IJNRD.ORG ISSN : 2456-4184
INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG
An International Open Access, Peer-reviewed, Refereed Journal

# "Formulation of Plant Based Hydrogel Against Biofilm Forming Pathogens"

**1. Miss Mitali S. Kankal , 2.Miss Rupali R. Vyavahare , 3.Dr. Rachana Pachori**

1. PG student, Microbiology Research laboratory, Rajasthan Aryan Mahavidyalaya Washim, Maharashtra, India. ,
2. Research scholars, Microbiology Research Laboratory, Rajasthan Aryans Mahavidyalaya, Washim. , 3. Professor and Head, U.G, P.G and Research section, Department of Microbiology Rajasthan Aryans Mahavidyalaya, Washim.

Department of Microbiology

Rajasthan Aryan Arts, Shri Mithulal Kacholia Commerce and Shri Satyanarayanji Ramkrishnaji Rathi Science College, Washim, Maharashtra

## ABSTRACT

Biofilm formation by pathogenic microorganisms poses a significant challenge in healthcare, contributing to persistent infection and increased antibiotic resistance. This research investigates the antibiofilm properties of novel hydrogel derived from natural plant sources. Neem extract was prepared by extracting bioactive compounds from the leaves of the Neem tree (Azadirachta indica). The persistence of bacterial infections is often attributed to biofilm development, which significantly reduces the effectiveness of antimicrobial therapies. Addressing this issue, the present study focused on designing a plant-based hydrogel with enhanced antibiofilm activity. The hydrogel was composed of Neem (Azadirachta indica) leaf extract and eucalyptus essential oil, integrated into a polymeric framework of sodium alginate, agar powder, glycerol, and calcium carbonate. Formulation procedures were carried out under aseptic conditions, and sterility tests using nutrient and potato dextrose media confirmed the absence of microbial contamination. The hydrogel exhibited high moisture retention with a swelling index of 7.96, indicating its capability for gradual release of active constituents. To evaluate its antibiofilm properties, the hydrogel was tested against six clinical isolates: *Staphylococcus aureus, Escherichia coli, Pseudomonas spp., Proteus spp., Salmonella spp., and Shigella spp*. Biofilms were induced using the crystal violet method and assessed at intervals of 24, 48, 72, and 96 hours. Substantial biofilm degradation was observed in *S. aureus and Shigella spp*., while *Pseudomonas and Salmonella spp*. displayed progressive but effective biofilm reduction. These outcomes suggest that the formulated hydrogel can be an effective natural strategy for managing infections where biofilm formation is a concern, offering a sustainable alternative to conventional treatments.

KEYWORDS: Neem extract, Eucalyptus oil, Biofilm inhibition, Plant-based hydrogel, Antimicrobial strategy, *Staphylococcus aureus, Shigella spp., Pseudomonas spp*.

# INTRODUCTION

The biofilm lifestyle of microorganisms was largely ignored until the 1970s, when Nils Høiby linked persistent infections in cystic fibrosis patients to bacterial aggregates. Since then, biofilms have been recognized as key in various infections, especially in chronic infections.Biofilms are complex microbial communities with bacteria encased in protective layers, making them hard to eliminate(Høiby, N.et.al 2017),(Costerton, J. Wet.al 1999),( Hall Stoodley et.al 2009),(Bjarnsholt et. al 2013) and (Latasa et.al 2005). Biofilms are

complex communities of microorganisms encased within a self-produced extracellular polymeric substance (EPS) matrix. Biofilms form on implants, creating a barrier against antibiotics and immune responses, leading to persistent infections. These infections are a major concern in biomedicine, contributing to many deaths worldwide. Both Gram-positive and Gram-negative bacteria can form biofilms on medical devices(Miño et.al 2024). Hydrogels are polymer-based materials that retain water and deliver therapeutic agents to targeted areas(Ahmed et.al 2015).These hydrogels exhibit viscosity, meaning they resist deformation(Choe et.al 2018).To test this hypothesis, we prepared a hydrogel from Neem and loaded it with essential oil (Eucalyptus Oil), calcium carbonate and alginate. Eucalyptus oil has antimicrobial and decongestant properties. When added to hydrogels, it enhances their therapeutic and aromatic effects, benefiting wound care, skin treatments, cosmetics, and drug delivery systems. Plant-based hydrogels, made from biocompatible and biodegradable materials like cellulose, chitosan, and alginate, offer eco-friendly solutions for treating biofilm-associated infections. They effectively disrupt pre-formed biofilms, making them a promising approach for next-generation therapies. Neem, native to the Indian subcontinent and Burma, is used in traditional medicine for its anti-inflammatory, anti-diabetic, anti-cancer, and other healing properties (Batra et al., 2022). Its leaves are a promising candidate for incorporation into hydrogels to combat biofilm-forming pathogens.

Biofilms are complex communities of microorganisms embedded within a self-produced extracellular polymeric substance (EPS) matrix. These structures enable pathogens to adhere to surfaces, evade host immune responses, and resist conventional antimicrobial treatments, contributing significantly to chronic and recurrent infections. Initially overlooked, the importance of biofilms in clinical settings was highlighted in the 1970s, particularly in cystic fibrosis-related infections (Høiby et al., 2017; Costerton et al., 1999). Biofilm-associated infections are especially problematic in medical devices such as catheters, prosthetics, and implants, where both Gram-positive and Gram-negative bacteria form resilient colonies (Miño et al., 2024). Traditional antibiotics are often ineffective against biofilms, necessitating alternative strategies.

Hydrogels have emerged as promising delivery systems in biomedical applications due to their high water content, biocompatibility, and capacity to encapsulate therapeutic agents (Ahmed et al., 2015). When enhanced with plant-based extracts and essential oils, hydrogels offer additional therapeutic properties such as antimicrobial, anti-inflammatory, and wound-healing effects. This study investigates the potential of a plant-based hydrogel composed of Neem (Azadirachta indica) leaf extract and eucalyptus essential oil, integrated with sodium alginate and other biocompatible materials. Neem is well-known in traditional medicine for its antimicrobial, antioxidant, and healing properties (Batra et al., 2022), while eucalyptus oil is valued for its broad-spectrum antimicrobial activity. Together, these components form a hydrogel designed to inhibit biofilm formation by pathogenic bacteria. By focusing on natural, eco-friendly components, this research aims to develop a sustainable alternative to synthetic antimicrobials. The hydrogel's antibiofilm efficacy was evaluated against clinically relevant pathogens such as *Staphylococcus aureus, Escherichia coli, Pseudomonas spp., Proteus spp., Salmonella spp., and Shigella spp.*, contributing to the development of next-generation antimicrobial therapies.

# NEED OF STUDY

1. Rising Threat of Biofilm-Associated Infections

Biofilms formed by bacteria are a major cause of chronic and device-related infections. They make microbes highly resistant to antibiotics, complicating treatment and increasing healthcare burdens.

2. Limitations of Conventional Antibiotics

Traditional antimicrobial treatments often fail against biofilms due to the protective extracellular matrix. This calls for alternative strategies that can disrupt biofilm structure and prevent bacterial resistance.

3. Need for Natural, Biocompatible Solutions

Plant-derived materials such as Neem and Eucalyptus oil are known for their antimicrobial properties. Using these in hydrogels offers a safer, more eco-friendly alternative to chemical-based treatments.

4. Lack of Research on Plant-Based Antibiofilm Hydrogels

There is limited scientific exploration of plant-based hydrogels targeting biofilms, especially using Neem and Eucalyptus in combination. This study addresses this gap by investigating their synergistic effects.

5. Potential for Sustainable Biomedical Applications

Developing a bioactive hydrogel from natural sources not only enhances antimicrobial action but also supports sustainable, low-cost options for wound care and infection control in clinical settings.

# OBJECTIVE OF THE STUDY

The goal of this study is to create a natural hydrogel using Neem extract that can help fight harmful bacteria forming biofilms. These biofilms often make infections harder to treat, so the aim is to develop a safer, plant-based option that could support better healing in such cases.

# METARIAL AND METHOD

# PREPARATION OF PLANT EXTRACT

The leaves of Neem (*Azadirachta indica*) were collected from town area. They were collected as fresh and healthy. The Neem leaves were brought aseptically to the laboratory and to carry further procedure. They are anti-inflammatory, antimicrobial, and antioxidant properties. It grows in tropical regions such as India.

Ensure the leaves are clean and free from contaminants, which have medicinal properties. Fresh 10g Neem leaves were weighed, sterilized with sodium hypochlorite, and then washed with sterile distilled water. The leaves were mixed with 50ml of sterile distilled water which are sterilized by autoclaving for 15 to 20 min. The mixture homogenate was filtered using filter paper(Harjai et.al 2013).

# FORMATION OF HYDROGEL

A hydrogel containing Neem extract, calcium carbonate, and alginate polymer, glycerine, agar, and essential oil was prepared in 50ml of sterile distilled water were placed in a beaker, and the alginate polymer was incorporated at a concentration of 3gm. The agar is prepared as 1.5gm with 50ml distelled water. Then autoclaved upto for 15 to 20 min. The sodium alginate polymer was taken at a concentration of 3gm which was sterilized in UV chamber. The plant extract was filtered in beaker and placed in water bath. Then the agar was mixed in beaker with the plant extract with continous stirring. Calcium carbonate in concentraction of 0.2 gm in 10ml distilled water was then added. Followed by the sodium alginate polymer was taken at a concentration of 3gm which was sterilized in UV chamber. The mixture was continuously stirred and boiled.The 7 drop of essential oil is added (Zhang et. al 2016)( Singh et.al 2016).The 2ml of glycerine is added.The Neem extract was integrated into the gel until fully mixed.The hydrogel is formed. Then it is cooled at room temperature and then refrigerated.

# ISOLATION AND IDENTIFICATION OF BIOFILM FORMING PATHOGEN

First the isolation of biofilm-forming pathogens proceeds with clinical samples such as wound swabs, blood, urine, sputum, or catheter tips. The samples were inoculated onto selective agar plates (e.g., MacConkey agar, blood agar) to culture the pathogens. The plates are incubated at the appropriate temperature for pathogen growth (typically 37°C for24hr). The pure colonies so obtained were examined for morphology (shape, size, color, texture). Identification is confirmed by gram staining, biochemical tests, IMVIC Tests, for specific pathogens. The pathogen isolated was *Escherichia coli, Pseudomonas spp, Salmonella spp, Shigella spp, Staphalococcus aureus, and Proteus species.*

# Sterility testing

Hydrogel sterility was evaluated by immersing 1x1 cm square blocks of the prepared hydrogel in 10 mL of sterile distilled water to detect bacterial contamination, respectively. Following incubation, samples were examined for turbidity or microbial growth. Additionally, hydrogel samples were incubated under optimal conditions to further assess sterility.

# Swelling test

To determine the swelling capacity (sc) of a hydrogel, we apply the following equation:

**SC = Wt−W0 / W0 × 100** was calculated by weighting the hydrogel sample in its dry state (W0) and when fully hydrated (Wt)( Galante et.al 2017). The hydrogel is immersed in a tube containing 3ml of distilled water for hydration. The weight is measured at regular intervals until it stabilize and no longer changes. During each measurement, the sample is gently blotted with paper to remove any excess water. All experiments are conducted at 25°C. All tests were performed at least in triplicate(Galante et.al 2018)to ensure reliability and accuracy.

# Qualitative Biofilm Inhibition Assay

Place the plant based hydrogel into each tube. Add 1-2ml of bacterial inoculums into each tube containing the hydrogel. Ensure that the hydrogel is submerged with the bacterial suspension. Immediately add 0.1ml of crystal violet stain (0.1%solution) to each tube and mix gently (Kamimura et. al 2022). After staining for 15 to 30 minutes, the excess crystal violet solution was discarded. The tubes were then washed three times with distilled water and allowed to dry. A positive result was indicated by the presence of a stained layer adhering to the inner wall of the tubes (Satish et.al 2017; Haney, E. F et.al 2018).

# Quantitative Biofilm Inhibition Assay

The activity of plant extracts against biofilm forming pathogens was carried by crystal violet staining (Olawuwo et.al 2022). A microtiter plate assay was performed to measure the amount of biofilm formation. Add 100 µL of bacterial suspension (at OD 0.1) to selected well of 96-well microtiter plate. Ensure separate and triplicate sets of wells for each microorganism and time point 24, 48, 72, 96 hours. For the control wells, include a negative control (no hydrogel) and a positive control where no biofilm distribution is expected. Incubate the plate at 37°C for 24, 48, 72 and 96 hour. After the incubation period, gently remove the culture medium and rise the wells with PBS to remove any non-adherent bacteria. Add 100 µL of 0.1% crystal violet solution to each well and incubate at room temperature for 15-20 minutes to allow the dye to bind to the biofilm. Carefully remove the crystal violet solution and wash the wells with PBS to remove excess stain. Next, 200 µL ethanol was added to each tube to dissolve the bound Crystal Violet dye from the biofilm. Using an ELISA plate reader, measuring absorbance at 590 nm for each well. The absorbance reading at 590nm will directly proportional to the biofilm biomass in each well. Higher absorbance values reflect more biofilm formation. Monitor absorbance at various time interval (24, 48, 72 and 96 hours) to analyze the growth pattern of the biofilm.

# RESULT AND CONCLUSION :

The present research aimed to develop a Neem-based hydrogel and assess its efficacy against biofilm-forming pathogens. Six bacterial species, namely *S. aureus*, *Salmonella* spp., *Proteus* spp., *Shigella* spp., *E. coli*, and *Pseudomonas* spp., were isolated from clinical sample including blood, urine, wound swabs, etc. The developed hydrogel was evaluated for sterility, swelling capacity, and antibiofilm activity. Sterility tests demonstrated that autoclaving effectively eliminated microbial contamination, as evidenced by the absence of microbial growth after incubation.

The swelling capacity (SC), representing the hydrogel's liquid absorption relative to its dry weight, was determined to be based on an initial dry weight (W0) of 0.25 grams and a swollen weight (Wt) of 1.24 grams. the SC was determined to be as:

$$SC = (Wt - W0)W0 \times 100 = (1.24\,g - 0.25\,g)0.25\,g \times 100 = 1.99\,g0.25\,g \times 100 = 796\%SC$$

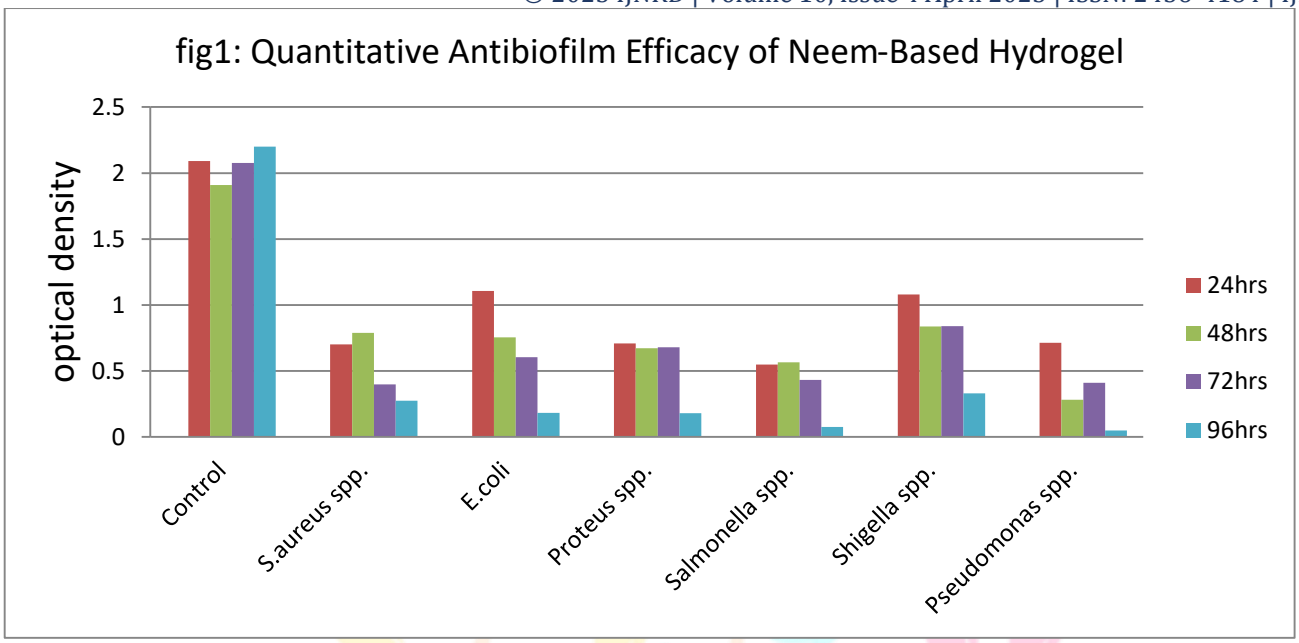
This result reveals that the hydrogel can absorb water up to 796% of its initial dry weight, equivalent to holding 7.96 times its own weight in water. Such high swelling capacities are typical of hydrogels, which are engineered to retain significant amounts of water, making them valuable for applications like wound care, where maintaining hydration is critical for tissue repair.

A crystal violet staining assay was used to assess the Neem-based hydrogel's ability to inhibit biofilm formation. The hydrogel significantly disrupted *Pseudomonas spp*, *Salmonella sp* , *Staphylococcus aureus* biofilm, resulting in substantially lighter staining compared to the control. Similarly, *Pseudomonas spp*, *Salmonella spp*, Shigella *spp* and *Staphylococcus aureus* biofilms were strongly inhibited, showing a significant reduction in staining. *Escherichia coli* exhibited moderate biofilm reduction, with a noticeable but less dramatic decrease in staining. *Proteus spp* showed a measurable reduction in biofilm formation, as indicated by decreased staining. These results demonstrate that the Neem-based hydrogel possesses varying levels of antibiofilm activity across different bacterial species, with particularly strong efficacy against *Pseudomonas spp*, *Staphylococcus aureus*, *Salmonella spp* and *Shigella spp*, while showing moderate effects on *E. coli* and *Proteus* spp.

The Quantitative antibiofilm potential of the Neem-based hydrogel was assessed over a period of 96 hours against several key biofilm-forming bacterial species, using crystal violet absorbance measurements at 570 nm (table 1 and fig 1). The study included both Gram-positive and Gram-negative bacteria viz. *Staphylococcus aureus, Escherichia coli, Proteus spp, Salmonella spp., Shigella spp., and Pseudomonas spp.*. The untreated control group consistently showed high optical density (OD) values, indicating significant biofilm formation, while the hydrogel-treated groups exhibited marked reductions in biofilm levels.

**Table 1: QUATITATIVE ANTIBIOFILM EFFICACY OF NEEM-BASED HYDROGEL**

| Time in hours | Control | *S. aureus* | *E.coli* | *Proteus spp.* | *Salmonella spp.* | *Shigella spp.* | *Pseudomonas spp.* |
|---|---|---|---|---|---|---|---|
| Optical Density | | | | | | | |
| 24hrs | 2.09 | 0.7 | 1.107 | 0.708 | 0.547 | 1.080 | 0.712 |
| 48hrs | 1.910 | 0.789 | 0.755 | 0.673 | 0.566 | 0.837 | 0.282 |
| 72hrs | 2.077 | 0.397 | 0.604 | 0.680 | 0.431 | 0.839 | 0.410 |
| 96hrs | 2.200 | 0.275 | 0.181 | 0.179 | 0.075 | 0.330 | 0.049 |




At 24 hours, the control group's OD was high (2.09), representing dense biofilm formation. In contrast, hydrogel-treated samples showed reduced OD values across all organisms. Salmonella spp. showed the most prominent response to the hydrogel, followed by *Pseudomonas spp. and S. aureus*, indicating early inhibition. *E. coli* showed relatively higher OD compared to the others, suggesting it was less affected in the initial phase.

By 48 hours, the control group still maintained elevated OD values, though a slight decrease was observed. In hydrogel-treated samples, *Pseudomonas spp.* showed a significant decline, with OD dropping to 0.282, reflecting high susceptibility. *S. aureus* also showed further inhibition. Moderate antibiofilm effects were observed for *Proteus spp., E. coli, and Salmonella spp.*, while *Shigella spp.* exhibited the least reduction in OD at this stage.

At 72 hours, hydrogel efficacy became more evident. While the control group's biofilm formation remained steady (OD ~2.07), the treated bacteria showed a sustained decline in OD. *S. aureus and Pseudomonas spp.* were the most inhibited, with OD values of 0.397 and 0.410, respectively. *Salmonella spp. and Proteus spp.* also showed substantial decreases. *E. coli and Shigella spp.* remained moderately inhibited, but less so compared to the other species.

At 96 hours, the hydrogel treatment showed its maximum impact. The control OD increased further (2.200), indicating persistent biofilm growth in the absence of treatment. Meanwhile, hydrogel-treated groups exhibited their lowest OD values across the board. *Pseudomonas spp.* (0.049) and *S. aureus* (0.275) were the most affected, showing strong suppression of biofilm formation. *Proteus spp. and Salmonella spp.* followed closely with ODs of 0.179 and 0.075, respectively. *E. coli and Shigella spp.* also displayed significant reduction, though they remained the least inhibited among the tested strains.

Conclusively, The Neem-based hydrogel demonstrated substantial antibiofilm activity, particularly against *Pseudomonas spp., S. aureus, Salmonella spp., and Proteus spp.* The consistent reduction in optical density over 96 hours indicates that the formulation can effectively interfere with biofilm development. These findings support the potential of Neem-derived hydrogels as promising candidates for managing infections associated with biofilm-forming pathogens. Further preclinical and clinical studies are warranted to validate its efficacy and safety in real-world healthcare settings.

# REFERENCES:


1. Latasa, C., Roux, A., Toledo-Arana, A., Ghigo, J. M., Gamazo, C., Penadés, J. R., & Lasa, I. (2005). BapA, a large secreted protein required for biofilm formation and host colonization of Salmonella enterica serovar Enteritidis. Molecular microbiology, 58(5), 1322-1339.

2. Choe, D.; Kim, Y. M.; Nam, J. E.; Nam, K.; Shin, C. S. & Roh, Y. H. Synthesis of high-strength microcrystalline cellulose hydrogel by viscosity adjustment. Carbohydr. Polym., 180:231-7, 2018.

3. Ahmed, E. M. Hydrogel: Preparation, characterization, and applications: A review. J. Adv. Res., 6(2):105-21, 2015

4. Altemimi, A.; Lakhssassi, N.; Baharlouei, A.; Watson, D. G. & Lightfoot, D. A. Phytochemicals: extraction, isolation, and identification of bioactive compounds from plant extracts. Plants (Basel), 6(4):42, 2017.

5. Zhang, X.; Zhao, C.; Xiang, N. & Li, W. Chain entanglements and hydrogen bonds in carbopol microgel reinforced hydrogel. Macromol. Chem. Phys., 217(19):2139-44, 2016.

6. Singh, M.; Kanoujia, J.; Singh, P.; Parashar, P.; Arya, M.; Tripathi, C. B.; Sinha, V. R. & Saraf, S. A. Development of an a-linolenic acid containing a soft nanocarrier for oral delivery-part II: buccoadhesive gel. RSC Adv., 6(103):101602, 2016.

7. Miño[1], K., Pérez, A., Iza-García, T. X., Cabascango, D. G., & Gordillo[1], C. (2024, November). Antimicrobial cellulose hydrogels against gram-positive and. In 1st Galapagos Soft Matter Conference-Research Topic (p. 68). Frontiers Media SA.

8. Olawuwo, O. S., Famuyide, I. M., & McGaw, L. J. (2022). Antibacterial and antibiofilm activity of selected medicinal plant leaf extracts against pathogens implicated in poultry diseases. Frontiers in veterinary science, 9, 820304.

9. Batra, N., Kumar, V. E., Nambiar, R., De Souza, C., Yuen, A., Le, U., ... & Vinall, R. L. (2022). Exploring the therapeutic potential of Neem (Azadirachta Indica) for the treatment of prostate cancer: a literature review. Annals of translational medicine, 10(13).

10. Harjai, K., Bala, A., Gupta, R. K., & Sharma, R. (2013). Leaf extract of Azadirachta indica (Neem): a potential antibiofilm agent for Pseudomonas aeruginosa. Pathogens and Disease, 69(1), 62-65.

11. Satish, J. V., Mohan, R. R., & Pavan, S. (2017). Tube adherence test as a screening tool for detection of biofilm formation among Staphylococcus. Indian J Microbiol Res, 4, 44-47.

12. Høiby, N. (2017). A short history of microbial biofilms and biofilm infections. *Apmis*, *125*(4), 272-275.

13. Vestby, L. K., Grønseth, T., Simm, R., & Nesse, L. L. (2020). Bacterial biofilm and its role in the pathogenesis of disease. *Antibiotics*, *9*(2), 59.

14. Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *science*, *284*(5418), 1318-1322.

15. Hall-Stoodley, L., & Stoodley, P. (2009). Evolving concepts in biofilm infections. *Cellular microbiology*, *11*(7), 1034-1043.

16. Bjarnsholt, T. (2013). The role of bacterial biofilms in chronic infections. *Apmis*, *121*, 1-58.

17. Haney, E. F., Trimble, M. J., Cheng, J. T., Vallé, Q., & Hancock, R. E. (2018). Critical assessment of methods to quantify biofilm growth and evaluate antibiofilm activity of host defence peptides. *Biomolecules*, *8*(2), 29.

18. Galante, R., Ghisleni, D., Paradiso, P., Alves, V. D., Pinto, T. J., Colaco, R., & Serro, A. P. (2017). Sterilization of silicone-based hydrogels for biomedical application using ozone gas: Comparison with conventional techniques. *Materials Science and Engineering: C*, *78*, 389-397.

19. Galante, R., Oliveira, A. S., Topete, A., Ghisleni, D., Braga, M., Pinto, T. J., ... & Serro, A. P. (2018). Drug-eluting silicone hydrogel for therapeutic contact lenses: Impact of sterilization methods on the system performance. *Colloids and Surfaces B: Biointerfaces*, *161*, 537-546.

20. Kamimura, R., Kanematsu, H., Ogawa, A., Kogo, T., Miura, H., Kawai, R., ... & Barry, D. M. (2022). Quantitative analyses of biofilm by using crystal violet staining and optical reflection. *Materials*, *15*(19), 6727