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ANTIBIOFILM PROPERTY OF GREEN SYNTHESIZED IRON OXIDE NANOPARTICLES FROM NEEM LEAVES

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Abstract: The production of nanoparticles usually utilizes a large quantity of organic solvents, toxic chemicals and non-biodegradable stabilizing agents. Recent research has focused on the production of nanoparticles using green, sustainable and more biodegradable technology. The use of green synthesized IONPs should reduce environmental impact of the production of nanoparticles besides being potentially attractive for invasive applications. *S. aureus* is known to be resistant to multiple types of drugs due to the bacteria's ability to form a biofilm. This study aims to investigate the antibiofilm activity of green synthesized IONPs extracted from neem leaves (*Azadirachta indica*) on the *S. aureus bacteria.* Insights into the cellular interactions and the cellular changes due to exposure towards the IONPs and the bacteria were observed and recorded. Firstly, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using a microdilution broth method. The antibiofilm activity of IONPs was then evaluated using a minimum biofilm inhibitory concentration (MBIC) assay. The morphology of the *S. aureus* biofilm after the treatment with $MBIC_{90}$ of IONPs was observed under a scanning electron microscope (SEM). From this study, green synthesized IONPs showed antibacterial activity with an MIC at 6.25 mg/ml and an MBC value at 25 mg/ml. The antibiofilm activity of IONPs displayed a dose-dependent pattern with $MBIC_{50}$ and $MBIC_{90}$, which were at 1.56 mg/ml and 12.5 mg/ml, respectively ($P<0.05$). The SEM image of the sample treated with IONPs at $MBIC_{90}$ showed significantly reduced biofilm formation and an abnormal morphology of the *S. aureus* was also observed, indicating good antibiofilm action. These findings suggest that the green synthesized IONPs have potential as an antibiofilm agent especially against the *S. aureus* bacteria and may prove useful in the future as an eco-friendly alternative in the fight against *S. aureus* infections.

Keywords: Biofilm, *S. aureus*, iron oxide nanoparticles, neem, *Azadirachta indica*.
Abbreviations: Iron oxide nanoparticles (IONPs), minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum biofilm inhibitory concentration (MBIC).

Introduction

S. aureus is a clinically relevant gram-positive pathogen found on human skin and mucous and the most frequent bacteria diagnosed for biofilm-related infections (Lister & Horswill, 2014). Various chronic infections that can cause by the establishment of mature biofilm of *S. aureus* are including osteomyelitis, immedicable wound infections such as pressure ulcers, diabetic foot ulcers and stasis ulcers, endocarditis, as well as ocular infections (Archer *et al*., 2011).

A study by Delgado *et al*. (2011) found that certain strains that cause mastitis, a type of breast infection, contained the gene icaA, which is responsible for the biofilm formation of *S. aureus*. The study also revealed that the strains responsible for human mastitis exhibited higher MICs than strains from bovine mastitis. Moreover, the isolates from human mastitis showed intermediate resistance to antibiotics such as erythromycin and chloramphenicol.

The estimated global prevalence of mastitis is reaching 33% (Boakes *et al*., 2018). Breast infections may occur via direct inoculation, Haematogenous dissemination, contiguous spread and ductal infections from the lymphatic route, which is the most frequent cause of mastitis (Mukerjee *et al*., 1974).

There are two types of breast infections: lactational and non-lactational, which commonly happen to women between the ages of 18 and 50. However, Park *et al*. (2018) reported in a case study, that *S. aureus* was the cause of breast abscesses suffered by two adolescent patients who were 13 and 15 years old and suffered from Atopic Dermatitis (AD). A breast abscess usually happens to lactating mothers, and patients suffering from obesity, diabetes mellitus and immune dysregulation. Biofilm-related infections usually need to be treated with the long-term use of antibacterial drugs in ever higher concentrations.

In an effort to address *S. aureus* infection rates and reduce or eliminate the use of antibacterial drugs and endure the; long term sustainability and viability of the production of these antibacterial agents, many researchers are exploring the potential of iron oxide nanoparticles (IONPs) as a means to stop the formation of biofilm by bacteria and to create new alternatives for anti-bactericidal material. IONPs used in combination with polymer brush coating on different biomaterial surfaces showed a significant reduction in biofilm formation (Thukkaram *et al.,* 2014). Similarly, biosurfactant-coated IOPNs demonstrated excellent anti-biofilm activity during the biofilm's formation as well as on pre-formed biofilms (Khalid *et al.,* 2019).

Nanoparticles are a viable candidate for bacterial infection treatments because they can directly interact with microbial cells by disrupting the cell envelope, interrupting the transfer process of transmembrane electrons, secretion of secondary products, and dissolve heavy metal ions (Vallabani & Singh, 2014). Iron oxide nanoparticles are one of the nanoparticles that is gaining attention from researchers because of its special properties such as its low toxicity, good stability, small size and superparamagnetic qualities that make them capable of carrying out multiple functions.

For instance, due to the small size, the IONPs possess a high surface area, high reactivity, and can easily pass through the cell membrane and blood-brain barrier (Sadeghi *et al*., 2015). Additionally, in terms of clinical use, IONPs are the only magnetic nanoparticles that have received the approval of the United States Food and Drug Administration (US FDA) as stated by Taib *et al*. (2014).

Several current studies found that nanoparticles derived from plants showed promising results as commercial synthetic nanoparticles that possess good antimicrobial properties. The green-facile approach to synthesizing nanoparticles is gaining traction as it is well-known as being safe for the environment (Thakur *et al*., 2019, Luo *et al*., 2019 & Guo *et al*., 2020). A study by Qasim *et al.* (2020) compared the biologically and chemically synthesized IONPs and found that IONPs synthesized via *Withania Coagulans* exhibit better characteristics in terms of compatibility, crystalline structure and stability. They have also been shown to be less toxic.

Qasim also reported that the green synthesized IONPs showed better antibacterial and photocatalytic activity than chemically synthesized IONPs. Thus, this research studies focusing on the antibiofilm of green synthesized IONPs (via *Adirachta indica*) tested on *S. aureus.*

Methodology

Preparation of Nanoparticles

The preparation of iron oxide nanoparticles (IONPs) was based on a previously published method (Taib *et al*., 2018). The IONPs were diluted in dimethylsulphoxide (DMSO) and ultrasonicated for one hour. In our previous work (Zambri *et al.,* 2019), we managed to synthesize iron oxide NPs (Fe_3O_4) nanoparticles from neem leaves extract with a one-pot-two-step co-

precipitation approach. The nanoparticles were characterized by UV–vis spectroscopy, TEM, and XRD. UV absorption peak was observed within 200-600 nm. XRD and TEM analysis revealed the Fe_3O_4-NPs to be crystalline, and the average size was 9-12 nm with irregular morphology.

Bacterial Suspension

S. aureus (ATCC 29213) of 0.5 McFarland standard, which is equivalent to $1x10^8$ CFU/ml (0.10 at 625 nm), was prepared in the biosafety cabinet with the application of sterile technique along with the procedure (Krishnan *et al*., 2015). Bacterial was inoculated from overnight culture into 10 ml Brain Heart Infusion (BHI) broth until the optical reading (OD) reached 0.2-0.24 nm. 1 ml of the bacterial suspension was transferred into 19 ml of new BHI broth (Missiakas & Schneewind, 2015).

Minimum Inhibitory Concentration (MIC)

The broth microdilution method was used to determine the minimum inhibitory concentration of IONPs on *S. aureus* using BHI broth in a 96-well plate. The concentrations of IONPs evaluated were 25, 12.5, 6.25, 3.125, 1.56 and 0.78 mg/ml prepared using a two-fold dilution in Brain Heart Infusion broth.

The addition into every 96-well plate is in the order of broth IONPs and this followed by a bacterial suspension. Columns 1-8 rows A through C consists of 100 µL of BHI broth, 100 µL of IONPs and 20 µL of 10^5 CFU/ml of the bacterial suspension. Columns 1-8 rows D, E and F are the positive controls consisting of 100 µL of BHI broth, 100 µL of antibiotics and 20 µL of bacterial suspension, while the negative control consisting of 100 µL of BHI broth and 20 µL, is in column 12 rows F, G and H. The optical reading (OD) for *S. aureus* (Muthukumar *et al*., 2017) was taken at 600 nm for 24 hours after incubation at 37°C.

Minimum Bactericidal Concentration (MBC)

Minimum bactericidal concentration (MBC) is the lowest concentration of the IONPs needed to kill the bacteria or reduce the viability of the initial bacterial inoculum by 99%. It is determined by subculturing from the minimum inhibitory concentration (MIC) test onto BHI agar plates and is incubated at 37°C for 24 hours (Krishnan *et al*., 2015).

Antibiofilm Assay by Using Crystal Violet

The *S.* aureus bacteria was treated with various concentrations of green synthesized IONPs ranging from (25-0.78 mg/ml) in 96 well plates with brain heart infusion broth. An aliquot of 50 µL was taken from bacterial suspensions and was pipetted into every well. The 96-well plate was incubated at 37°C for 24 hours. The planktonic cells were removed, and the wells were washed with sterile distilled water. The wells were allowed to air-dry and then stained with 0.4% crystal violet. The plate was incubated for 15 minutes in the incubator (37°C). The unbound crystal violet was removed by washing with tap water and allowed to air-dry for 30 minutes. Biofilm-bound crystal violet was extracted with 20% (v/v) glacial acetic acid solution. For biofilm quantification, the reading of the wells was measured at 570 nm (Subramenium *et al*., 2018). The percentage of biofilm inhibition was calculated by using the formula below (Namasivayam *et al*., 2012):

$$\text{Percentage of inhibition} = \frac{OD\ negative\ control - OD\ test}{OD\ negative\ control} \times 100$$

Morphology Viewing by Scanning Electron Microscopy (SEM): Biofilm Cultivation

Changes in morphology and density of biofilm formed by the *S. aureus* bacteria were observed after 24 hours of treatment with nanoparticles at an MBIC90 concentration in line with the method described by Konrat *et al.* (2016) with a slight modification.

The modification in question was the addition of 3 mm and 5 mm glass beads which were autoclaved and served as the substrate for the biofilm (Merck KGaA, Darmstadt, Germany). The beads were then placed in the wells of a 24-well microplate (one bead per well). A Brain heart infusion (BHI) overnight culture of *S. aureus* was diluted in BHI to approximately. $1x10^6$ bacteria mL-1 and dispensed into the bead-containing 24 well microplates (1 mL per well).

One ml of nanoparticle solution (25 mg/ml) was added to the treated well to give the final concentration of $MBIC^{90}$ (12.5 mg/ml).

The biofilm inhibition concentration (MBIC90) is defined as the concentrations that showed a 90% inhibition of biofilm formation. Data on MBIC truly reflects the nanoparticles ability to inhibit biofilm formation and are not due to the death of the bacteria by the test compound. The microplate was then placed in the incubator and incubated at 37°C for 24 hours.

Biofilm Processing for Scanning Electron Microscopy (SEM)

Beads from the experiments were processed for SEM viewing according to the method described by Shafiei *et al*. (2016) with slight modification. Briefly, three glass beads for each experiment were fixed with 1 ml of 4% glutaraldehyde solution (prepared in sodium cacodylate buffer, pH 7.4) and kept at 4°C until the subsequent analysis. Prior to the analysis, 4% glutaraldehyde solution was pipetted out and washed twice with sodium cacodylate buffer for 15 minutes, followed by post fixing with 2% (vol/vol) osmium tetroxide in 1% (wt/vol) sodium cacodylate buffer solution for one hour and kept at 4°C. Then, the glass beads were rinsed with deionized water twice for 15 minutes before the dehydration process.

Ethanol was used at 30%, 50%, 70%, 80% and 95% concentrations at an interval of 15 minutes was used for the dehydration process. This was followed by dehydration carried out twice for 15 minutes, each using 100% ethanol. Ethanol was gradually replaced with acetone in the following ratios (vol/vol); Ethanol: Acetone 3:1, 1:1, 1:3 each for 20 minutes and finally followed by 100% acetone three times for 20 minutes.

The dehydrated samples were then subjected to Critical Point Drying (CPD) for 1 hour 40 minutes in liquid CO_2 under 95 bar pressure. The glass beads were then kept in a tight container in a desiccator. Prior to SEM viewing, the beads were gold-coated under low pressure with an ion sputter coater (Joel JFC1100, Japan). The beads were viewed for cell population at 10,000X magnification using Scanning Electron Microscope. Surface morphological changes and bacterial cell densities were observed and compared with 0.1% DMSO diluent controls.

Results and Discussion

MIC and MBC of Green Synthesized IONPs

The antimicrobial activity of green synthesized iron oxide nanoparticles was tested against *S. aureus* by MIC and MBC, and the results were summarized in Figure 1. The MIC value was found to be at a concentration of 6.25 mg/ml, while the MBC value was at concentration 25 mg/ml of green synthesized IONPs. Meanwhile, the MIC and MBC of positive control (amoxicillin) against *S. aureus* is at a concentration 1.95 mg/ml.

S. aureus is one of the major pathogens of chronic infections, and it can attach and produce biofilm on medical devices and implants. The biofilm helps to improve the *S. aureus' ability* to withstand oxygen radicals, nutrient deprivation and antibiotics. The problem in eradicating the biofilm is one that has come to attention in the medical industry. The application of nanoparticles to prevent and cure infections is a new approach as nanoparticles have many good properties, such as high surface-to-volume ratios and nanoscale sizes (Shi *et al*., 2016).

MIC can be defined as the lowest IONPs concentration that can prevent the growth of *S. aureus* after a 24-hour incubation in BHI. Meanwhile, MBC is the lowest concentration

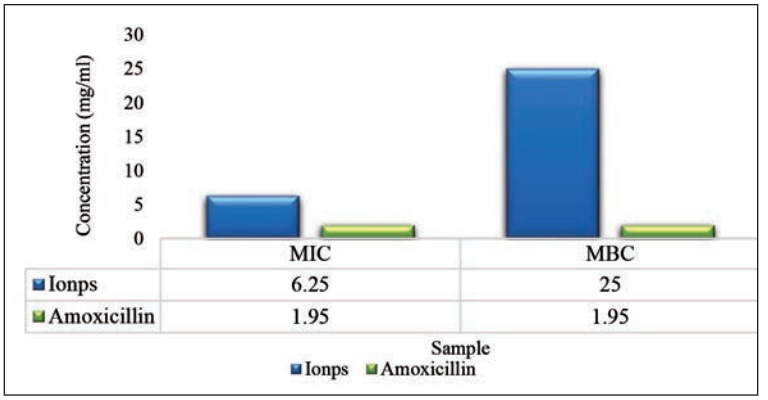

Figure 1: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of green synthesized IONPs against *S. aurues*

of IONPs required to kill all the bacteria. Researchers have proposed several mechanisms that explain the inhibitory effects of IONPs on bacteria. Several factors contribute to the bactericidal properties of IONPs. One of the common mechanisms that assist in IONPs antimicrobial activity relates to the release of oxidative stress by Reactive Oxygen Species (ROS) (Kohanski *et al*., 2007).

The components within ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals (-OH), superoxide radical (O_2^-), and singlet oxygen($^1O_2$) can cause destruction to protein and DNA in bacteria (Wang *et al*., 2017). Tran *et al*. (2010) reported that metal oxides such as IONPs could be the factor that produced ROS leading to the prevention of *S. aureus* growth. Each of the components of ROS played different roles such as hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-) result in less severe stress reactions and can be counterbalanced by endogenous antioxidants. Meanwhile, hydroxyl radicals (-OH) and singlet oxygen($^1O_2$) give rise to acute microbial death (Wang *et al*., 2017).

Irshad *et al.* (2017) described a similar mechanism of action in which the excited electrons of biosynthesized IONPs lead to the production of reactive oxygen species. Irshad et al. suggested that biosynthesized IONPs interact with the surface of bacterial cells and cause intracellular ROS generation and discharged cytoplasmic materials. Then the reactive oxygen species (ROS) induced oxidative stress and might also destroy the biomolecules such as protein and DNA. Several studies reported that the bactericidal effects of IONPs can also be caused by the small size of the nanoparticles.

Taib *et al*. (2014) reported that the size of IONPs used in this study has an average diameter of around 11 nm. The smaller sized IONPs showed better antibacterial activity because they have a higher surface-area-to -mass ratio for interaction with the cells (Wang *et al*., 2017).

Results of antibacterial activity from this study were comparable with the findings of Mansour *et al.* (2019) who found the efficacy of IONPs synthesized by using Persea Americana extracts inhibited *S. aureus* with the MIC at the 12.5 mg/ml level.

Another study by Jagathesan and Rajiv (2018) found the antibacterial activity of IONPs synthesized by using *Eicchornia crassipes* leaf extract against several bacterial strains, including *S. aureus* at concentration 100 µg/ml. A research study by Ullah *et al.* (2018) also demonstrated the antibacterial activity of IONPs synthesized using *Agrewia optiva* and *Prunus Persica* phyto species against *S. aureus* by agar diffusion method. The previous and current data indicates a considerable antimicrobial tendency of green synthesized IONPs against *S. aureus.*

Antibiofilm Activity of Green Synthesized IONPs

The inhibition effect of green synthesized IONPs against biofilm formation of *S. aureus* was evaluated by the microtiter plate assay using crystal-violet staining. The crystal-violet stained biofilm cells were quantified by measuring OD at 570 nm and the percentage of inhibition was calculated. The results were summarized in Figure 2. There were five concentrations of IONPs used to test on *S. aureus* biofilm (1.56 mg/ml, 3.13 mg/ml, 6.25 mg/ml, 12.50 mg/ml and 25 mg/ml) and the percentage of inhibition was 58.00%, 58.37%, 80.97%, 91.89% and 94.20% respectively. The results were analysed by Kruskal-wall were tested using SPSS, version 20.0 and there was significantly difference ($p<0.05$) for percentage of inhibition between different value of concentrations of IONPs.

Ultrastructural Changes of S. aureus

SEM analysis showed morphological changes of *S. aureus* biofilm when comparing results for different treatments. Large cluster of cells can be seen on the negative control group (Figure 3a and 3b).

For IONPs treated group, as observed in Figure 3 (c) the biofilm thickness decreased and the area covered by the biofilm was reduced. The cell aggregates covered a smaller area and were scattered, with a greater distance between cells Higher magnification (10 000 x) shows that the cell surface morphology was irregular and wrinkled.

The percentage of inhibition showing the increase in the percentage with an increase in the concentration of green synthesized IONPs from 0.78 mg/ml to 25 mg/ml. The results indicate that the antibiofilm potential was dose-dependent. A study conducted by Subhi *et al*. (2018) found the antibiofilm activity of IONPs-chitosan composite at 50 mg/ml with 48.2% of inhibition and 5 mg/ml with 21.2% of inhibition against *S. aureus*. IONPs at higher concentrations shows a significant reduction in biofilm growth compared to lower concentrations, as were observed in both studies.

Comprehensive literature studies have reported the effectiveness of IONPs synthesized via various methods by using chemicals, microorganisms as well as plants to inhibit the biofilm of *S. aureus* and other bacterial strains. Salman *et al.* (2015) conducted a study that displayed the antibiofilm activity of IONPs on coated catheters against *S. aureus* with 33.97% percentage of biofilm inhibition. Velusamy *et al*. (2016) synthesized oleic acid coated IONPs with good antibiofilm activity against *S. aureus*

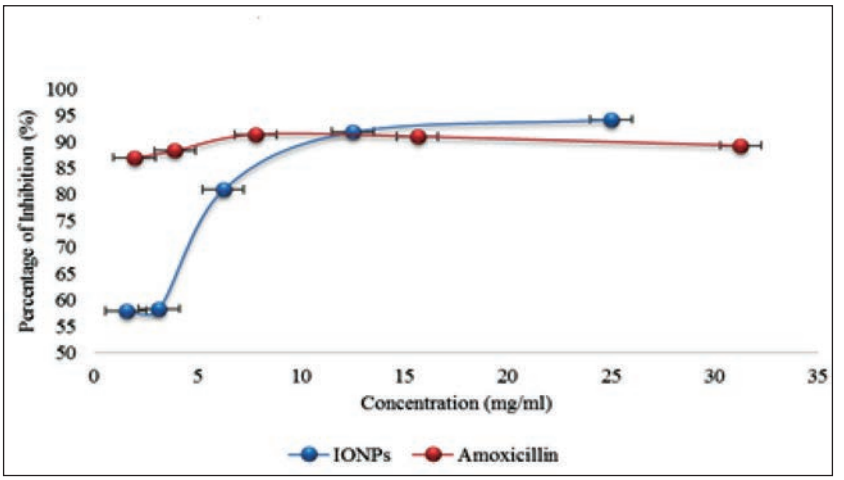

Figure 2: Percentage of inhibition of green synthesized IONPs and Amoxicillin (positive control) against the biofilm formation of *S. aureus*

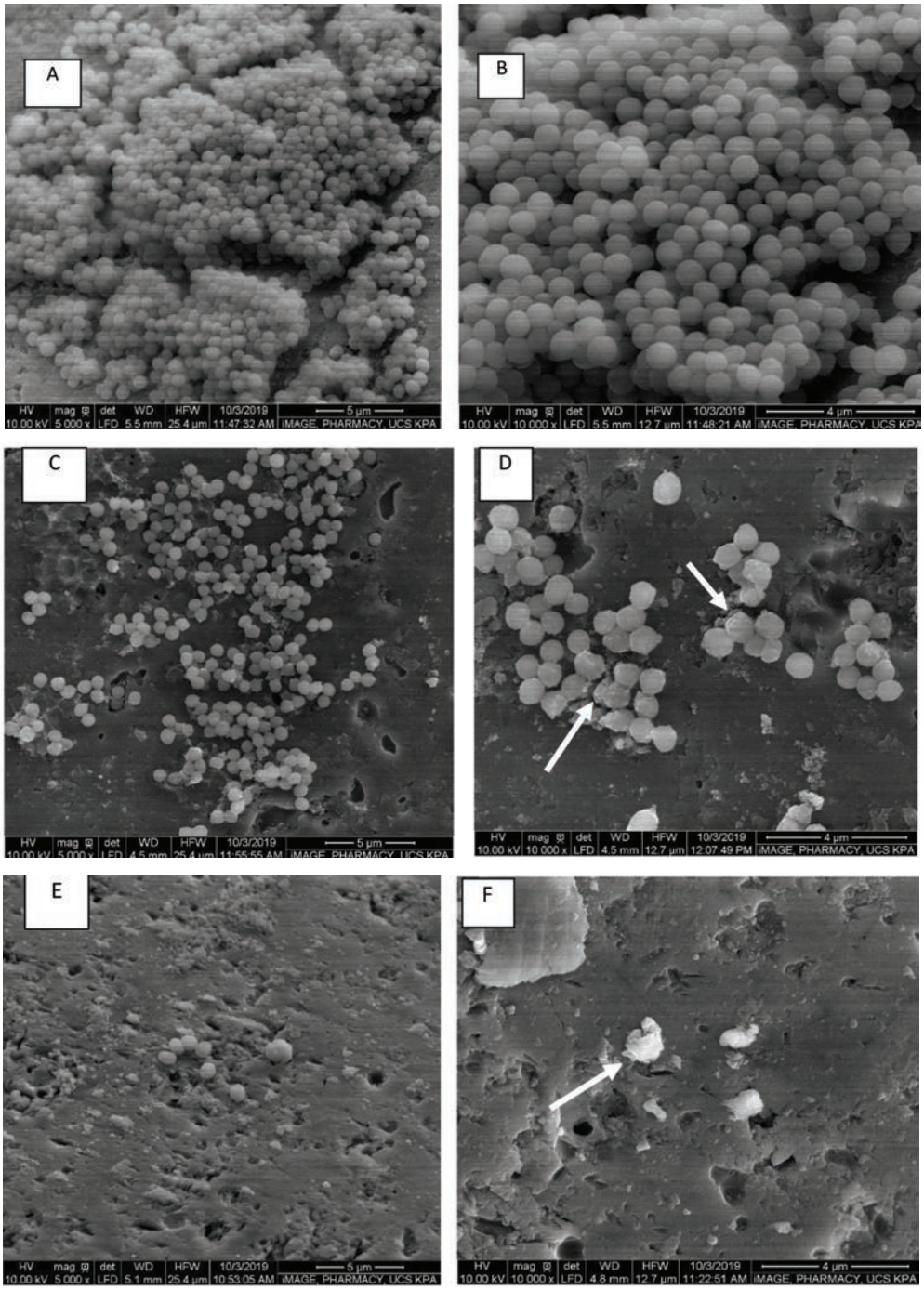

Figure 3: Scanning Electron Microscope (SEM) images of *S. aureus* (a) negative control at 5 000x magnification (b) negative control at 10 000x magnification (c) green synthesized IONPs at $MBIC_{90}$ (12.5 mg/ml) at 5 000x magnification (d) green synthesized IONPs at $MBIC_{90}$ (12.5 mg/ml) at 10 000x magnification (e) amoxicillin (positive control) at 5 000x magnification (f) amoxicillin (positive control) at 10 000x magnification . Arrows indicate the altered morphology of the bacterial cells

and *P. aeruginosa*. Another study by Shi *et al*. (2016) investigated the antibiofilm efficacy of IONPs coated with chitosan by static biofilm formation assay on polystyrene culture plates. Significant biofilm inhibition was observed against *S. aureus* at a concentration of 4 mg/ml.

In the antibiofilm activity, three important sequential mechanisms involved in the interactions between nanoparticles and biofilms include the movement of nanoparticles to the biofilm-fluid interface, adherence process to the outer surface of biofilm and movement into a deeper area of the biofilm (Ikuma *et al*., 2015). According to Fulaz *et al*. (2019), extracellular matrix (EPS) is one of the biofilm features consist of a complex mixture of biomacromolecules that helps the bacteria to resist antimicrobial action. The local environmental conditions such as biochemical and physicochemical factors continuously influenced the structure, material and chemical properties of the bacterial biofilm. Transportation of nanoparticles into biofilm is influenced by the viscosity of EPS matrix, cell density, liquid flow and the properties of pores or water spaces in the EPS matrix (Fulaz *et al.,* 2019).

Cell surface hydrophobicity (CSH) play a significant role in the adhesion process of bacteria on solid surfaces. In the beginning of the adhesion phase of the biofilm formation, the hydrophobicity of the surface influences the attachment of microbes by strengthening the attachment and reducing the force of repulsion between the surface and the bacteria (Jamal *et al*., 2018).

According to Pessan *et al.* (2018), physicochemical properties of nanoparticles such as surface charge, hydrophobicity, and high surface area ratio by volume enable the nanoparticles to diffuse and penetrate the biofilms. Ramalingam *et al.* (2019) demonstrated that the percentage of hydrophobicity of three bacterial strains were reduced after treatment with IONPs at 200 ug/ml. The hydrophobicity on the cell wall occurs due to the rise of interaction with hydrocarbons when FeO-NPs adhered to the cell wall of biofilm.

Hence, with support from previous research, probably the disruption of EPS and hydrophobicity are the mechanisms for the inhibition of *S. aureus* biofilm that was observed in this study.

The antibiofilm efficacy of green synthesized IONPs was further confirmed by visualizing the untreated and treated *S. aureus* biofilm using Scanning Electron Microscopy. The results clearly showed that the presence of IONPs caused physical damage to the biofilm of the *S. aureus* bacteria. The differences in the morphology between untreated and treated cultures demonstrated good antibiofilm efficacy of green synthesized IONPs at 12.5 mg/ml. The results are in accordance with a previously research study by Shi *et al*. (2016), in which a significant reduction of biofilm formation was observed after incubated with chitosan-coated IONPs at 4 mg/ml.

Shi *et al*. (2016) also stated that the nanoparticles' size may contribute to the antibacterial and antibiofilm efficacy. The study demonstrated that the chitosan-coated IONPs with smaller size (15–25 nm) exhibited better antibiofilm activity than larger chitosan nanoparticles (200-500 nm).

According to Neihaya *et al*. (2018) who investigated the antibiofilm effect of silver nanoparticles (AgNPs), water channels that exist within the biofilm helps the nanoparticles to inhibit the existing biofilm. The AgNPs migrate through the water channel that is used to transport nutrients and spread throughout the exopolysaccharide layer. The size of the nanoparticles makes it capable of diffusing into the biofilm matrix and attaching to the bacterial cells, which leads to biofilm inhibition. A study by Kalishwaralal *et al.* (2010) also stated that there is a high possibility, the presence of the water channels within the biofilm is the cause of the inhibitory effect of nanoparticles on the forming biofilm. The outcome of this study may help researchers develop green synthesized IONPs as efficient nano systems to combat the infections caused by *S. aureus*.

Conclusion

The IONPs synthesized by using neem leaf effectively inhibited the biofilm formation of *S. eu aureus*. When observed under SEM, the morphology of the bacterial cells showed that the number of treated bacterial cells reduced significantly exhibit abnormal morphology compared to the untreated sample. The outcome from this study suggested that green synthesized IONPs using *A. indica* extract have the potential to be further explored as an alternative treatment or management of *S. aureus* infections.

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References

Abd Elrahman, A. A., & Mansour, F. R. (2019). Targeted magnetic iron oxide nanoparticles: Preparation, functionalization and biomedical application. *Journal of Drug Delivery Science and Technology*, *52*(May), 702-712.

Abdul, F. R., Subhi, H. T., Taher, N. A., & Raheem, I. A. (2019). Activity of Iron oxide nanoparticles on bacterial biofilm formation, *11*(3), 1126-1130.

Archer, N. K., Mazaitis, M. J., William Costerton, J., Leid, J. G., Powers, M. E., & Shirtliff, M. E. (2011). *S. aureus*biofilms: Properties, regulation and roles in human disease. *Virulence*, *2*(5), 445-459.

Balakrishnan, V., Ab Wab, H. A., Abdul Razak, K., & Shamsuddin, S. (2013). *In Vitro* evaluation of Cytotoxicity of Colloidal Amorphous Silica Nanoparticles designed for drug delivery on human cell lines. *Journal of Nanomaterials*, *2013*, 729306.

Boakes, E., Woods, A., Johnson, N., & Kadoglou, N. (2018). Breast infection: A review of diagnosis and management practices. *European Journal of Breast Health*, (12), 136-143.

Burdall, S. E., Hanby, A. M., Lansdown, M. R. J., & Speirs, V. (2003). Breast cancer cell lines: Friend or foe?, 89-95.

Comşa, Ş., Cîmpean, A. M., & Raica, M. (2015). The story of MCF-7 Breast Cancer Cell Line: 40 years of experience in research, *3154*, 3147-3154.

Delgado, S., García, P., Fernández, L., Jiménez, E., Rodríguez-Baños, M., del Campo, R., & Rodríguez, J. M. (2011). Characterization of *S. aureus*strains involved in human and bovine mastitis. *FEMS Immunology and Medical Microbiology*, *62*(2), 225-235.

Fulaz, S., Vitale, S., Quinn, L., & Casey, E. (2019). Nanoparticle-Biofilm Interactions: The role of the EPS Matrix. *Trends in Microbiology*, *27*(11), 915-926.

Gholami, L., Kazemi, R., Tafaghodi, M., & Ramezani, A. (2018). Green facile synthesis of low-toxic superparamagnetic iron oxide nanoparticles (SPIONs) and their cytotoxicity effects toward Neuro2A and HUVEC cell lines. *Ceramics International*, *44*(8), 9263-9268.

Guo X., Zheng S., Luo Y., Pang H., (2020). Synthesis of confining cobalt nanoparticles within SiOx/nitrogen-doped carbon framework derived from sustainable bamboo leaves as oxygen electrocatalysts for rechargeable Zn-air batteries. *Chemical Engineering Journal*, (401), 126005.

Ikuma, K., Decho, A. W., & Lau, B. L. T. (2015). When nanoparticles meet biofilms - Interactions guiding the environmental fate and accumulation of nanoparticles. *Frontiers in Microbiology*, *6*(MAY), 1-6.

Irshad, R., Tahir, K., Li, B., Ahmad, A., R. Siddiqui, A., & Nazir, S. (2017). Antibacterial activity of biochemically capped iron oxide nanoparticles: A view towards green chemistry. *Journal of Photochemistry and Photobiology B: Biology*, *170*(December 2016), 241-246.

Jagathesan, G., & Rajiv, P. (2018). Biocatalysis and Agricultural Biotechnology Biosynthesis and characterization of iron oxide nanoparticles using Eichhornia crassipes leaf extract and assessing their antibacterial activity. *Biocatalysis and Agricultural Biotechnology*, *13*(August 2017), 90-94.

Jamal, M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Asif, M., & Atif, M. (2018). ScienceDirect Bacterial biofilm and associated infections. *Journal of the Chinese Medical Association*, *81*(1), 7-11.

Kalishwaralal, K., BarathManiKanth, S., Pandian, S. R., Deepak, V., & Gurunathan, S. (2010). Silver nanoparticles impede the biofilm formation by Pseudomonas aeruginosa and Staphylococcus epidermidis. *Colloids and surfaces. B, Biointerfaces*, *79*(2), 340-344.

Kanagesan, S., Hashim, M., Tamilselvan, S., Alitheen, N. B., Ismail, I., Hajalilou, A., & Ahsanul, K. (2013). Synthesis, characterization, and cytotoxicity of iron oxide nanoparticles. *Advances in Materials Science and Engineering*.

Karthick, S., Namasivayam, R., Aroma, R., Manikanta, M., Gopinath, P., & Francis, A. L. (2016) Evaluation of enzyme activity inhibition of biogenic silver nanoparticles against microbial extracellular enzymes. *International Journal of PharmTech Research*, *9*(2), 40-47.

Khalid, H. F., Tehseen, B., Sarwar, Y., Hussain, S. Z., Khan, W. S., Raza, Z. A., Bajwa, S. Z., Kanaras, A. G., Hussain, I., & Rehman A. (2019) Biosurfactant coated silver and iron oxide nanoparticles with enhanced anti-biofilm and anti-adhesive properties. *Journal of Hazardous Mate*rials, *15*(364), 441-448.

Kohanski, M. A., Dwyer, D. J., Hayete, B., & Lawrence, C. A. (2007). A common mechanism of Cellular Death Induced by bactericidal antibiotics, 797-810.

Konrat, K., Schwebke, I., Laue, M., Dittmann, C., Levin, K., Andrich, R., Arvand, M., & Schaudinn, C. (2016). The Bead Assay for Biofilms: A quick, easy and robust method for testing disinfectants. *PloS one*, *11*(6), e0157663.

Krishnan, R., Arumugam, V., & Vasaviah, S. K. (2015). The MIC and MBC of Silver Nanoparticles against Enterococcus faecalis - *Nanomedicine & Nanotechnology*, *6*(3).

Liao, C., Li, Y., & Tjong, S. C. (2019). Bactericidal and cytotoxic properties of silver nanoparticles. *International Journal of Molecular Sciences*, *20*(2).

Lister, J. L., & Horswill, A. R. (2014). *S. aureus*biofilms: Recent developments in biofilm dispersal. *Frontiers in Cellular and Infection Microbiology*, *4*(DEC), 1-9.

Luo Y., Guo X., Yuan M., Yan Y., Chen C., and Pang H., (2019). γ-MnOOH Nanowires Hydrothermally reduced by leaves for high-efficiency electrocatalysis of the glucose oxidation reaction. *ACS Sustainable Chemistry & Engineering*, *7*(9), 8972-8978

Missiakas, D. M., & Schneewind, O. (2015). Growth and laboratory maintenance of *S. aurues*, *344*(6188), 1173-1178.

Mukerjee, P., George, M., Maheshwari, H. B., & Rao, C. P. (1974). Tuberculosis of the breast. *Journal of the Indian Medical Association*, *62*(12), 410-412.

Mustafa, T., Zhang, Y., Watanabe, F., Karmakar, A., Asar, M. P., Little, R., & Biris, A. S. (2013). Iron oxide nanoparticle-based radio-frequency thermotherapy for human breast adenocarcinoma cancer cells. *Biomaterials Science*, *1*(8), 870-880.

Muthukumar, H., Chandrasekaran, N. I., Naina Mohammed, S., Pichiah, S., & Manickam, M. (2017). Iron oxide nanomaterial: Physicochemical traits and in vitro antibacterial propensity against multidrug resistant bacteria. *Journal of Industrial and Engineering Chemistry*, *45*, 121-130.

Namasivayam, S. K. R., Preethi, M., Bharani, A., Robin, G., & Latha, B. (2012). Biofilm inhibitory effect of silver nanoparticles coated catheter against *S. aureus*and evaluation of its synergistic effects with antibiotics. *Int J Biol Pharm Res*, *3*(2), 259-265.

Neihaya, H. Z., & Zaman, H. H. (2018). Microbial Pathogenesis Investigating the effect of biosynthesized silver nanoparticles as antibiofilm on bacterial clinical isolates, *116*(February), 200-208.

Panda, J., Satapathy, B. S., Majumder, S., Sarkar, R., Mukherjee, B., & Tudu, B. (2019). Engineered polymeric iron oxide nanoparticles as potential drug carrier for targeted delivery of docetaxel to breast cancer cells. *Journal of Magnetism and Magnetic Materials*, *485*, 165-173.

Park, S. M., Choi, W. S., Yoon, Y. S., Jung, G. H., Lee, C. K., Ahn, S. H., Yoo, Y. (2018). Breast abscess caused by *S. aureus*in 2 adolescent girls with atopic dermatitis. *Korean Journal of Pediatrics*, *61*(6), 200-204.

Patil, R. M., Thorat, N. D., Shete, P. B., Bedge, P. A., Gavde, S., Joshi, M. G., … Bohara, R. A. (2018). Comprehensive cytotoxicity studies of superparamagnetic iron oxide nanoparticles. *Biochemistry and Biophysics Reports*, *13*(January), 63-72.

Pessan, J. P., Paula, A., Vieira, M., Maria, T., Lima, T. De, Carlos, A., & Delbem, B. (2018). Iron oxide nanoparticles for biomedical applications: A perspective on synthesis, drugs, antimicrobial activity, and toxicity.

Pirayesh Islamian, J., Hatamian, M., Aval, N. A., Rashidi, M. R., Mesbahi, A., Mohammadzadeh, M., & Asghari Jafarabadi, M. (2017). Targeted superparamagnetic nanoparticles coated with 2-deoxy-d-gloucose and doxorubicin more sensitize breast cancer cells to ionizing radiation. *Breast (Edinburgh, Scotland)*, *33*, 97-10.

Qasim, S., Zafar, A., Saqib, M., Ali, Z., Nazar, M., Waqas, M., & Iqbal, F. (2020). Green synthesis of iron oxide nanorods using Withania coagulans extract improved photocatalytic degradation and antimicrobial activity. *Journal of Photochemistry & Photobiology, B: Biology*, *204*(January), 111784.

Raghunath, A., & Perumal, E. (2017). Metal oxide nanoparticles as antimicrobial agents: A promise for the future. *International Journal of Antimicrobial Agents*, *49*(2), 137-152.

Ramalingam, V., Dhinesh, P., & Sundaramahalingam, S. (2019). Green fabrication of iron oxide nanoparticles using grey mangrove Avicennia marina for antibiofilm activity and in vitro toxicity. *Surfaces and Interfaces*, *15*(November 2018), 70-77.

Rosli, N. S. B., Rahman, A. A., Aziz, A. A., & Shamsuddin, S. (2015, April). Determining the size and concentration dependence of gold nanoparticles in vitro cytotoxicity (IC50) test using WST-1 assay. In *AIP Conference Proceedings*, *1657*(1), 060001

Sadeghi, L., Tanwir, F., Yousefi, V., & Yousefi Babadi, V. (2015). Experimental and toxicologic pathology in vitro toxicity of iron oxide nanoparticle: Oxidative damages on Hep. *Experimental and Toxicologic Pathology*, *67*(2), 197-203.

Salman, Jehan A. S., Mohammed F. Al -Marjani, Raghad A. Abdulrazaq, I. A. S., & N. B. K. (2015). Antibiofilm effect of iron oxide nanoparticles synthesized by Lactobacillus Fermentum on Catheter. *World Journal of Pharmaceutical Research*, *4*(8), 317-328.

Seifi Mansour, S., Ezzatzadeh, E., & Safarkar, R. (2019). In vitro evaluation of its antimicrobial effect of the synthesized Fe3O4 nanoparticles using Persea Americana extract as a green approach on two standard strains. *Asian Journal of Green Chemistry*, *3*(3), 353-365.

Shafiei, Z., Abdul Rahim, Z. H., Philip, K., & Thurairajah, N. (2016). Antibacterial and anti-adherence effects of a plant extract mixture (PEM) and its individual constituent extracts (Psidium sp., Mangifera sp., and Mentha sp.) on single- and dual-species biofilms. *PeerJ*, *2016*(10).

Shi, S. F., Jia, J. F., Guo, X. K., Zhao, Y. P., Chen, D. S., Guo, Y. Y., & Zhang, X. L. (2016). Reduced *S. aureus*biofilm formation in the presence of chitosan-coated iron oxide nanoparticles. *International Journal of Nanomedicine*, *11*, 6499-6506.

Subhi, H. (2018). Activity of Iron Oxide Nanoparticles-Chitosan Composite on Bacterial Biofilm Formation, (Icpas), 7-10.

Subramenium, G. A., & Vijayakumar, K. (2018). Limonene inhibits streptococcal biofilm formation by targeting surface-associated virulence factors, (2015), 879-890.

Taib, N. I., Latif, F. A., Mohamed, Z., Diyana, N., & Zambri, S. (2018). Green Synthesis of Iron Oxide Nanoparticles (Fe 3 O 4 -NPs) using *Azadirachta Indica* Aqueous Leaf Extract, *7*, 9-13.

Thakur, B. K., Kumar, A., & Kumar, D. (2019). Green synthesis of titanium dioxide nanoparticles using Azadirachta indica leaf extract and evaluation of their antibacterial activity. *South African Journal of Botany*, *124*, 223-227.

Thoidingjam, S., & Tiku, A. B. (2017). New developments in breast cancer therapy: Role of iron oxide nanoparticles. *Advances in Natural Sciences: Nanoscience and Nanotechnology*, *8*(2), 023002.

Thukkaram, M., Sitaram, S., Kannaiyan, S. K., Subbiahdoss, G. (2014). Antibacterial efficacy of iron oxide nanoparticles against biofilms on different biomaterial surfaces. *International Journal of Biomaterials*, *2014*, 716080.

Tran, N., Mir, A., Mallik, D., Sinha, A., Nayar, S., & Webster, T. J. (2010). Bactericidal effect of iron oxide nanoparticles on *S. aureus*. *International Journal of Nanomedicine*, *5*(1), 277-283.

Ullah, A., Kareem, A., Nami, S. A. A. A., Shoeb, M., Rehman, S., Mirza, A. U., Nishat, N. (2018). Biogenic synthesis of iron oxide nanoparticles using Agrewia optiva and Prunus persica phyto species: Characterization, antibacterial and antioxidant activity. *Journal of Photochemistry & Photobiology, B: Biology*, *185*(April), 262-274.

Vallabani, N. V. S., & Singh, S. (2018). Recent advances and future prospects of iron oxide nanoparticles in biomedicine and diagnostics. *3 Biotech*, *8*(6).

Velusamy, P., Chia-Hung, S., Shritama, A., Kumar, G. V., Jeyanthi, V., & Pandian, K. (2016). Synthesis of oleic acid coated iron oxide nanoparticles and its role in anti-biofilm activity against clinical isolates of bacterial pathogens. *Journal of the Taiwan Institute of Chemical Engineers*, *59*, 450-456.

Wang, L., Hu, C., & Shao, L. (2017). The antimicrobial activity of nanoparticles: Present situation and prospects for the future. *International Journal of Nanomedicine*.

Zambri, N. D. S., Taib, N. I., Abdul Latif, F., Mohamed Z. (2019). Utilization of Neem Leaf extract on biosynthesis of iron oxide nanoparticles. *Molecules*, *24*, 3803.

Zhi, D., Yang, T., Yang, J., Fu, S., & Zhang, S. (2020). Targeting strategies for superparamagnetic iron oxide nanoparticles in cancer therapy. *Acta biomaterialia*, *102*, 13-34.