RESEARCH
www.commprac.com
ISSN 1462 2815

EFFICACY OF NOVEL COMBINATION OF NANO-SILVER INFUSED PEPPERMINT OIL (Ag-Np PEO) FOR ITS INHIBITORY EFFECT ON DENTAL E-FECALIS BIOFILMS: A INVITRO STUDY

Tahoora Taskeen [1], Aparna Mohan [2]*, Lakshmi Pathy [3] and Sunnypriyatham Tirupathi [4]

[1,2,3] Department of Conservative Dentistry and Endodontics, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai.
Email: [1]151901044.sdc@saveetha.com, [2]aparnamohane@gmail.com (*Corresponding Author), [3]lakshmipathym.sdc@saveetha.com
[4] Department of Pedodontics and Preventive Dentistry, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai.
Email: dr.priyatham@gmail.com

DOI: 10.5281/zenodo.11545447

Abstract

Introduction: Essential oils are aromatic oily liquids extracted from aromatic plant materials. Plant essential oils have been considered natural and safe alternatives to synthetic antiseptics and antibacterial drugs for their broad-spectrum antimicrobial effects. Peppermint essential oil (PEO) contains most of the biological substances in mint, such as menthol and menthone, and is a potential candidate to extend the shelf-life of food due to its good inhibitory effect on foodborne pathogens and spoilage bacteria. This study aims to evaluate the antibiofilm effect of peppermint oil in combination with nano silver towards dental applications. **Materials and methods:** The study design was done in Saveetha Dental College and Hospitals, Chennai, India. The synthesis of silver nanoparticles (AgNPs) with peppermint oil and preparation of inoculum and antibiofilm assay. **Result: Antibiofilm assay:** The absorbance of 570 nm was just 0.1 in 0.003mg/ml with 35% of peppermint oil with nanoparticles which shows that compared to the control group the vitality of *E fecalis* with peppermint oil nanoparticles has been tremendously reduced. **Conclusion:** Ag-Np PEO was effective in preventing biofilm formation, as confirmed by the SEM observations. Ag-Np PEO inactivated a mature *E fecalis* biofilm. The integration of biologically produced peppermint oil with silver nanoparticles presents a promising avenue for addressing biofilm-related challenges in dental settings

Keywords: Peppermint Oil Nanoparticle, Peppermint Oil, Essential Oil, Antibiofilm Assay, Ag-Np, Silver Nano Particles, Peppermint Extract, Biofilm, Dental

Short-Title: Ag-Np PEO for antibiofilm effect.

INTRODUCTION

Nanomedicine represents a relatively recent advancement in the field of medicine, and metal nanoparticles, particularly silver nanoparticles, have emerged as noteworthy contributors with demonstrated antibacterial, antifungal and antiviral properties(3). The resurgence of silver nanoparticles in the medical field is attributed to recent technological progress. Notably, these nanoparticles exhibit low toxicity to mammalian cells while displaying potent antimicrobial activity, making them versatile across various disciplines(1,2). In particular, silver nanoparticles have found application in treating biofilms associated with medical devices, posing a threat to life. Biofilms, formed when bacteria adhere to surfaces and create structured formations, serve as the natural survival strategy for bacterial invasion(3). These biofilm structures prove to be more resilient against commonly used antimicrobial treatments, rendering their control more challenging and leading to the escalation of infections(3,4).The rise of

bacterial resistance to antibiotics, coupled with the ability to colonize non-living surfaces through biofilm formation, constitutes significant factors contributing to infections associated with medical implants. Such infections not only prolong hospital stays but also pose risks to patient mortality(3–5). To counteract these challenges, various strategies have been implemented in medical settings to prevent and manage infections linked to biofilm formation. The utilization of silver nanoparticles stands out as a promising avenue in this context due to their effectiveness and compatibility with medical applications(6). Antimicrobial effects of peppermint oil are well-known. The antibacterial and antifungal properties of its constituents, such as limonene and menthol, have been researched. Thanks to its cool flavor and possible dental health benefits, peppermint oil is frequently found in mouthwash formulations(6,7). The capacity of nano silver particles to prevent the growth of bacteria, viruses, and fungi makes them useful in a variety of applications due to their antibacterial qualities(8). Because of its potential for both infection prevention and treatment, nano silver has been investigated for use in dental and medical fields. The precise combination and its efficacy in dental applications would probably rely on a number of parameters, even though both peppermint oil and nano silver have individually shown antibacterial qualities(8,9). The way in which the two components are combined, the concentrations used, and the overall formulation can impact their efficacy. Nano silver formulations can vary in terms of stability, and the addition of peppermint oil may influence the stability of the overall product. It's important to consider the safety of the combination, especially in oral applications where the product may come into direct contact with sensitive tissues. Scientific studies and clinical trials are crucial to validate the efficacy and safety of any novel combination (10,11). The prevention of new bacterial colonization within biofilms is a crucial consideration, and exposure to silver nanoparticles appears to be an effective strategy in hindering this process(12). Consequently, the search for anti-biofilm molecules that can efficiently reduce and eliminate biofilms linked to infections is of paramount importance. In this context, our investigation has focused on whether silver nanoparticles could prevent the formation of biofilms in hospital isolates or dental infections(12,13).

The primary objective of our study was to comprehensively evaluate the antibacterial and anti-biofilm potential of biologically produced peppermint oil combined with silver nanoparticles (Ag-Np-PEO) against E. faecalis. By targeting this specific bacterium, our research aimed to provide valuable insights into a strategy for controlling biofilm formation associated with dental diseases, such as chronic pulpitis. The integration of biologically produced peppermint oil with silver nanoparticles presents a promising avenue for addressing biofilm-related challenges in dental settings. Thus the study aims to evaluate the antibiofilm effect of peppermint oil in combination with nano silver towards dental applications.

MATERIALS AND METHODS

Synthesis of silver nanoparticle: In a 250mL Erlenmeyer flask, 100mL of a $1x10^{3}$ M silver nitrate (AgNO3) solution was prepared. To this solution, 10mL of deionized milli water containing 0.01 g of gallic acid was added with continuous stirring. Simultaneously, 1M NaOH was added drop-wise to the solution to adjust its pH to 11 and peppermint oil added. This experimental procedure likely aimed to create a specific chemical environment or reaction for further studies or applications involving silver nanoparticles.

Preparation of inoculum:

In a 250 mL Erlenmeyer flask, 100 mL of Brain Heart Infusion (BHI) broth was prepared. This broth was then inoculated with 4-5 individual colonies each of Enterococcus faecalis. The inoculated culture was allowed to incubate overnight at 37°C in an incubator.

After incubation, the log phase cultures were obtained, with an optical density at 600 nm (OD600) of 0.1, and these log phase cultures were used for subsequent experiments. This process likely aimed to cultivate and prepare bacterial cultures in a standardized manner for further experimental investigations.

Antibiofilm assay:

In the experimental process, the samples (PEO and Ag-Np-PEO) were diluted to the desired concentrations, ranging from 0.1 ml to 0.003 ml. These diluted samples were then added to Brain Heart Infusion (BHI) broth in wells. Subsequently, the samples in the wells were inoculated with 50 µL of the broth culture and left to incubate for 48 hours at 37℃. Following the incubation period, the broth was aspirated from the wells using a sterile pipette, and a wash with PBS solution was performed.

Then, 150 µL of crystal violet (0.2%) was added to each well and allowed to stand for 15-20 minutes. Afterward, the dye was removed, and another wash with PBS was conducted to eliminate unbound and excess dye. To dissolve the dye, 150 µL of glacial acetic acid (30%) was added to each well.

Finally, readings were taken using an ELISA Plate Reader at 570 nm, and the absorbance values were recorded. This series of steps is likely part of a bioassay or experiment designed to assess the impact or interaction of the samples (PEO and Ag-Np-PEO) on the bacterial culture in the BHI broth which was analyzed in SEM and EDS.

Statistical analysis:

The obtained data from antibiofilm assay, SEM and EDS was evaluated in microsoft Excel and association graph obtained by student t test with SPSS 26.0.

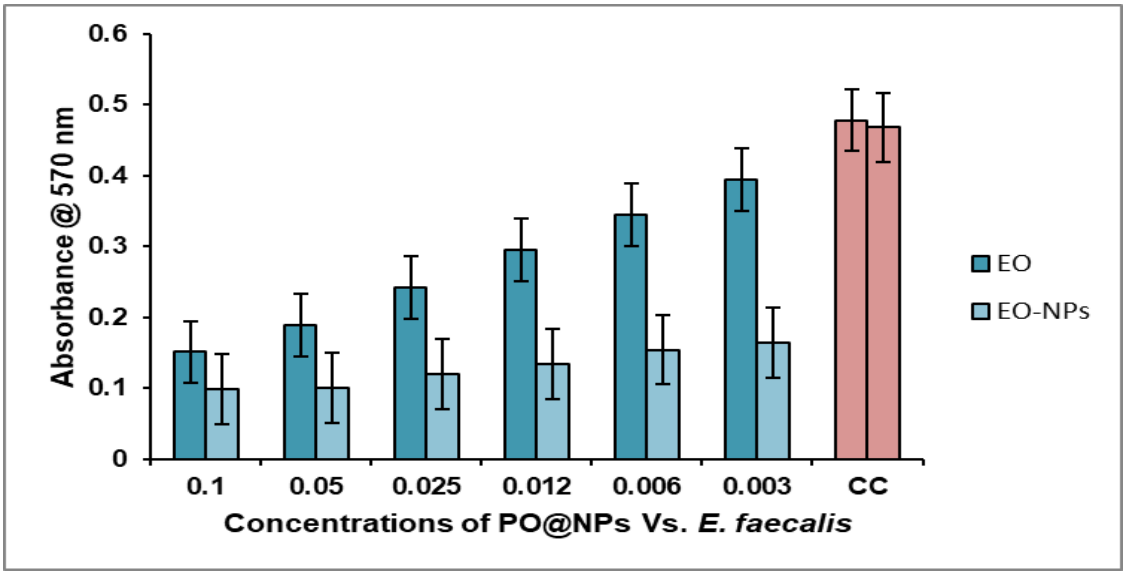
RESULTS

Antibiofilm assay:

The antibiofilm assay of peppermint oil with nanoparticle (PEO-NP) and peppermint oil (PEO) against *E.faecalis* has shown that the control group (CC) has 0.5 absorbance rate of fluorescence which on addition of PEO and PEO-NP in concentration 0.1 ,0.05, 0.025, 0.012, 0.006, 0.003 which has demonstrated a gradual decrease in absorbance rate. In the PEO concentration 0.1 (31.6%), 0.05 (39.5%), 0.025(50.6%), 0.012 (61.7%), 0.006 (72.2%), 0.003 (82.4%) showed the above mentioned absorbance rate which signifies that as the concentration increased the biofilm formation decreased when only peppermint oil was used whereas when PEO-NP concentration 0.1 (20.9%), 0.05 (21.4%), 0.025(25.6%), 0.012 (28.6%), 0.006 (32.9%), 0.003 (35%) showed the above mentioned absorbance rate which signifies that as the concentration increased the biofilm formation decreased when only peppermint oil with nanoparticle was used which deliberately shows that PEO-NP is having more efficacy as antibiofilm agent than PEO against *E.feacalis* (Graph 1 and table 1) (Figure 3,4&5).

SEM and EDS:

SEM confirmed the shape of Ag-NP was more of square or diamond with sharp end which can help it to destroy the bacterial cell wall and cause cytoplasmic content leakage leading to bacterial cell lysis (Figure 1). EDS confirmed the present of Ag 43%, O2 (34%), C(21.9). The oxygen and carbon may be absorbed from the atmosphere but the maximal percentage of Ag confirms presence of silver nanoparticle in the compound assessed (Figure 2).



Graph 1: Illustrates absorbance recorded against different concentrations of peppermint oil with nanoparticle (PEO-NP) and peppermint oil (PEO) in E.faecalis. The control group (CC) showed an absorbance of 0.5 which on the addition of PEO and PEO-NP in concentrations of 0.003, 0.006, 0.012, 0.025, 0.05, and 0.1 (units required) decreased gradually, the maximum absorbance was seen for PEO with the concentration of 0.003 (units) and the least was seen for PEO-NP having a concentration of 0.1(units).

Table 1: Table showing the percentage of biofilm formation in varied concentration of PEO and PEO-NP:

% Biofilm formation		
Concentration	**PEO**	**Ag-Np PEO**
0.1	31.6	20.9
0.05	39.5	21.4
0.025	50.6	25.6
0.012	61.7	28.6
0.006	72.2	32.9
0.003	82.4	35.0

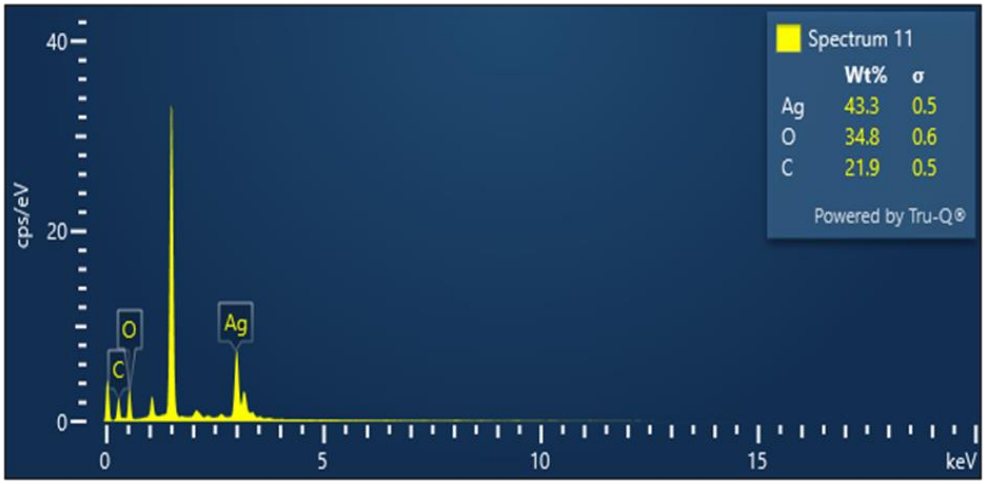


Figure 1: Illustrates the EDS confirming presence of Ag, C and O

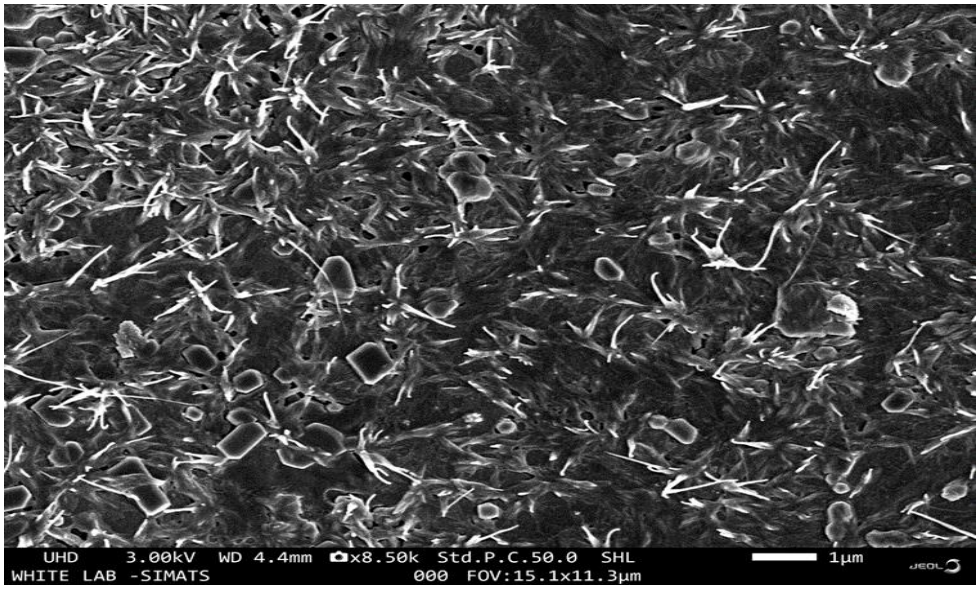


Figure 2: illustrates the morphology of Ag-NP nanoparticle synthesized

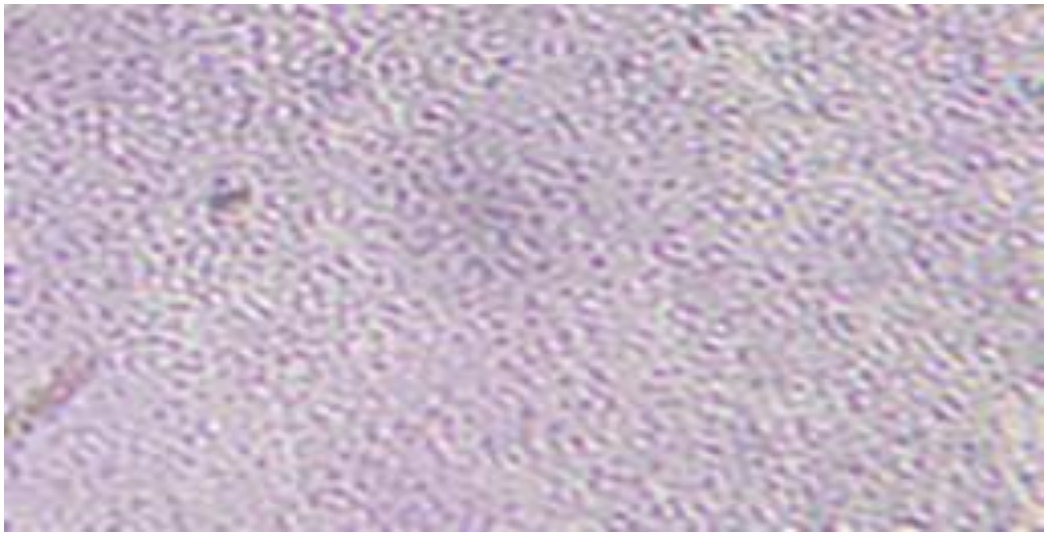


Figure 3: Illustrates the control biofilm under 40s microscope

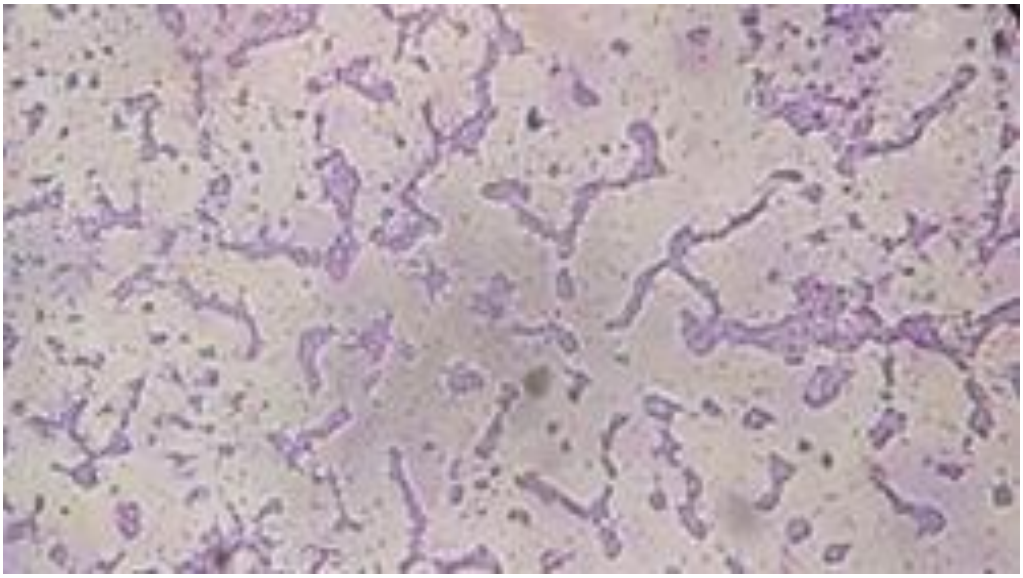


Figure 4: illustrates the peppermint oil exposed biofilm under 40s microscope

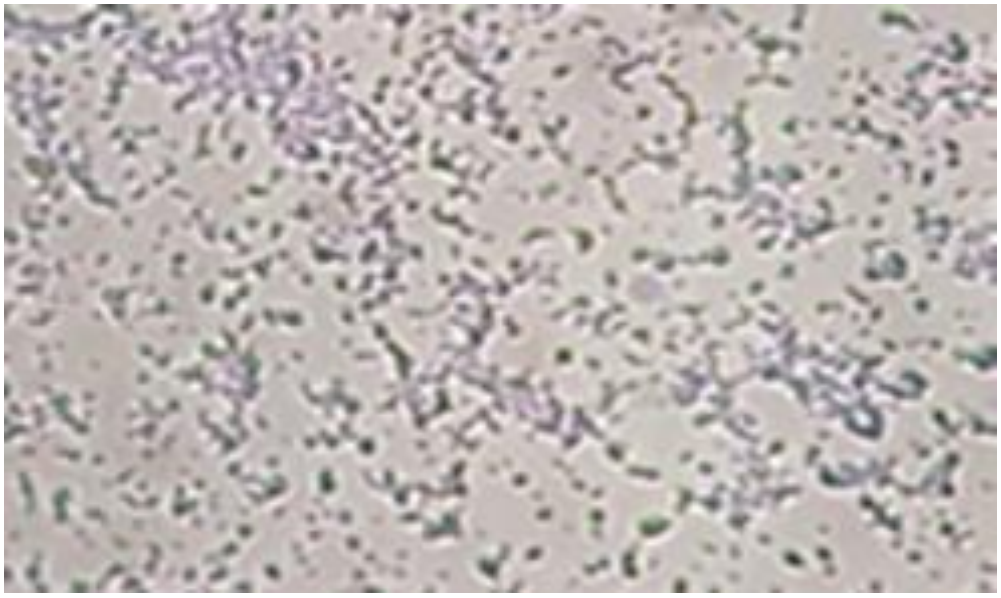


Figure 5: illustrates the peppermint oil with Ag-NP exposed biofilm under 40s microscope

DISCUSSION

In the investigation of the anti-biofilm efficiency, silver nanoparticles were tested against E. coli U12 on Congo Red Agar (CRA) both enriched with and without silver nanoparticles, using the Crystal Violet (CV) assay method. In the control group without silver nanoparticles, E. coli U12 formed black, dry crystalline colonies, indicating the necessity of exopolysaccharide generation (EPS) for biofilm formation. However, when treated with silver nanoparticles, the bacterial growth and biofilm production were inhibited at all concentrations. These results align with findings by Lahiri et al.(14), who observed that E. coli and K. pneumoniae, when treated with silver nanoparticles, did not establish biofilms over CRA medium due to the inhibition of glycocalyx matrix and exopolysaccharide synthesis. Furthermore, the current study demonstrated a gradual decrease in E. coli U12 biofilm development in microtitre plate

wells as silver nanoparticles concentrations increased. This trend is in line with the observations of Ansari et al (15), who noted that increasing silver nanoparticles concentrations from 12.5 to 100 µg/ml prevented biofilm development in Klebsiella pneumoniae, Escherichia coli, and Pseudomonas aeruginosa within 24 hours. Additionally, a previous study indicated that silver nanoparticles concentrations ranging from 7.5 to 35 mg/L significantly reduced and impaired the development of biofilms generated by uropathogenic E. faecalis by 97%. These collective findings underscore the potential of silver nanoparticles in inhibiting biofilm formation across different bacterial strains(16). Similarly in present study the antibiofim efficacy of peppermint oil test showed that peppermint oil with nanoparticle (Ag-Np PEO) and peppermint oil (PEO) against E.faecalis has shown that the control group (CC) has 0.5 absorbance rate of fluorescence which on addition of PEO and PEO-NP in concentration 0.1 ,0.05, 0.025, 0.012, 0.006, 0.003 which has demonstrated a gradual decrease in absorbance rate. In the PEO concentration 0.1 (31.6%), 0.05 (39.5%), 0.025(50.6%), 0.012 (61.7%), 0.006 (72.2%), 0.003 (82.4%) showed the above mentioned absorbance rate which signifies that as the concentration increased the biofilm formation decreased when only peppermint oil was used whereas when Ag-Np PEO concentration 0.1 (20.9%), 0.05 (21.4%), 0.025(25.6%), 0.012 (28.6%), 0.006 (32.9%), 0.003 (35%) showed the above mentioned absorbance rate which signifies that as the concentration increased the biofilm formation decreased when only peppermint oil with nanoparticle was used which deliberately shows that Ag-Np PEO is having more efficacy as antibiofilm agent than PEO against E.feacalis (Graph -1 and table -1) (Figure 3,4&5).

The transmission electron microscopy (TEM) study revealed that a concentration of 85 µg/ml of silver nanoparticles had a significant impact on E. coli U12 cells over 24 hours. This resulted in the rupture of the cell wall and cell membrane, leading to the release of cell contents. Similarly, Ansari et al. observed changes in the shape of E. coli biofilms on glass slides after 24 hours of exposure to silver nanoparticles, with a concentration of 20 µg/ml affecting the roughness of the cell surface. In the case of K. pneumonia, Skora et al.(17), conducted SEM investigations, demonstrating that silver nanoparticles suppressed bacterial growth and exopolysaccharide development on glass slides within 24 hours. Another study by Kostenko et al. reported that, upon inspection with SEM, bacterial cells of P. aeruginosa and E. coli exhibited morphological changes, including apparent membrane pores, leakage of intracellular content, and cell lysis, following treatment with silver nanoparticles (18). These microscopy studies collectively provide visual evidence of the impact of silver nanoparticles on bacterial cells, highlighting alterations in morphology, membrane integrity, and cellular content release, supporting the antimicrobial and anti-biofilm properties of silver nanoparticles(19). Similarly in present study Scanning electron microscopy showed the shape of Ag-NP was more of square or diamond with sharp end which can help it to destroy the bacterial cell wall and cause cytoplasmic content leakage leading to bacterial cell lysis (Figure 1). EDS confirmed the present of Ag 43%, O2 (34%), C(21.9). The oxygen and carbon may be absorbed from the atmosphere but the maximal percentage of Ag confirms presence of silver nanoparticles in the compound assessed. The limitations of the study are inclusion of more criteria's, further anti-inflammatory and cytotoxicity properties not tested. Hence more studies has to be done to generalize the results whereas the future scope of the study is that depending on the cytotoxicity, anti-inflammatory property, antioxidant property

CONCLUSION

Ag Np PEO had potent antibacterial and anti-biofilm activities against E-fecalis. The cell membranes of E.feacalis exhibited irreversible damage, disruptive effect was confirmed by increased cell membrane permeability, increased leakage of nucleic acids, proteins and ATP, decreased cell viability, and changes in bacterial morphology. Ag-Np PEO was effective in preventing biofilm formation, as confirmed by the SEM observations. Ag-Np PEO inactivated a mature *E fecalis* biofilm. The integration of biologically produced peppermint oil with silver nanoparticles presents a promising avenue for addressing biofilm-related challenges in dental settings

References

1) Bruna T, Maldonado-Bravo F, Jara P, Caro N. Silver Nanoparticles and Their Antibacterial Applications. Int J Mol Sci. 2021 Jul 4;22(13):7202. doi: 10.3390/ijms22137202.

2) Kannan KP, Gunasekaran V, Sreenivasan P, Sathishkumar P. Recent updates and feasibility of nanodrugs in the prevention and eradication of dental biofilm and its associated pathogens-A review. J Dent. 2024 Apr;143:104888. doi: 10.1016/j.jdent.2024.104888.

3) Kaul S, Ahmed S, Nandini VV, Lathief J, Boruah S. Evaluation of Physical Properties of Denture Base Resins Containing Silver Nanoparticles of Aloe barbadensis Miller, Morinda citrifolia, and Boesenbergia rotunda and Its Anti-microbial Effect: An In Vitro Study. Cureus. 2023 Nov;15(11):e48260.

4) Ye M, Yang W, Zhang M, Huang H, Huang A, Qiu B. Biosynthesis, characterization, and antifungal activity of plant-mediated silver nanoparticles using fruit extract. Front Microbiol. 2023 Nov 20;14:1291030.

5) Al-Badwy AH, Khalil AM, Bashal AH, Kebeish R. Polysaccharides from Spirulina platensis (PSP): promising biostimulants for the green synthesis of silver nanoparticles and their potential application in the treatment of cancer tumors. Microb Cell Fact. 2023 Dec 5;22(1):247.

6) Tidke S, Chhabra GK, Madhu PP, Reche A, Wazurkar S, Singi SR. The Effectiveness of Herbal Versus Non-Herbal Mouthwash for Periodontal Health: A Literature Review. Cureus. 2022 Aug 13;14(8):e27956. doi: 10.7759/cureus.27956.

7) Rosu G, Muresan EI, Spac AF, Diaconu M, Ciolacu DE, Danila A, et al. Aromatherapeutic and Antibacterial Properties of Cotton Materials Treated with Emulsions Containing Peppermint Essential Oil (). Polymers [Internet]. 2023 May 17;15(10). Available from: http://dx.doi.org/10.3390/polym15102348

8) Liu Q, Gao Y, Fu X, Chen W, Yang J, Chen Z, et al. Preparation of peppermint oil nanoemulsions: Investigation of stability, antibacterial mechanism and apoptosis effects. Colloids Surf B Biointerfaces. 2021 May;201:111626.

9) Arbab S, Ullah H, Bano I, Li K, Ul Hassan I, Wang W, et al. Evaluation of in vitro antibacterial effect of essential oil and some herbal plant extract used against mastitis pathogens. Vet Med Sci. 2022 Nov;8(6):2655–61.

10) Lai H, Liu Y, Huang G, Chen Y, Song Y, Ma Y, et al. Fabrication and antibacterial evaluation of peppermint oil-loaded composite microcapsules by chitosan-decorated silica nanoparticles stabilized Pickering emulsion templating. Int J Biol Macromol. 2021 Jul 31;183:2314–25.

11) Neagu R, Popovici V, Ionescu LE, Ordeanu V, Popescu DM, Ozon EA, et al. Antibacterial and Antibiofilm Effects of Different Samples of Five Commercially Available Essential Oils. Antibiotics (Basel) [Internet]. 2023 Jul 14;12(7). Available from: http://dx.doi.org/10.3390/antibiotics12071191

12) Shetta AAA. Encapsulation of Essential Oils in Chitosan Nanoparticle Formulations and Investigation on Their Antioxidant and Antibacterial Properties. 2018. 230 p.

13) Goyal MR, Kulkarni S. Advances in Green and Sustainable Nanomaterials: Applications in Energy, Biomedicine, Agriculture, and Environmental Science. CRC Press; 2023. 422 p.

14) Lahiri D, Nag M, Ray RR, Ghosh S. Biofilm Associated Antimicrobial Resistance and Its Recovery. CRC Press; 2023. 391 p.

15) Ansari MA, Khan HM, Khan AA, Cameotra SS, Saquib Q, Musarrat J. Gum arabic capped-silver nanoparticles inhibit biofilm formation by multi-drug resistant strains of Pseudomonas aeruginosa. J Basic Microbiol. 2014 Jul;54(7):688–99.

16) Rodríguez-Serrano C, Guzmán-Moreno J, Ángeles-Chávez C, Rodríguez-González V, Ortega-Sigala JJ, Ramírez-Santoyo RM, et al. Biosynthesis of silver nanoparticles by Fusarium scirpi and its potential as antimicrobial agent against uropathogenic Escherichia coli biofilms. PLoS One. 2020 Mar 12;15(3):e0230275.

17) Skóra B, Krajewska U, Nowak A, Dziedzic A, Barylyak A, Kus-Liśkiewicz M. Noncytotoxic silver nanoparticles as a new antimicrobial strategy. Sci Rep. 2021 Jun 29;11(1):13451.

18) Kostenko V, Lyczak J, Turner K, Martinuzzi RJ. Impact of silver-containing wound dressings on bacterial biofilm viability and susceptibility to antibiotics during prolonged treatment. Antimicrob Agents Chemother. 2010 Dec;54(12):5120–31.

19) Kumar G, Rajula MP, Rao KS, Ravishankar PL, Albar DH, Bahammam MA, Alamoudi A, Alzahrani KJ, Alsharif KF, Halawani IF, Alzahrani FM, Alnfiai MM, Baeshen HA, Patil S. Antimicrobial Efficacy of Blended Essential Oil and Chlorhexidine against Periodontal Pathogen (P.gingivalis)-An In Vitro Study. Niger J Clin Pract. 2023 May;26(5):625-629. doi: 10.4103/njcp.njcp_787_22.