Combination of Leucine- Fluoroquinolone in Inhibiting Dental Pathogen Biofilms

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KEYWORDS
Dental caries susceptibility, gram-positive bacteria, essential amino acids, fluoroquinolones, mouthwashes, toothpastes.

ABSTRACT
Purpose: Leucine, an essential amino acid, has shown potential in modulating bacterial growth, with potential applications in dentistry and oral health. When used in conjunction with fluoroquinolones, which are antibiotics known for their broad-spectrum activity, it is effective against a variety of bacterial pathogens. The principal aim of the study was to assess the combinatorial efficacy of leucine - fluoroquinolone antibiotics in the deterring of biofilm formed by prominent dental pathogens.
Materials & Methods: 100 mL of Brain Heart Infusion broth was inoculated with 4-5 colonies of *S.mutans* and *E.faecalis* and incubated at 37 degree C overnight in an incubator. Leucine & Leucine - fluoroquinolone was diluted to desired concentrations and added to BHI broth in the wells & was inoculated with 50 mL of broth culture. The Broth was aspirated, washed, & 150 µL of crystal violet was added to each well & allowed to stand for 15-20 minutes. The dye was removed, washed, and then dissolved by adding 150 µL of 30% glacial acetic acid in each well. Reading was taken using ELISA plate reader at 570 nm.
Results: The optical density of leucine was comparatively higher than the combination of leucine and ciprofloxacin in case of both *Streptococcus mutans* and *Enterococcus faecalis*, demonstrating that proportion of biofilm formation with S.mutans & E.faecalis had decreased when treated with leucine-ciprofloxacin antibiotic combination than with leucine alone.
Conclusion: Leucine has potential anti-biofilm activity if used as an adjuvant along with an antibiotic against drug resistant strains of bacteria such as S.mutans & E.faecalis.

1. Introduction

The need for efficient methods to prevent oral infections and preserve dental health has increased due to the emergence of bacterial strains that are resistant to antibiotics. One of the significant challenges in this realm is the formation of biofilms by pathogenic bacteria that contribute to dental caries and other oral diseases [1]. Among these pathogens, *Streptococcus mutans* and *Enterococcus faecalis* are particularly common, playing critical roles in the development and persistence of dental caries and endodontic infections, respectively [2]. *Streptococcus mutans* play a central role in the development of dental caries, a common and significant dental health issue [3]. This bacterium is highly adept at adhering to tooth surfaces and forming biofilms, commonly known as dental plaque. *S. mutans* is particularly effective at adhering to the enamel of teeth due to its ability to produce adhesive polysaccharides [4]. These polysaccharides form a biofilm structure that enables *S. mutans* to adhere firmly to the tooth surface, establishing a stable growth environment. It often coexists with other oral bacteria in a complex microbial community. Its presence may exacerbate the cariogenic process by affecting the composition and actions of other microorganisms.

Groups of bacteria, protozoa, and fungus that stick to surfaces and are covered in a matrix of extracellular polymeric materials that are rich in water is known as biofilm [5]. Bacterial resistance to host defenses and therapeutic interventions is facilitated by the intricate process of biofilm formation. Quorum sensing plays an important role in regulating the biofilm formation [6]. Quorum sensing is a process that allows bacteria to communicate and coordinate their activities by producing, releasing, detecting, and responding to chemical signals called autoinducers[7]. It allows bacteria to adjust their

gene expression and coordinate behaviors like sporulation, bioluminescence, and biofilm formation. Traditional methods for managing oral bacterial load, such as mouthwashes and toothpaste, have limitations in preventing biofilm development [8]. Recent research suggests that combining different therapeutic agents could enhance efficacy [9].

Leucine, an essential amino acid, has shown potential in modulating bacterial growth, with several important roles and potential applications in dentistry and oral health [10]. Leucine plays a key role in protein synthesis, which is vital for the repair and regeneration of tissues [11]. When used in conjunction with fluoroquinolones—antibiotics known for their broad-spectrum activity and effectiveness against a variety of bacterial pathogens—this combination may offer a novel approach to combating biofilms formed by *S. mutans* and *E. faecalis*. The principal aim of the study was to assess the combinatorial efficacy of leucine - fluoroquinolone antibiotics in the deterring of biofilm formed by prominent dental pathogens.

2. Materials and Methods

Preparation of Inoculum:

To prepare the inoculum, 100 mL of Brain Heart Infusion (BHI) broth was inoculated with 4-5 individual colonies of *Streptococcus mutans* and *Enterococcus faecalis*. The inoculated broth was then incubated overnight at 37°C in an incubator to allow the microorganisms to grow and reach the desired density.

Antibiofilm Assay:

1. **Dilution of Compounds**: Leucine and Leucine-fluoroquinolone were diluted to the required concentrations and mixed with BHI broth.
2. **Inoculation**: Each well of the microtiter plate was filled with the prepared BHI broth containing the test compounds and inoculated with 50 µL of the overnight broth culture.
3. **Biofilm Formation**: The plate was incubated under suitable conditions to allow biofilm formation by the microorganisms.
4. **Washing**: After incubation, the broth was carefully aspirated from the wells, and the wells were washed with phosphate-buffered saline (PBS) to remove non-adherent cells and residual broth.
5. **Staining with Crystal Violet**: 150 µL of crystal violet solution was added to each well to stain the biofilms. The plate was left undisturbed for 15-20 minutes to allow proper staining of the adhered biofilm matrix. After staining, the dye was aspirated, and the wells were washed thoroughly with PBS to remove unbound or excess crystal violet.
6. **Dissolving Bound Dye**: To quantify the biofilm, the bound dye was dissolved by adding 150 µL of 30% glacial acetic acid to each well.
7. **Measurement**: The optical density (OD) of the dissolved dye, which corresponds to the biofilm mass, was measured at 570 nm using an ELISA plate reader.

3. Results

The results obtained from the study were plotted in the form of graphs. At 0.1 concentration, the biofilm formation was 29% with leucine and 11.4% with the combination of leucine and antibiotic (ciprofloxacin) against *S.mutans*. 67.4% & 26.9% of biofilm formation was observed against *S.mutans* at 0.003 concentration with leucine and leucine-ciprofloxacin combination respectively. Figure 1 depicts the comparison of effect of leucine & leucine fluoroquinolone antibiotic and its antimicrobial photodynamic therapy on *Streptococcus mutans* biofilm. Y-axis represents the absorbance at 570 nm and X-axis represents the concentration of leucine and leucine-fluoroquinolone antibiotics. Figure 2 illustrates the effect of leucine & leucine fluoroquinolone antibiotic and its antimicrobial photodynamic therapy on *Enterococcus faecalis* biofilm. Y-axis represents the absorbance at 570 nm

and X-axis represents the concentration of leucine and leucine-fluoroquinolone antibiotics. Tables 1 & 2 depicts the percentage of biofilm formation with leucine & leucine-ciprofloxacin combination against *S.mutans* & *E.faecalis* respectively.

4. Discussion

The optical density of leucine was comparatively higher than the combination of leucine and ciprofloxacin (antibiotic) in case of both *S.mutans* and *E.faecalis*, which demonstrates that the proportion of biofilm formation with S.mutans & E.faecalis had significantly decreased when treated with leucine-ciprofloxacin antibiotic combination than with leucine alone.

A vast number of agents have been established to deter the formation of biofilm due to dental pathogens like *S.mutans, P. aeruginosa, E.faecalis*. The present study demonstrates that the use of leucine in combination with ciprofloxacin showed synergistic effects at different stages of biofilm development. These include the inhibition of biofilm formation and adhesion[12], downregulation of bacterial cell-cell communication (quorum sensing) [13], & killing of preformed biofilm. Leucine and ciprofloxacin together disrupt the first stages of biofilm formation by decreasing bacterial adherence to surfaces, which stops biofilm formation. The research conducted against strains of gram-negative and gram-positive organisms such as *P. aeruginosa, A. baumannii, K.pneumoniae, E. coli* with an antibiotic combination of Ciprofloxacin, ceftazidime and tobramycin revealed an efficacy rate of 98-100% [14]. The results of another study revealed that fluoroquinolones reveal strong bactericidal activity to biofilm bacteria regardless of the growth rate[15]. Fluoroquinolones are broad-spectrum antibiotics that work by inhibiting bacterial topoisomerase action. This leads to DNA damage and reactive oxygen species (ROS) production, which causes oxidative stress[16]. Ciprofloxacin is known to have inhibitory effects in the formation of biofilm. Previous researches indicate that the decrease in virulence factors and biofilm formation resulted from the inhibition of the quorum sensing mechanism, as shown by the reduced production of quorum sensing signal molecules by certain bacteria, when exposed to subinhibitory concentrations of ciprofloxacin [17].

Leucine, on the other hand, is an essential amino acid that helps regulate many cellular processes, including protein synthesis, tissue regeneration, and metabolism. According to the results, Leucine by itself inhibits biofilm formation to a certain extent. Studies have revealed that amino acids have the ability to enhance the solubility of many poorly soluble drugs, and when the synergistic effect of combining D-amino acids with Cip was investigated as a strategy to overcome antimicrobial resistance in these biofilms, it showed that the amino acids alone were able to significantly disperse established biofilms and inhibit new biofilm formation in the absence of an antibiotic, but disclosed 96.89% inhibition along with the presence of an antibiotic like ciprofloxacin[18].

The future scope of this study is that the combination of amino acid & fluoroquinolone can aid in the formulation of intracanal medicaments, mouth rinse, for the prevention of caries or formation of biofilm. Further research can be carried out by the employment of various other essential amino acids along with fluoroquinolone with various caries-causing bacteria.

5. Conclusion

Leucine has potential anti-biofilm activity if used as an adjuvant along with an antibiotic against drug resistant strains of bacteria such as S.mutans & E.faecalis.

Authorship Contribution:

Bianca has contributed to data analysis & interpretation, preparation of original draft & funding acquisition. Dr. Sandhya has done review of literature, conceptualization & design, methodology & validation, formal analysis & supervision. Dr. S. Jayalakshmi carried out project administration, methodology & validation.

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Statements and Declarations:

The authors have no conflicts of interest to declare.

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Tables and Figures:

Table 1: The percentage of biofilm formation with leucine & leucine-ciprofloxacin combination at 0.1, 0.05, 0.025, 0.012, 0.006 and 0.003 concentrations with *Streptococcus mutans. LE - Leucine; LE-AB - Leucine Antibiotic combination*

% Biofilm Formation		
CONC	LE	LE-AB
0.1	29.0	11.4
0.05	33.4	15.2
0.025	38.6	17.3
0.012	47.0	22.9
0.006	52.6	25.0
0.003	67.4	26.9

Table 2 - Percentage of biofilm formation with leucine & leucine-ciprofloxacin combination at 0.1, 0.05, 0.025, 0.012, 0.006 and 0.003 concentrations with *Enterococcus faecalis*. *LE - Leucine; LE-AB - Leucine Antibiotic combination*

% Biofilm Formation		
CONC	LE	LE-AB
0.1	24.4	13.6
0.05	28.9	15.7
0.025	34.0	16.8
0.012	38.9	20.3
0.006	45.7	22.3
0.003	55.5	25.2

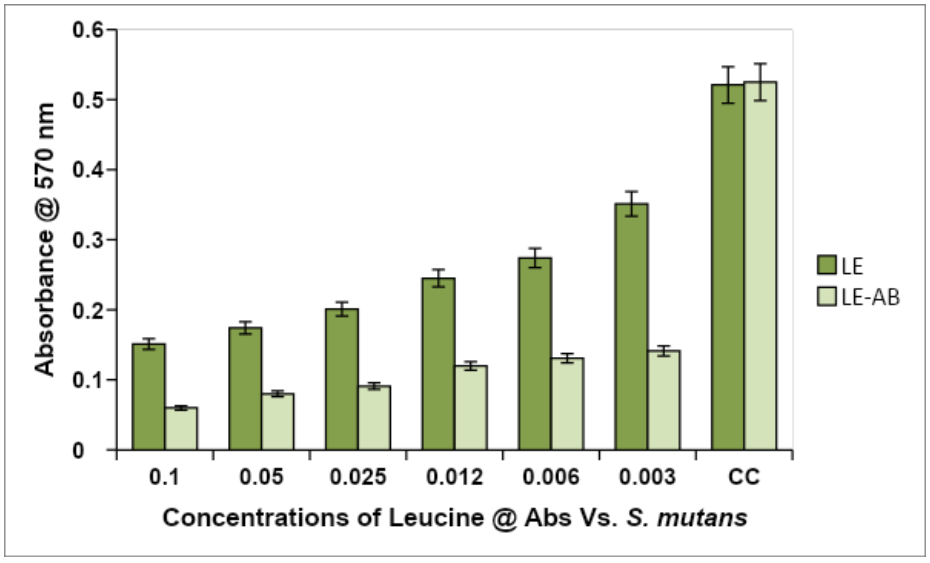

Figure 1: Comparison of effect of leucine & leucine fluoroquinolone antibiotic and its antimicrobial photodynamic therapy on *Streptococcus mutans* biofilm.

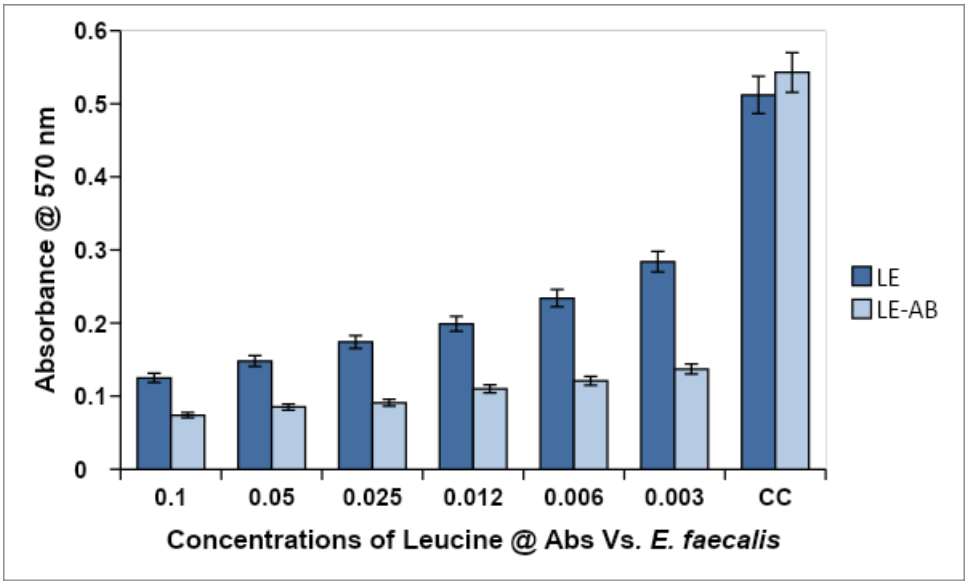

Figure 2: Effect of leucine & leucine fluoroquinolone antibiotic and its antimicrobial photodynamic therapy on *Enterococcus faecalis* biofilm