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Exploring the Antimicrobial and Antibiofilm Activities of *Luffa cylindrica* against *Pseudomonas aeruginosa* and *Enterococcus faecalis*

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Abstract

Biofilm-producing Pseudomonas aeruginosa (P. aeruginosa) and Enterococcus faecalis (E. faecalis) pose a significant risk to patients due to their enhanced resistance to antibiotics and the immune response. Studies show that preventing the generation of virulence factors and the formation of biofilms can mitigate the pathogenesis of P. aeruginosa and E. faecalis. The natural medicinal plant extract of Luffa cylindrica (L. cylindrica) has the potential to inhibit the quorum sensing (QS) system in both PAO1 and E. faecalis at low concentrations. The study, conducted over one year from April 2023 to April 2024, involved a series of in-vitro investigations designed to evaluate the antimicrobial and antibiofilm activities of L. cylindrica extract. These investigations included determining the antimicrobial analysis, antibiotic susceptibility testing, Minimum Inhibitory Concentration (MIC), performing crystal violet biofilm inhibition assays, evaluating bacterial growth curves, and quantifying extracellular polymeric substances (EPS) at specific concentrations. The results demonstrated that the ethanol extract from L. cylindrica inhibited the proliferation of PAO1 at 10 mg/mL and E. faecalis at 2.5 mg/mL. Subsequent antibiofilm studies revealed that L. cylindrica extract inhibited biofilm formation in PAO1 at 2.5 mg/mL and in E. faecalis at 0.625 mg/mL. Additionally, the L. cylindrica extract significantly reduced the production of EPS in both organisms. These findings highlight the potential of L. cylindrica as an antipathogenic compound capable of preventing QS-dependent pathogenicity in PAO1 and E. faecalis.

Keywords: *Antibiotic Resistance, Biofilm, E. faecalis, L. cylindrica, PAO1, Virulence Factors.*

Introduction

The potential for clinical pathogens to spread a wide range of infections poses a serious threat to human health. Treatment and control of infectious diseases become more difficult when clinical pathogens develop and acquire antimicrobial resistance [1]. Bacteria classified as critical importance include *Acinetobacter, Pseudomonas* and *some Enterobacteriaceae spp* [2]. These pathogens exhibit resistance to a range of antibiotics, posing a significant risk leading to serious and deadly infectious diseases such as bloodstream infections and pneumonia [3]. Bacteria with a high priority category include *Enterococcus faecalis* (*E. faecalis*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) both are resistant to a wide range of antibiotics, including quinolones, aminoglycoside, β-lactams, and cephalosporins [4,5].

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The presence and pathogenicity of *P. aeruginosa* are dependent on the synthesis of lytic enzymes such as protease, elastase, pyocyanin pigment, exopolysaccharide (EPS) [6–8]. Uses of four primary quorum sensing (QS) systems by which each of these systems is linked with its unique autoinducer: 3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL), 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal - PQS), N-butanol homoserine lactone (C4-HSL) and 2-(2-hydroxy-phenyl)-thiazole-4-carbaldehyde (integrated quorum sensing signal - IQS), respectively [9]. When these AHL molecules bind to their respective receptors, LasR and RhlR, induce the expression of pathogen phenotypes. [6,10]. The expression of rhamnolipid is controlled by the RhlI/RhlR system, in the LasI/LasR system also plays a key role in virulence factors [11].

One key step in the development of *E. faecalis* is the ability to attach to host tissues, especially in case of urinary tract infection (UTI) [12]. *E. faecalis* plays a significant role in dental caries, especially in the context of persistent infections in root canals [13]. The pathogenicity of *P. aeruginosa* is regulated by a variety of virulence factors [14]. There are several virulence factors such as aggregation substance (Agg) to initiate adherence in response to the induction of pheromone another factor associated with adherence to collagen (Ace). Both Agg and Ace play important roles in host tissue adherence and colonization. [15,16]. Furthermore, cytolysin, gelatinase enzyme (GelE), serine protease (SprE), gelatinase and casein play a significant function in damaging the host tissue and spreading Enterococci in their host. [17]. The primary component of the QS is the fsr regulator locus which is comprised of fsrA, fsrB, and fsrC genes, these genes play a key role in controlling the expression of gelatinase and serine protease [18]. The coordinated expression of these factors in *P. aeruginosa* and *E. faecalis* helps the bacteria adapt to population density and environmental changes. Eliminating these QS systems via antimicrobial compounds will be a viable option for eliminating *P. aeruginosa* and *E. faecalis* infections. Due to the production of virulence factors, most of the antibiotics seem to lose effectiveness and, in turn, enhance bacterial pathogenesis resulting in a death rate ranging from 18% to 61% of patients [19]. Therefore, the search for alternative antimicrobial agents has become essential.

Natural products have proven to be a valuable source of novel pharmacological compounds, and there is renewed interest in investigating them as potential drug candidates, particularly in the context of combating antimicrobial resistance. Plant-based bioactive compounds inhibit disease pathogenesis genes by disrupting QS-associated virulence factors. In developing nations, it is of utmost importance to prioritize the documentation of medicinal plants as a matter of great urgency [20]. As a result, there is an urgent need to identify effective quorum sensing inhibitors (QSIs), preferably derived from medicinal resources.

Luffa cylindrica (L. cylindrica) is a medicinal plant, member of the *cucurbitaceous* family widely distributed in the humid tropics and Asia. [21]. It has been traditionally used for culinary purposes sometimes referred to as the ridge gourd, sponge gourd, or loofah and with fruits containing black seeds has shown promising bioactive properties. The herb of *L. cylindrica* is known to harbour a neutral bitter principle along with an alkaloid, saponins, and flavonoids. Due to their anticarcinogenic, antimicrobial, antifungal, anti-inflammatory, wound healing, and hypoglycaemic properties, the fruits, and seeds have been widely used in traditional medicine. In Korea, the pulp of the *L. cylindrical* fruit is employed for various purposes, including alleviating fever, promoting blood clotting, encouraging menstrual flow, reinforcing blood vessels, revitalizing blood, and clearing respiratory mucus [22].

L. cylindrica showed a strong affinity for the substrate-binding pocket of SARS-CoV-2 Mpro, as reflected by docking energy scores of -7.54, -7.47, -7.29, and -7.13 kcal/mol. These scores were notably comparable to the binding ability of the N3 protease inhibitor (−7.51 kcal/mol), a well-established inhibitor [23]. Altogether, *L. cylindrical* holds great promise as a source of beneficial phytochemicals, with potential applications for improving human health. [21]. This study introduces *L. cylindrica* extract as a novel antimicrobial and antibiofilm agent effective at low concentrations against *P. aeruginosa* and *E. faecalis* by inhibiting QS thus presenting a promising natural alternative for combating antibiotic-resistant infections.

Here, we aimed to investigate the antibiofilm properties of *L. cylindrica* against the bacterial strains *P. aeruginosa* and *E. faecalis*. Our objective was to evaluate the effectiveness of *L. cylindrica* in preventing and disrupting biofilm formation by these common pathogens.

Materials and Methods

Study Period

The one-year study, conducted from April 2023 to April 2024, investigates the antimicrobial and antibiofilm properties of *Luffa cylindrica* (*L. cylindrical*) extract against *Pseudomonas aeruginosa* (PAO1) and *Enterococcus faecalis* (*E. faecalis*). The study encompasses various methods including solvent extraction, antimicrobial activity assays, antibiotic susceptibility testing, Minimum Inhibitory Concentration (MIC) determination, biofilm inhibition assays, bacterial growth curve analysis, and EPS quantification.

Sample Collection

L. cylindrical seed samples were obtained from the indigenous botanical garden located in Chennai, Tamil Nadu, India. The authenticity of the seeds was verified by a qualified botanist. Seeds were rinsed with distilled water and dried at room temperature for eight days. The dried seeds were ground into a powder and used for extraction.

Solvent Extraction

20 grams of *L. cylindrical* seed powder was subjected to a cold maceration process in 100 mL of ethanol. The mixture was divided into two maceration containers and incubated for 48 h. Following extraction, the suspension was filtered through No. 1 filter paper (Whatman, Maidstone, England) layered over a funnel with a white muslin cloth. The filtrate was concentrated using a hot water bath at 50°C. The resulting dried extract was weighed and stored at 4°C for later use [Figure 1].

Figure 1. Ethanol Extract of *L. cylindrical*

L. cylindrical = Luffa cylindrica

Bacterial Strain and Growth Condition

E. faecalis was obtained from Saveetha Dental College and Hospital, Tamil Nadu, India, while the PAO1 strain was gifted by Dr. Busi Siddhardha, Pondicherry University, Puducherry. *E. faecalis* was sub-cultured in

Brain Heart Infusion (BHI) broth (HiMedia, India) and PAO1 in Luria Bertani (LB) broth (HiMedia, India). Both cultures were incubated at 37^0 C with shaking at 100rpm for 24 h. Preliminary identification of the bacterial strains was confirmed through standard microbiological methods [24].

Antimicrobial Activity of *L. cylindrical*

Antimicrobial activity was assessed using the agar well-diffusion method. Bacterial cultures of PAO1 and *E. faecalis* were spread onto Mueller Hinton agar (MHA) plates using a sterile swab. [25]. Two wells (8mm diameter) were punched into the agar, filled with 40 µL of *L. cylindrica* extract in one well, and the other well served as a control. Plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured with a vernier calliper.

Antibiotic Susceptibility Testing

Antimicrobial susceptibility was determined using the Kirby-Bauer disk diffusion method. Bacterial suspensions were spread onto MHA plates, and antibiotic disks were placed on the agar. Plates were incubated at 37°C for 24 hours, and the inhibition zones were measured to assess susceptibility.

Evaluation of Minimum Inhibitory Concentration

The MIC of *L. cylindrica* extract was determined using a two-fold broth dilution method. BHI and LB broths were inoculated with bacterial cultures to achieve a 0.5 McFarland turbidity standard (1.5 × 10^8 CFU/mL). Extract concentrations ranged from 10 mg/mL to 0.019 mg/mL. After incubation at 37°C for 24 hours, 40 µL of 2,3,5-triphenyl tetrazolium chloride (TTC) was added to each tube. The MIC was determined as the lowest concentration with no colour change, indicating no bacterial growth [26].

Crystal Violet Biofilm Inhibition Assay

Biofilm formation was assessed using crystal violet staining. Overnight cultures of PAO1 and *E. faecalis* (20 µL) were added to a microtiter plate containing 180 µL of fresh BHI or LB medium. Extract concentrations ranged from 5 mg/mL to 0.009 mg/mL for PAO1 and 1.25 mg/mL to 0.002 mg/mL for. Plates were incubated at 37°C for 48 hours. Biofilms were stained with 0.1% crystal violet solution, washed, and eluted in 200 µL of 70% ethanol. The optical density (OD) was measured at 520 nm using a UV-Vis spectrophotometer. The percentage of biofilm inhibition was calculated as follows:

$$Control\ OD\ 520\ nm - Treated\ OD\ 520\ nm / Control\ OD\ 520\ nm \times 100$$

Bacterial Growth Curve

The effect of *L. cylindrica* extract on bacterial growth was evaluated by measuring OD at 600nm every 60 minutes for 24 hours. Cultures were incubated at 37^0C with extract concentrations of 2.5 mg/ml for PAO1 and 0.625 mg/ml for *E. faecalis.*

Estimation of Exopolysaccharide (EPS)

EPS was extracted using a method described previously. PAO1 and *E. faecalis* were grown in LB and BHI broths supplemented with 1% glucose and treated with *L. cylindrica* extract (5 mg/mL to 0.009 mg/mL for PAO1 and 1.25 mg/mL to 0.002 mg/mL for E. faecalis). After incubation at 37°C for 24 hours, cultures were centrifuged at 10,000 rpm for 15 minutes. The supernatant was mixed with ethanol (three times the volume of the supernatant) and centrifuged at 10,000 rpm for 30 minutes. EPS was resuspended in Milli-Q water. One mL of EPS was mixed with 1 mL of cold 5% phenol and 5 mL of concentrated sulfuric acid. The red colour intensity was measured at OD 490 nm using a UV-Vis spectrophotometer.

Statistical Evaluation

All experiments were performed in triplicate. Statistical significance for growth curve analysis and biofilm quantification was determined using the student's T-test. A

significance level of $P < 0.05$ was considered statistically significant (*).

Results

Antimicrobial Susceptibility

The zone of inhibition was measured and was as follows: PAO1- 10mm and *E. faecalis* 8mm our findings suggest that *L. cylindrical* has antimicrobial activity against PAO1 as well as *E. faecalis*.

Antibiotic Sensitivity Testing (ABST)

ABST was performed by the Clinical and Laboratory Standards Institute (CLSI) Guidelines 2022 (CLSI. 2022). In our finding PAO1 is resistant to aminoglycosides, azlocillin, piperacillin, and carbenicillin and *E. faecalis* resistant to cephalosporins, aminoglycosides, trimethoprim and clindamycin.

At a Minimal Concentration *L. cylindrical* Inhibited PAO1 and *E. faecalis*

By employing a two-fold serial dilution approach, the antibacterial activity of the *L. cylindrica* extract was investigated ranging from (10 mg/mL to 0.019 mg/mL). We found that *L. cylindrica* inhibited the growth of PAO1 at 10 mg/mL and at a dose of 2.5 mg/mL, *L. cylindrica* inhibited the growth of *E. faecalis.* **[**Table 1**]**. Hence, sub-MIC concentration of the, *L. cylindrica* was used to determine the anti-biofilm and anti-virulence activities.

Table 1. Minimum Inhibitory Concentration of Ethanol Extract *L. cylindrica* Against PAO1 and *E. faecalis.*

S.no	**Two-fold dilution concentration (mg/mL)**	**Growth Measured**	
		PAO1	***E. faecalis***
1	10	-	-
2	5	+	-
3	2.5	+	-
4	1.25	+	+
5	0.62	+	+
6	0.312	+	+
7	0.156	+	+
8	0.078	+	+

9	0.039	+	+
10	0.019	+	+

Note: "- "Growth inhibited," +" Grow

L. cylindrical Inhibited the Biofilm in PAO1 and *E. faecalis*

By applying 0.1% crystal violet dye to a static microtiter plate, we investigate the biofilm inhibitory effect of *L. cylindrica* against the PAO1 and *E. faecalis*. Spectrophotometric analysis shows that the concentrations of 2.5 mg/mL, 1.25 mg/mL, and 0.625 mg/mL ethanol extract of *L. cylindrica* drastically decrease the biofilm formation to 77.22%, 38.22%, and 15.11% [Figure 2A;2B]. At a concentration of 0.625 mg/mL, 0.312 mg/mL, and 0.156 mg/mL *L. cylindrica* extract decreases the biofilm formation in *E. faecalis* to 50.11%, 21.55%, and 11.11% [Figure 3A; 3B].

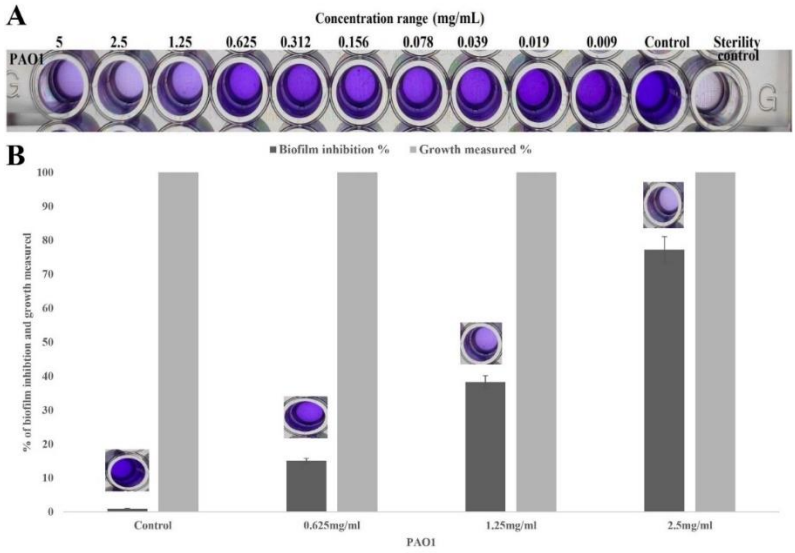

Figure 2. Crystal violet biofilm inhibition assay. **(A)** Ethanol extract of *L. cylindrica* extract inhibited PAO1 biofilm at sub-inhibitory concentrations of 2.5 mg/mL, 1.25 mg/mL, and 0.625 mg/mL. (**B)** Graphical Representation. 77.22%, 38.22%, and 15.11% of biofilm inhibition in PAO1 at sub-inhibitory concentrations of 2.5 mg/mL, 1.25 mg/mL, and 0.625 mg/mL *L. cylindrica* extract.

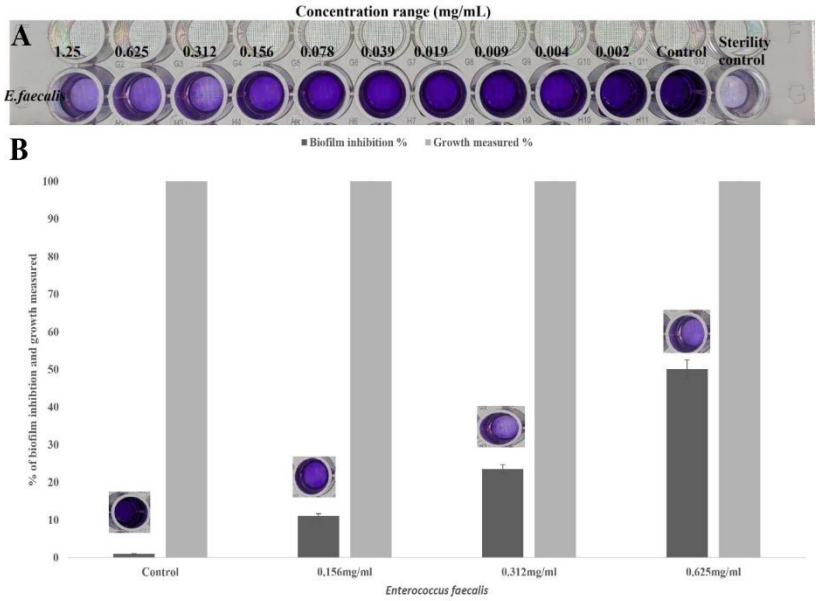

Figure 3. Crystal violet biofilm inhibition assay. **(A)** Ethanol extract of *L. cylindrica* extract inhibited *E. faecalis* biofilm at sub-inhibitory concentrations of 0.625 mg/mL,0.312 mg/mL, and 0.156. **(B)** Graphical Representation. 50.11%, 21.55%, and 11.11% of biofilm inhibition in PAO1 at sub-inhibitory concentrations of 0.625mg/mL, 0.312mg/mL, and 0.156 mg/mL of *L. cylindrica* extract.

Bacterial Growth Curve Analysis

The growth curve was performed in the presence and absence of PAO1 and *E. faecalis*. The results revealed that *L. cylindrica* does not inhibit bacterial growth of PAO1 at 2.5 mg/mL [Figure 4A], and *E. faecalis* at 0.625 mg/mL [Figure 4B]. The spectrophotometric analysis shows that there is no difference between the control and treated bacterial cells at 600nm. This suggests that at these concentrations, the *L. cylindrica* extract does not exhibit bactericidal or bacteriostatic effects on planktonic cells.

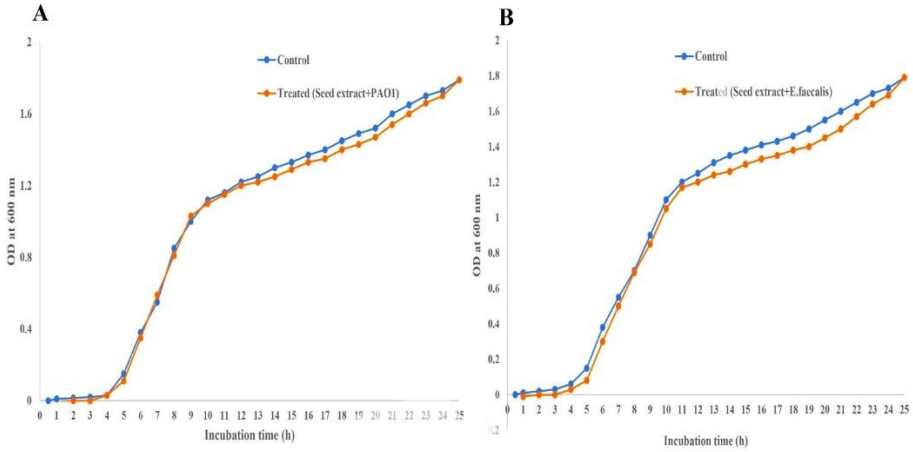

Figure 4. Growth curve analysis: **(A)** PAO1 grown without (control) and in the presence of *L. cylindrica* extract at the concentration of 2.5 mg/mL. **(B)** Growth curve analysis: *E. faecalis* grown without (control) and in the presence of *L. cylindrica* extract at the concentration of 0.625 mg/mL.

Effects of *L. cylindrical* on QS-mediated Virulence Factor in PAO1 and *E. faecalis*

EPS is one of the key constituents in both providing and maintaining the structural framework of the biofilm. The study revealed that a concentration of 2.5 mg/mL, 1.25 mg/mL, and 0.625 mg/mL ethanol extract of *L. cylindrica* inhibited EPS production of PAO1 to a level of 47.55%, 18.91%, and 4.66% respectively. At a concentration of 0.625 mg/mL, 0.312 mg/mL, and 0.156 mg/mL *L. cylindrica* extract drastically decrease the EPS production at a level of 23.12%, 10.22%, and 3.71% respectively (Figure 5A;5B).

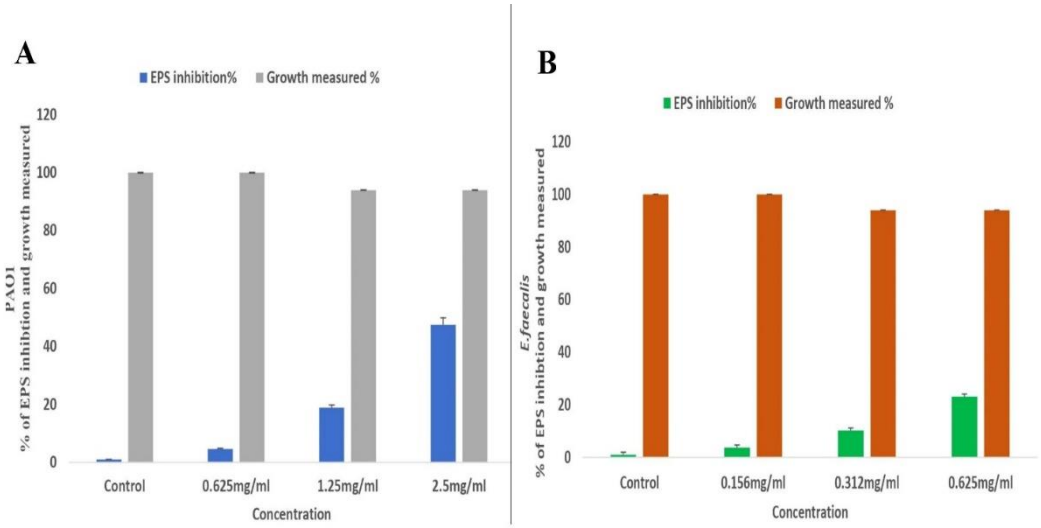

Figure 5. Graphical Representation of EPS inhibition and cell growth. **A)** 47.55%, 18.91, and 4.66% of EPS inhibition in PAO1 when treated with 2.5 mg/mL, 1.25 mg/mL, and 0.625 mg/mL of *L. cylindrica* extract. **B)** Graphical Representation. 23.12, 10.22%, and 3.71% of EPS inhibition in *E. faecalis* when treated with 0.625 mg/mL, 0.312 mg/mL, and 0.156 mg/mL *L. cylindrica* extract.

Discussion

Pseudomonas aeruginosa and *Enterococcus faecalis*, classified as opportunistic pathogens, pose a risk to individuals with compromised immune systems. The production of lytic enzymes, pigment synthesis, and QS systems are necessary for *P. aeruginosa* to be infectious [7]. Additionally, *E. faecalis* demonstrates virulence factors that play a crucial role in dental caries by promoting tissue adherence and colonization in host tissues [12,15]. Our investigation explores the involvement of QS systems in both of these pathogens, highlighting the potential for disrupting these systems as an effective strategy for addressing infections. The control of virulence factors and the significance of adherence factors in PAO1 and *E. faecalis* underscore the interplay of molecular pathways in pathogenicity [11,15]. In this study, we introduce the medicinal plant *L. cylindrica* with promising bioactive properties as a potential source of antimicrobial compounds.

The current study demonstrates the antimicrobial and antibiofilm activities. Of *L. cylindrica* extract against PAO1 and *E. faecalis*. To ascertain the extract's direct inhibitory effects on planktonic bacterial cells, antibacterial activity was evaluated. Initial results showed that *L. cylindrica* extract inhibited bacterial activity at the lowest concentration of 10 mg/mL for PAO1 and 2.5 mg/mL for *E. faecalis*. The extract's ability to stop bacterial growth was confirmed through the agar well-diffusion method. Recent studies, such as those by Marcoux et al. [29], have shown that cinnamon oil exhibits strong microbicidal activity against *E. faecalis* with MIC values ranging from 1.56 to 25µg/mL. Similarly, Vambe et al. [30] Reported that the leaf extract of *Searsia lancea* has antibacterial properties with effective MIC levels. Additionally, Nwanekwu and colleagues have found that *Ocimum gratissimum* and *Hibiscus sabdariffa* extracts possess strong bactericidal activity against *P. aeruginosa*. [31].

The antibiofilm activity of *L. cylindrica* extract was assessed to gauge its effectiveness in preventing biofilm formation and proliferation. Biofilms, which are complex bacterial communities embedded in a self-produced extracellular matrix, exhibit significantly higher resistance to both the host immune system and antibiotics compared to planktonic cells. Evaluating the antibiofilm properties of *L. cylindrica* extract is essential

for developing treatments that can reduce biofilm-associated resistance and prevent chronic infections. [19]. Our study found that at concentrations below the minimum inhibitory concentration (sub-MIC), *L. cylindrica* extract inhibited the QS-dependent biofilm formation of PAO1 and *E. faecalis* in a dose-dependent manner. The crystal violet assay showed that *L. cylindrica* extracts drastically reduced biofilm formation in PAO1 and *E. faecalis* without affecting planktonic cell growth. This dual assessment underscores the potential of *L. cylindrica* extract as both a long-term antibacterial agent and an immediate antibacterial agent in preventing biofilm-related complications. This is particularly important for developing new strategies to combat antibiotic resistance and chronic illnesses. Previous studies reported that *L. cylindrica* leaf extract inhibits biofilm formation in *E. coli* and *S. aureus* at low concentrations. [32]. Similarly, Zhou et al. [33] Reported that the compound hordenine from *Hordeum vulgare* reduces AHL levels, thereby inhibiting *P. aeruginosa* biofilm formation by up to 69%. Majik et al. [34] Found that R)-Bgugaine, a natural pyrrolidine alkaloid from *Arisarum vulgare,* inhibits pyocyanin pigmentation, Lasa protease, and rhamnolipid production, reducing biofilm density by 83%. Recent research on Juglone derivatives (2-ethoxy-6-acetyl-7-methyljuglone and 2-methoxy-6-acetyl-7-methyl juglone) results in 70% of biofilm inhibition at a concentration of 10Mm [35].

Phytochemicals in *L. cylindrica* seed extract are essential for preventing the growth of microorganisms. Notable phytochemicals include saponins, which disrupt cell membranes and cause cell lysis; flavonoids, which interfere with bacterial cell wall and nucleic acid synthesis; alkaloids, which disrupt DNA and RNA synthesis; and terpenoids, which interfere with metabolism and inhibit bacterial growth. Bacterial cell membrane disruption is a significant mechanism as phytochemical interactions with the lipid bilayer may increase membrane permeability, leading to cell death due to leakage of cellular contents [36]. These membrane-targeting properties likely contribute to the antimicrobial activity of *L. cylindrica* extract against PAO1 and *E. faecalis*. The growth curve analysis, measuring OD at 600 to track bacterial biomass over time, indicates that the extract inhibited biofilm formation at sub-MIC concentrations but did not affect bacterial growth compared to the control (shown in Figure 4).

The study offers a thorough understanding of the interactions between *L. cylindrical* extracts and bacteria, assessing both antibacterial and antibiofilm activities. The extract's potential to disrupt QS systems and prevent biofilm formation in PAO1 and *E. faecalis* highlights its potential role in combating bacterial infections. Further studies are needed to isolate and characterise the active components of *L. cylindrica* responsible for these activities and to evaluate their efficacy in broader clinical contexts.

Conclusion

In conclusion, this study insights valuable knowledge into the complex interplay of disease-causing pathogens, antimicrobial resistance, and the potential of natural products in combating infections. Our results suggest that the natural medicinal plant *L. cylindrica* seed has the potential to inhibit the QS system in PAO1 and in *E. faecalis* potentially being explored via *in vitro* studies. Further expanded research with different formulations would be helpful to enhance the pharmacological therapeutic applications of the identified compounds, specifically their anti-quorum sensing and anti-biofilm properties.

Conflict of Interest

The authors declare there is no conflict of interest.

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