infections, and respiratory tract infections, are frequently associated with biofilm-forming pathogens. In experimental models, bacterial cultures treated with *Piper betle* leaf extracts demonstrate a significant decrease in biofilm biomass when measured through crystal violet staining. The assay quantifies adherent biofilm cells by staining them with crystal violet dye, followed by solubilization and spectrophotometric measurement. A reduction in absorbance values compared to untreated controls confirms inhibition of biofilm formation. These findings indicate that betel leaf extracts interfere with microbial adhesion and matrix development, critical steps in biofilm establishment.

The combined inhibition of coagulase activity, EPS-mediated capsule formation, and biofilm development highlights the multi-targeted antivirulence potential of *Piper betle* L. Such multi-pathway interference reduces the likelihood of resistance development, as pathogens would need to simultaneously adapt to multiple disrupted mechanisms. Furthermore, because these strategies attenuate pathogenicity without directly killing bacterial cells, they exert lower selective pressure compared to conventional antibiotics.

Experimental studies involving clinically relevant pathogens such as *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* have supported these findings, demonstrating significant reductions in virulence factor expression following exposure to betel leaf extracts. These results reinforce the concept that *Piper betle* L. phytochemicals function as effective antivirulence agents capable of complementing existing antimicrobial therapies.

2. Materials and Methods

1) Preparation of *Piper betle* L. Leaf Extract

Fresh leaves of *Piper betle* L. were collected, authenticated, washed thoroughly with distilled water, and shade-dried at room temperature. The dried leaves were pulverized into fine powder using a motor-pestle. The powdered material was extracted using ethanol (or methanol/aqueous solvent as applicable) through Soxhlet extraction for 6–8 hours. The extract was filtered using Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary

evaporator. The dried crude extract was stored at 4°C until further use.

Stock solutions were prepared by dissolving the extract in dimethyl sulfoxide (DMSO) and diluted to required concentrations using sterile nutrient broth. The final DMSO concentration did not exceed 1% (v/v) to avoid solvent-induced effects.

2) Microbial Strains and Culture Conditions

Laboratory cultures of *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumonia* were used in the study. Bacterial strains were cultured in nutrient broth at 37°C for 18–24 hours before experimentation. Cultures were standardized to 0.5 McFarland turbidity standard (~1 × 10^8 CFU/mL) prior to assays.

3) Coagulase Inhibition Test

The coagulase inhibition assay was performed to evaluate the effect of *Piper betle* extract on coagulase enzyme activity in *Staphylococcus aureus*.

Tube Coagulase Test

Overnight cultures of *S. aureus* were incubated with different concentrations of *Piper betle* extract for 4 hours at 37°C. After incubation, 0.5 mL of treated bacterial suspension was mixed with 0.5 mL of 1:10 Diluted plasma (anticoagulated with EDTA) in sterile test tubes.

Control Tubes included:

- Positive control: Untreated bacterial suspension with plasma
- Negative control: Plasma without bacterial inoculation

The tubes were incubated at 37°C and examined at 1, 2, and 4 hours for clot formation. The absence or reduction of clot formation compared to the positive control indicated inhibition of coagulase activity.

Results were recorded qualitatively (presence/absence of clot) and semi-quantitatively based on clot firmness.

4) Capsule Inhibition Test

Microscopic Method

Capsule staining (using Manewal's staining method) was performed on treated and untreated bacterial cells. Smears were prepared on clean glass slides, stained appropriately, and observed under oil immersion microscopy (1000× magnification). Reduction in capsule thickness or absence of clear halo surrounding bacterial cells was interpreted as capsule inhibition.

5) Crystal Violet Assay

a) Sterile glass test tubes containing 5 mL of nutrient broth were prepared as follows:

- **Control Tube (Positive Control):** Broth + bacterial inoculum (no extract)
- **Test Tubes:** Broth + bacterial inoculum + varying concentrations of *Piper betle* extract (Undiluted, 1:2, 1:4, 1:6)
- **Negative Control:** Broth only (no bacteria)

Each tube was inoculated with 1% (v/v) standardized bacterial suspension.

b) Incubation

All tubes were incubated statically at 37°C for 24 hours to allow biofilm formation on the inner surface of the tubes.

c) Removal of Planktonic Cells

After incubation:

- Broth cultures were gently discarded.
- Tubes were washed carefully three times with sterile PBS to remove non-adherent (planktonic) cells.
- Tubes were inverted and air-dried for 30–60 minutes.

d) Staining with Crystal Violet

- Each tube was filled with 0.1% crystal violet solution.
- Tubes were allowed to stand at room temperature for 15 minutes.
- Excess stain was discarded.
- Tubes were rinsed gently with distilled water to remove unbound dye.
- Tubes were air-dried in an inverted position.

e) Semi-Quantitative Measurement

To quantify biofilm biomass:

- Add 5 mL of 95% ethanol (or 33% acetic acid) to each stained tube.
- Incubate for 10–15 minutes to solubilize the bound dye.
- Transfer the solution to cuvettes.
- Measure absorbance at 570 nm using a spectrophotometer.

Calculation of Percentage Biofilm Inhibition

$$Biofilm\ Inhibition\ (\%) = \frac{(OD\ control - OD\ test)}{(OD\ Control)} \times 100$$

Where:

- OD control = Absorbance of untreated control
- OD test = Absorbance of extract-treated sample

3. Results

1) Preparation of Betle leaves extract:

Fig. 2 Betle Leaves Extract

2) Coagulase Inhibition Assay

The coagulase inhibition test demonstrated a significant reduction in clot formation in extract-treated samples compared to the positive control.

- The untreated control showed firm clot formation within 2–4 hours, confirming active coagulase enzyme production.
- In contrast, extract-treated samples exhibited complete absence of clot formation.

3) Capsule Inhibition Test

Capsule inhibition by the *Piper betle* leaf extract was evaluated using both cultural and microscopic methods against *Klebsiella pneumoniae*.

a) **MacConkey Agar Observation**

On *MacConkey agar*, untreated *Klebsiella pneumoniae* cultures produced large, glistening mucoid colonies, characteristic of capsulated strains.

In contrast, extract-treated cultures showed:

- Marked reduction in mucoid appearance
- Formation of small, dry, non-mucoid colonies

This change indicates suppression of extracellular polysaccharide (capsule) production.

b) Microscopic Analysis

Capsule staining further supported these findings:

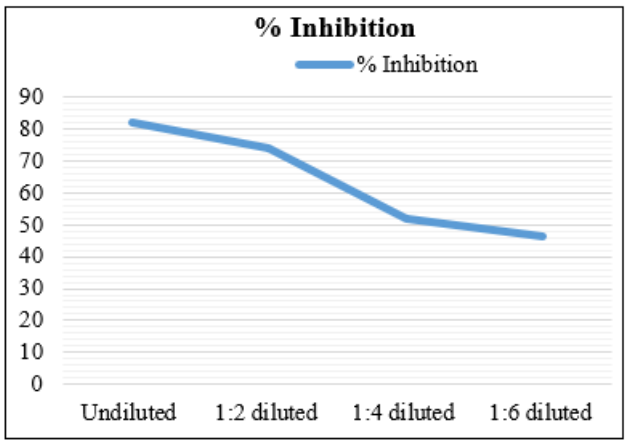
- Untreated cells displayed a well-defined capsule, visible as a clear halo surrounding the bacterial cells.
- Extract-treated cells exhibited:
  - Significantly reduced capsule thickness

4) Biofilm Inhibition (Crystal Violet Assay)

The crystal violet assay demonstrated a strong inhibitory effect of the extract on biofilm formation in all tested bacterial strains.

- The positive control showed dense biofilm formation, indicated by high absorbance values at 570 nm.
- Extract-treated samples showed a progressive decrease in biofilm biomass with increasing extract concentration.

Concentration of Betel leaves extract	% Inhibition
Undiluted	82.2
1:2	74.0
1:4	52.0
1:6	46.6



4. Discussion

The present study highlights the significant antimicrobial and antivirulence activity of Piper betle leaf extract against major bacterial pathogens. The complete inhibition of coagulase activity in Staphylococcus aureus suggests interference with an important virulence enzyme responsible for immune evasion. Similarly, capsule inhibition in Klebsiella pneumoniae, evidenced by loss of mucoid colonies on MacConkey agar and reduced halo in microscopy, indicates suppression of protective polysaccharide layers. The extract also demonstrated strong antibiofilm activity, with maximum inhibition of 82.2%, confirming its ability to disrupt bacterial adherence and colonization. These combined effects suggest that the extract targets multiple virulence factors simultaneously. Such multi-target action is particularly important in overcoming antimicrobial resistance. Overall, the findings support the therapeutic potential of Piper betle in controlling pathogenic bacteria.

5. Conclusion

In conclusion, the *Piper betle* leaf extract exhibited potent antimicrobial and antivirulence properties against the tested organisms. It effectively inhibited coagulase activity in *Staphylococcus aureus*, reduced capsule formation in *Klebsiella pneumoniae*, and significantly decreased biofilm formation across all tested strains. The highest biofilm inhibition of 82.2% at undiluted concentration highlights its strong efficacy. These results indicate that the extract can impair key mechanisms involved in bacterial pathogenicity and survival. Therefore, *Piper betle* may serve as a promising natural source for developing alternative antimicrobial agents. However, further studies including compound isolation, toxicity analysis, and in vivo validation are required. This study provides a strong foundation for future research in plant-based antimicrobial therapies.

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